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This information is current as of October 23, 2012.
Cardiovascular responses to hydrogen peroxide into the nucleus tractus solitarius

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1Department of Physiology, Federal University of São Paulo, São Paulo-SP, Brazil; 2Department of Physiology and Pathology, São Paulo State University, Araraquara-SP, Brazil; 3Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas; and 4Department of Biological Sciences, Federal University of Ouro Preto, Ouro Preto-MG, Brazil

Cardoso LM, Colombari DSA, Menani JV, Toney GM, Chianca Jr. DA, Colombari E. Cardiovascular responses to hydrogen peroxide into the nucleus tractus solitarius. Am J Physiol Regul Integr Comp Physiol 297: R462–R469, 2009. First published June 10, 2009; doi:10.1152/ajpregu.90796.2008.—The nucleus tractus solitarius (NTS), a major hindbrain area involved in cardiovascular regulation, receives primary afferent fibers from peripheral baroreceptors and chemoreceptors. Hydrogen peroxide (H2O2) is a relatively stable and diffusible reactive oxygen species (ROS), which acting centrally, may affect neural mechanisms. In the present study, we investigated effects of H2O2 alone or combined with the glutamatergic antagonist kynurenate into the NTS on mean arterial pressure (MAP) and heart rate (HR). Conscious or anesthetized (urethane and α-chloralose) male Holtzman rats (280–320 g) were used. Injections of H2O2 (125 to 1500 pmol/40 nl) into the intermediate NTS of anesthetized rats evoked dose-dependent and transient hypotension (−18 ± 3 to −55 ± 11 mmHg) and bradycardia (−16 ± 5 to −116 ± 40 bpm). Injection of the catalase inhibitor 3-amino-1,2,4-triazole (100 nmol/40 nl) into the NTS also produced hypotension and bradycardia. Previous injection of the ionotropic L-glutamate receptor antagonist kynurenate (7 nmol/40 nl) attenuated by 48% the bradycardic response, without changing the hypotension evoked by H2O2 (500 pmol/40 nl) in anesthetized rats. The antioxidant L-ascorbate (600 pmol/80 nl) injected into the NTS attenuated the bradycardiac (42%) and hypotensive (67%) responses to H2O2 (500 pmol/40 nl) into the NTS. In conscious rats, injection of H2O2 (50 nmol/100 nl) into the NTS also evoked intense bradycardia (−207 ± 8 bpm) and hypotension (−54 ± 6 mmHg) that were abolished by prior injection of kynurenate (7 nmol/100 nl). The results show that H2O2 into the NTS induces hypotension and bradycardia probably due to activation of glutamatergic mechanisms.

reactive oxygen species; catalase inhibition; kynurenic acid; blood pressure; heart rate
tigated yet. In this study, we investigated whether increasing H$_2$O$_2$ levels in the NTS acutely would affect arterial blood pressure and heart rate and whether these effects were linked to activation of ionotropic glutamate receptors in the same area.

**MATERIALS AND METHODS**

**Animals**

Male Holtzman rats (280 to 320 g, n = 27) from the main breeding stock of the Dentistry School-São Paulo State University animal facility were used. Animals were housed in individual cages in a room with controlled temperature (22 ± 3°C) and humidity (40 to 60%) and received rat chow (Guabi Rat Chow, Paulinia, SP, Brazil) and water ad libitum. Lights were on from 7 AM to 7 PM. All experiments were done in accordance with the Brazilian Society for Neuroscience and Behavior Guidelines for Animal Experimentation and had the approval of the institutional animal care and use committee of the Federal University of São Paulo/Escola Paulista de Medicina.

**Drugs**

Hydrogen peroxide (H$_2$O$_2$) was prepared in saline 0.9% (vehicle) from a stock solution (30% wt/wt; Sigma, St. Louis, MO) to final concentrations ranging from 3.1 to 500 mmol/l. Ascorbic acid and kynurenic acid (Sigma) were neutralized with sodium bicarbonate (1 mol/l) and diluted in vehicle to the desired final concentrations.

**Animal Instrumentation**

For experiments conducted in anesthetized rats, femoral artery and vein polyethylene cannulas (PE-10 connected to PE-50; Clay Adams, Parsippany, NJ) were implanted in animals anesthetized with ketamine (80 mg/kg) and xylazine (7 mg/kg) delivered by intraperitoneal injection. The free end of each catheter was tunneled subcutaneously and exteriorized at the back of the neck.

