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Central antioxidant therapy inhibits parasympathetic baroreflex control in conscious rats

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ABSTRACT

Baroreceptor reflex is an important system for neural control of blood pressure. Recently, reactive oxygen species (ROS) have been shown to play an important role in neuronal activity of central areas related to blood pressure control. The aim of this study was to investigate the effects elicited by ascorbic acid (AAC) and N-acetylcysteine (NAC) injections into the 4thV on the parasympathetic component of the baroreflex. Male Wistar rats were implanted with a stainless steel guide cannula into the 4thV. One day prior to the experiments, the femoral artery and vein were cannulated for pulsatile arterial pressure, mean arterial pressure and heart rate measurements and drug administration, respectively. After baseline recordings, the baroreflex was tested with a pressor dose of phenylephrine (PHE, 3 μg/kg, i.v.) and a depressor dose of sodium nitroprusside (SNP, 30 μg/kg, i.v.) before (control) and 5, 15, 30 and 60 min after AAC or NAC into the 4thV. Control PHE injection induced baroreflex-mediated bradycardia (−93 ± 13 bpm, $n = 7$). Interestingly, after AAC injection into the 4thV, PHE injection produced a transient tachycardia at 5 (40 ± 23 bpm), 15 (26 ± 22 bpm) and 30 min (59 ± 21 bpm). No changes were observed in baroreflex-mediated tachycardia evoked by SNP after AAC injection on 4thV (control: 151 ± 23 bpm vs. 135 ± 18 bpm at 5 min after AAC, $n = 7$). In the NAC treated group, PHE induced a reduction in reflex bradycardia at 5 min when compared to control (−11 ± 17 bpm vs. −83 ± 15 bpm, $n = 7$). No changes were observed in baroreflex-mediated tachycardia evoked by SNP after NAC injection on 4thV. The antioxidants AAC and NAC may act in the central nervous system affecting the parasympathetic component of the cardiac baroreflex.

Baroreceptor reflex is an important mechanism involved in blood pressure control. Afferent information from arterial baroreceptors rises to the dorsal medulla and is integrated in the ventrolateral medulla. These areas modulate the cardiovascular function through the autonomic nervous system [5]. The sensitivity of the baroreceptor reflex seems to be attenuated in different models of hypertension [2,22], likely due to resetting of arterial baroreceptor to high blood pressure levels, which reduce their responsiveness to changes in arterial pressure [11].

Several studies have documented an important role for reactive oxygen species in the neural control of blood pressure both in health and disease [12,13]. Reactive oxygen species (ROS), as superoxide ($O2^{\bullet -}$) and hydrogen peroxide (H_2O_2), are produced during normal cellular metabolism. The excessive production of them or their ineffective removal from the organism can result in tissue damage, which often involves generation of highly reactive species as the hydroxyl radical ($^{\bullet -}OH$) and other oxidants. An imbalance between the antioxidant and the pro-oxidant mechanisms is linked to pathophysiological conditions such as hypertension, neurodegenerative disorders, malignancies and atherosclerosis [17,27,19,3,25].

The most common antioxidants for clinical use and experimental trials related to cardiovascular system are ascorbic acid (AAC) and N-acetylcysteine (NAC). It has been reported that either AAC or NAC treatment delays or prevents the development of different types of experimental hypertension [2,25,18].

Considering that reactive oxygen species seems to play an important role in autonomic regulation during health and disease and that the effects of antioxidant therapy on brainstem areas involved in baroreflex control are not completely understood, in this study we sought to investigate the effects of AAC and NAC injected into the fourth ventricle (4thV) on the baroreceptor reflex.

Adult male Wistar rats, weighting 300–350 g, were obtained from the Animal Care Facility from School of Medicine of ABC. Ani-

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mals were housed in individual cages in a temperature- (20–24° C) and humidity-controlled room (60–70%) with light-dark cycle of 12:12 h. Rats had access to standard rat chow pellets (Nuvilab Chow, Nuvital, Colombo, PR, Brazil) and water *ad libitum*. All experiments were performed according to the Guide for Care and Use of Laboratory Animals endorsed by The American Physiological Society and by The Brazilian College of Animal Experimentation (COBEA), and approved by the Animal Ethics Committee of the School of Medicine of ABC (protocol# 002/2009).

Surgical procedures were performed under intraperitoneal ketamine (Dopalen®, Vet brand, Jacarei, SP, Brazil) (50 mg/kg body weight) combined with intramuscular xylazine (Anasedan®, Vetbrand, Jacarei, SP, Brazil) (10 mg/kg body weight).

Rats were placed in a stereotaxic apparatus (Stoelting Co., Wood Dale, IL, USA), where bregma and lambda were positioned at the same horizontal level. A stainless steel guide cannula (12.0 mm × 0.6 mm) was implanted into 4thV using the following coordinates: 12.0 mm caudal to bregma, 0.0 mm lateral and 6.3 mm below the skull surface. The guide cannula was positioned and fixed to the skull bone with dental acrylic cement. At the end of the surgery, rats received a prophylactic injection of Veterinary Pentabiotic (2000 IU, IM, Fort Dodge, Campinas, SP, Brazil).

Three days after brain surgery, polyethylene tubing (PE-10 connected to PE-50, Clay Adams, Parsippany, NJ, USA), filled with heparinized saline (125 IU/mL), were inserted into the femoral artery and vein for measurement of pulsatile arterial pressure (PAP) and administration of drugs, respectively. The free ends of the catheters were tunneled subcutaneously and exteriorized at the back of the animal neck. This surgical procedure was performed under the same anesthetic protocol as described above.

During the experiments, the polyethylene tubing previously inserted into the femoral artery was connected to pressure transducer, and the signal was amplified and digitalized in a BIOPAC data acquisition system (Sta. Barbara, CA, USA). The pressure transducer was located approximately 40 cm above the animal, allowing it to move around without having its arterial line twisted. In addition, the noise in the recording room was kept to a minimum in order to prevent unnecessary stress to the animal. Heart rate (HR) and mean arterial pressure (MAP) were derived on-line from the pulsatile arterial pressure signals using Acqknowledge 3.8.1 for windows software (Biopac Systems, Inc., USA). All experiments were performed in unanesthetized freely moving rats, approximately 24 h after the vessel catheters implantation. Before the experiments, rats were allowed to adapt to the new environment for at least 30 min. A 20–30-min recording period without any interference was allowed to elapse in order to determine baseline MAP and HR values.

Injections into the 4thV were made with a 10 μL Hamilton syringe connected by polyethylene tubing (PE-10) to an injection needle. Injections in the 4thV were made in a volume of 1.0 μL for about 5–10 s. In a group of seven animals, ascorbic acid (Sigma–Aldrich) was used in the concentration of 500 nmol/μL (dissolved in NaCl 0.9% and neutralized with 1 M of NaOH). N-Acetylcysteine (Sigma–Aldrich) was used for treatment in seven animals, in the concentration of 250 nmol/μL (dissolved in NaCl 0.9% and neutralized with 1 M of NaOH). In a control group of five animals, saline (0.9%) was injected in the 4thV before testing baroreceptor reflex.

After basal MAP and HR recordings, seven animals received intravenous injections of phenylephrine (3 μg/kg) or sodium nitroprusside (30 μg/kg) to determine the baroreceptor reflex control responses. At least 15 min later, AAC or NAC, or saline was injected into the 4thV. Afterwards, the baroreflex responses were tested 5, 15, 30 and 60 min after 4thV injection of the drugs. Baroreceptor reflex responses of heart rate were evaluated at the maximum (peak changes) responses.

After the experiments, the animals were deeply anesthetized with sodium thiopental (70 mg/kg of body weight, IV). Afterwards, the rats were perfused with 10% formalin solution. The brain was removed and maintained in the same formalin solution for at least 24 h. After this period, the brains were frozen, coronally cut in 40 μm sections, stained with 2% neutral red solution (Sigma–Aldrich, St. Louis, USA) and analyzed by light microscopy to evaluate the track of the injector through the cerebellum towards the 4th ventricle without trespassing the dorsal surface of the brain stem.