After a recovery period of 48 h, the arterial cannula was connected to a Statham-Gould pressure transducer. The transducer signal was amplified and directed to an analog-to-digital converter for data acquisition (PowerLab 16Sp; ADInstruments, Sydney, Australia). Data were digitized at 1,000 Hz. Heart rate (HR) and mean arterial pressure (MAP) were derived online from the pulsatile arterial pressure signal with Chart 5 for Windows software (ADInstruments).

For experiments conducted in conscious rats, stainless-steel guide cannulas directed to the NTS were implanted in rats anesthetized with ketamine (80 mg/kg ip) combined with xylazine (7 mg/kg ip) (Cristália Produtos Químicos e Farmacêuticos, Itapira, SP, Brazil). Rats were placed in a Stoelting stereotaxic instrument, and an incision was made through the skin to expose bregma and lambda that were positioned at the same horizontal plane. A stainless-steel cannula (14.0 mm × 0.6 mm OD) was implanted so that its tip was located 14.5 mm caudal and 7.5 mm ventral to bregma and 0.5 mm lateral to the midline. Two jeweler screws were implanted in the skull, and the cannula was anchored to the screws with acrylic cement. At the end of the surgery, rats received an intramuscular injection with 30,000 IU of penicillin (Fort Dodge Saúde Animal, Campinas, SP, Brazil) and were housed in individual cages with chow and water ad libitum.

Three days after stereotaxic surgery, also under ketamine/xylazine anesthesia, PE cannulas (PE-10 connected to PE-50, Clay Adams) filled with heparinized saline (125 IU/ml) were inserted into the aorta through the right femoral artery for measurement of pulsatile arterial pressure, MAP, and HR, according to procedures indicated above.

**Experimental Protocols in Anesthetized Rats**

After an initial recording of MAP and HR performed in freely moving conscious rats, anesthesia was induced and maintained with a mixture of urethane (830 mg/kg) plus α-chloralose (55 mg/kg iv) through the venous line. The level of anesthesia was adjusted for each animal to completely inhibit corneal and withdraw reflexes. Rats were tracheotomized to facilitate spontaneous respiration. The rats were then placed in a stereotaxic instrument (David Kopf), and a longitudinal incision was performed on the dorsal part of the neck between the occipital and the second vertebrae. The muscle was retracted so that the occipital bone and the atlanto-occipital membrane were easily visualized. Part of the atlanto-occipital membrane was removed, and a partial occipital craniotomy was performed. After removing the pia-mater, the dorsal surface of the medulla was completely exposed, allowing clear visualization of the area postrema and easy access to the injection site in the NTS. Injections were performed 0.50 mm rostral and 0.40 to 0.50 mm lateral to callamus scriptorius, and 0.40 to 0.50 mm ventral to the medulla surface using a glass micropipette coupled to a nitrogen pressure injection system (Picospitzer).

**Dose-response curve for H$_2$O$_2$.** In a group of seven rats, after arterial pressure and HR stabilized, vehicle and L-glutamate (500 pmol) were injected (40 nl) unilaterally into the NTS. Thereafter, different doses of H$_2$O$_2$ (125, 250, 500, 1000, and 1,500 pmol/40 nl/rat) were randomly injected into the same site. The interval separating each injection was 10 min.

**Catalase inhibition in the NTS.** To test whether endogenously generated H$_2$O$_2$ could produce cardiovascular responses similar to those produced by exogenous H$_2$O$_2$, the catalase inhibitor 3-amino-1,2,4-triazole (ATZ) was injected (100 nmol/40 nl) unilaterally into the NTS of another group of rats (n = 4). The dose of ATZ was selected based on published data, indicating the tissue concentration of ATZ needed to inhibit up to 95% of brain catalase activity (5). Injections of vehicle were performed prior to injections of ATZ.

**Effects of H$_2$O$_2$ after blockade of ionotropic glutamate receptors in the NTS.** To determine whether blood pressure and HR effects of H$_2$O$_2$ in the NTS were linked to changes in glutamatergic transmission, the effect of H$_2$O$_2$ (500 pmol/40 nl) was tested again, after injection of the ionotropic glutamate receptor antagonist kynurenate (7 nmol/40 nl) in another group of rats (n = 7). Injections of H$_2$O$_2$ were performed 15 min before and after injection of kynurenate into the same site.

**Effects of H$_2$O$_2$ after local injection of L-ascorbate.** To confirm that effects of H$_2$O$_2$ in the NTS were linked to its oxidant action, H$_2$O$_2$ (500 pmol/40 nl) was injected 15 min before and 20 min after injection of the endogenous antioxidant L-ascorbate (600 pmol/80 nl) in another group of rats (n = 6).