The results were reported as mean ± standard error of mean (S.E.M.). Analyses of variance (ANOVA) for repeated measures followed by the Tukey post test were used. Significance level was set as $p < 0.05$.

The central injection of ascorbic acid did not cause changes in baseline MAP and HR and neither in the pressor dose of PHE. Fig. 1A shows representative tracings illustrating the changes in heart rate in response to PHE (i.v.) before and after AAC injection in 4thV. The reflex bradycardic response to pressor doses of PHE

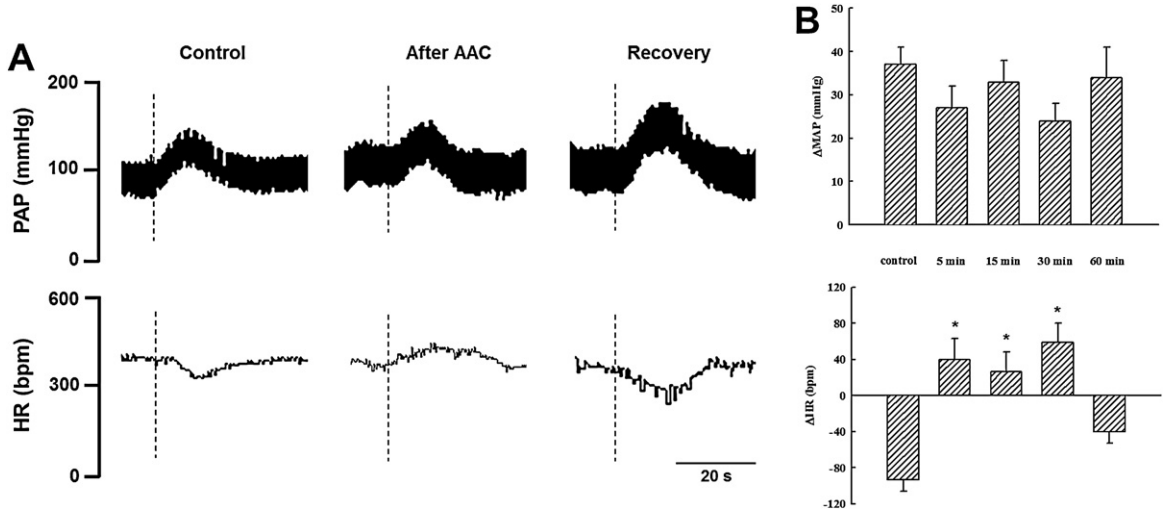

Fig. 1. (A) Representative tracings illustrating the changes in heart rate (HR, bpm) in response to a pressor dose of phenylephrine (PHE, 3 μg/kg, i.v.) before (control) and after ascorbic acid (AAC), and after the recovery period. PAP means pulse arterial pressure. (B) Changes in the mean arterial pressure (ΔMAP, mm Hg) and in the heart rate (ΔHR, bpm) in response to phenylephrine (3 μg/kg, i.v.) before and 5, 15, 30 and 60 min after the microinjection of ascorbic acid (AAC) in the 4th ventricle. * means $p < 0.05$ compared to control.

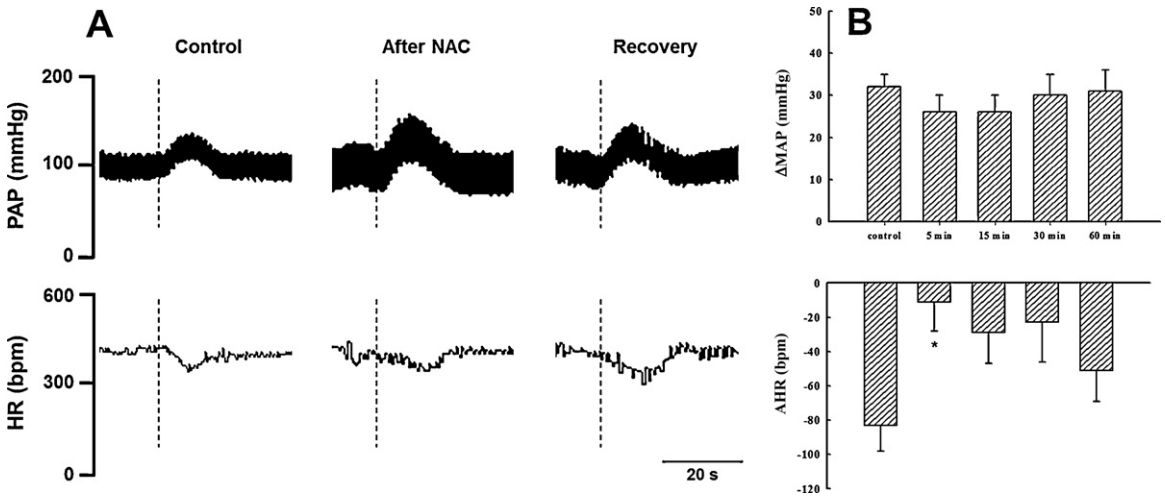

Fig. 2. (A) Representative tracings illustrating the changes in heart rate (HR, bpm) in response to a pressor dose of phenylephrine (PHE, 3 μg/kg, i.v.) before (control) and after N-acetylcysteine (NAC), and after the recovery period. PAP means pulse arterial pressure. (B) Changes in the mean arterial pressure (ΔMAP, mm Hg) and in the heart rate (ΔHR, bpm) in response to phenylephrine (3 μg/kg, i.v.) before and 5, 15, 30 and 60 min after the microinjection of N-acetylcysteine (NAC) in the 4th ventricle. * means $p < 0.05$ compared to control.

(-93 ± 13 bpm) was completely abolished and turned into tachycardia as observed at 5 (40 ± 23 bpm), 15 (26 ± 22 bpm) and 30 (59 ± 21 bpm) min after AAC injection in 4thV (Fig. 1B). No significant changes were observed in tachycardia induced after baroreflex testing with sodium nitroprusside (data not shown).

The central injection of N-acetylcysteine did not cause changes in baseline MAP and HR and neither in the pressor response to PHE. Fig. 2A illustrates the changes in heart rate in response to a pressor dose of PHE (i.v.) before and after NAC injection in 4thV. A significant reduction in the reflex bradycardia was observed at 5 min after the injection N-acetylcysteine compared to control (-11 ± 17 vs. -83 ± 15 bpm, $p < 0.05$) (Fig. 2B).

Furthermore, hypotension and reflex tachycardia elicited by sodium nitroprusside at 5, 15, 30 and 60 min after N-acetylcysteine into the 4thV were not different compared to control responses (data not shown). At the same site of injections saline did not change the reflex bradycardia and tachycardia induced by either PHE or SNP injection, respectively (data not shown).

The main finding of the present study was that central antioxidant administration to the fourth ventricle impaired the parasympathetic component of the cardiac baroreflex in conscious rats. To our knowledge, this is the first study to investigate the effects elicited by the AAC and NAC administered in the fourth ventricle on the parasympathetic component of the cardiac baroreflex. Our results suggest that AAC and NAC may affect central pathways related to parasympathetic component that control blood pressure, indicating that the inhibition of endogenous reactive oxygen species (ROS) might affect the autonomic control of heart rate.

There is compelling experimental evidence that oxidative stress has a role in brain areas involved in the neural control of blood pressure in health and disease [12,13]. For instance, scavenging of superoxide in the subfornical organ or in the rostral ventrolateral medulla prevents angiotensin-II-dependent hypertension [27,16]. In addition, administration of tempol, a superoxide dismutase mimetic, in the RVLM of normotensive rats attenuates the pressor response to peripheral chemoreflex activation [15]. Moreover, overexpression of CuZn superoxide dismutase or inhibition of Rac 1 expression on the nucleus tractus solitarius (NTS) reduces blood pressure, heart rate and urinary norepinephrine excretion in spontaneously hypertensive stroke prone rats [14]. Interestingly, administration of hydrogen peroxide in the NTS induces hypotension and bradycardia, being the bradycardia inhibited by pretreatment with either AAC or kynurenate, suggesting that free radical may activate glutamatergic pathways in the NTS [4].

Our data showed that the baroreflex-mediated bradycardia induced by PHE was completely abolished after 4thV treatment with AAC and attenuated after NAC. These findings are in accordance with those data from Cardoso et al. [4]. Therefore, scavenging of endogenous ROS in the NTS could affect glutamatergic neurotransmission and reduce the baroreflex-mediated bradycardia, since the central processing of the baroreflex is dependent on glutamatergic synapses within the NTS [23]. In addition to the NTS, we cannot rule out the possibility that AAC or NAC could be acting in other brain areas involved in the processing of the baroreflex-mediated bradycardia such as the nucleus ambiguous (NA) by its diffusible molecules (i.e. dehydroascorbic acid). The NA is known for holding cardiac premotor vagal neurons. Administration of sodium nitroprusside or l-arginine in the NA decreases heart rate *in vivo* [20], probably by increasing gabaergic and inhibiting glutamatergic currents as demonstrated by electrophysiological studies using brain slices [10]. Therefore, scavenging superoxide would lead to an increase in the bioavailability of nitric oxide in NA, resulting in inhibition of the parasympathetic neurons in response to baroreflex activation. Although an interesting possibility, this is still matter for further investigation.

We also observed tachycardia after PHE injection at 5, 15 and 30 min after treatment with AAC. This tachycardia could be explained by a direct activation of alpha-1 receptors on the sinoatrial node. This possibility is corroborated by a study performed by Saitoh et al. [21] where authors demonstrated that, under autonomic blockade with atropine and propranolol, administration of phenylephrine induces tachycardia in humans by its action on alpha-1 adrenergic receptors. In addition to its possible effects on alpha-1 receptors in the heart, PHE is also known for having little beta-agonistic action, which could partially explain its positive chronotropism. Another possibility for the PHE-mediated tachycardia observed in our experiments is that PHE could lead to an increase in automaticity caused by mechanical stretching as suggested by Jose [9].

Regarding the treatment with NAC, we observed that at 5 min after treatment the reflex bradycardia was reduced. The reduction in the baroreflex-mediated bradycardia by NAC injected within the brain is different from what is described regarding its effects in the periphery [1,6,7]. For instance, Girouard et al. [7] described that chronic peripheral treatment with NAC given in the drinking

water over 4 weeks reduces blood pressure and heart rate in spontaneously hypertensive rats (SHR). In addition, in the same study the authors described that NAC improved baroreflex-mediated bradycardia and tachycardia, and normalized glutathione peroxidase activity in plasma of SHR, but not in normotensive animals. In our study, administration of NAC in the 4thV reduced the baroreflex-mediated bradycardia in normotensive rats. Whether administration on NAC in the 4thV would differentially affect the baroreflex responses in hypertensive animals is still matter for further investigation.

In summary, our results suggest a possible role for endogenous ROS involved in the release of neurotransmitters responsible for cardiovascular modulation in the brain stem. Indeed, central treatment with antioxidants can inhibit the action of endogenous ROS and, consequently, the release of glutamate that would explain the blockade of the reflex bradycardia. Despite the actions of reactive oxygen species on neurotransmitters dynamics and their behavior related to neural modulation of blood pressure, our results can be supported by previous studies showing that ROS might stimulate autonomic discharges, probably through release of neurotransmitters or causing some delay in their reuptake, as occurs with glutamate [26]. They can also promote an imbalance in the central nitrergic pathways (related to the availability of NO^-), involved in baroreflex control in the NTS as well as in the NA [20,10,24,8].

We conclude that antioxidant therapy may act centrally in areas responsible for parasympathetic modulation of baroreceptors reflex.

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