**Experimental Protocols in Conscious Rats**

Experiments were performed in unanesthetized freely moving rats, ~48 h after cannulation surgery. To minimize any impact of stress on experimental outcomes, rats were allowed to stabilize for a period of at least 1 h before experiments started.

Unilateral injections into the NTS were made with a 5-μl Hamilton syringe connected by PE-10 tube to an injector cannula made of 32-gauge stainless-steel tubing. The injector, when fully inserted, protruded 2 mm beyond the tip of the guide cannula. Injections of 100 nl each were performed over a period of 5 to 10 s.

**Effects of H$_2$O$_2$ before and after local blockade of glutamate receptors in the NTS of conscious rats.** Effects of H$_2$O$_2$ (50 nmol/100 nl) in the NTS of conscious freely moving rats was tested after the injection site was identified as a site from which injection of L-glutamate produced a robust decrease in mean arterial pressure and HR (20). After site identification, H$_2$O$_2$ (50 nmol/100 nl) was injected into the NTS. Cardiovascular responses to H$_2$O$_2$ were then tested again 5 and 60 min after the injection of kynurenate (7 nmol/100 nl) into the same site. To confirm the efficacy of kynurenate, L-glutamate was injected into the same site 10 min later (i.e., 5 min after injection of H$_2$O$_2$).

**Histology**

At the end of each experiment, injection sites were marked with 2% Evan’s Blue solution (40 to 100 nl, depending on the protocol). Immediately after the injection, animals were deeply anesthetized.
with sodium thiopental (70 mg/kg of body wt iv) and perfused through the heart with 0.9% NaCl (30 ml) followed by 10% buffered formalin (300 ml). Brains were removed and kept in 10% buffered formalin solution for at least 24 h. Coronal slices of 50 μm were cut on a freezing microtome, mounted on glass slides, stained with Neutral red, and viewed under light microscopy to evaluate the location and distribution of dye at the injection site. If the site encompassed the NTS, the histology was considered positive, and the results from those animals were used for data analysis.

Data Analysis

Data analysis typically consisted of measuring the maximal change in MAP and HR, taking as baseline 20 s of recording just before each NTS microinjection. Results are reported as means ± SE. ANOVA for repeated measures followed by post hoc pairwise multiple comparisons Student-Newman-Keuls test, or paired t-tests were used. Dose-response curves were determined by a nonlinear fit using a sigmoidal dose-response curve with a variable slope. All data were statistically analyzed using Prism software version 5.00 (GraphPad Software, San Diego CA). Differences were considered significant when the probability of a Type I error was less than 5% (P < 0.05).

RESULTS

Cardiovascular Responses to H₂O₂ Injected Into the NTS in Anesthetized Rats

Urethane combined with α-chloralose anesthesia did not affect baseline MAP (126 ± 2 mmHg) vs. preanesthesia (122 ± 2 mmHg, n = 24) but produced a small bradycardia (361 ± 7 bpm) vs. preanesthesia (387 ± 10 bpm, paired t-test, P < 0.05).

The plot of the changes in MAP × log dose H₂O₂ showed a sigmoid dose-dependent pattern with a maximum hypertensive response of −56 ± 13 mmHg and a slope of −3.2 mmHg/pmol. The ED₅₀ was 525 pmol, and the E₉₀ was −18 ± 10 mmHg pmol. The plot of the changes in HR × log dose H₂O₂ showed a dose-dependent transient bradycardia without a plateau (Fig. 1).

Injections of H₂O₂ (500, 1,000, and 1,500 pmol/40 nl) into the intermediate NTS produced depressor responses (−35 ± 5, −52 ± 10, and −55 ± 11 mmHg, respectively, vs. vehicle: H₂O₂ (1,000 and 1,500 pmol/40 nl) into the NTS also produced bradycardia (−81 ± 23 and −116 ± 40 bpm, respectively, vs. vehicle: −5 ± 2 bpm) (Fig. 1). Hypotensive and bradycardic responses were transient, reaching a peak within 20 s, with full recovery of baseline MAP and HR levels within 30 to 90 s.

The changes in MAP produced by H₂O₂ at the doses of 125 and 250 pmol/40 nl into the intermediate NTS (−18 ± 3 and −22 ± 4 mmHg, respectively), and the changes in HR produced by H₂O₂ at the doses of 125, 250, and 500 pmol/40 nl (−16 ± 5, −30 ± 7, and −47 ± 12 bpm, respectively) did not reach statistical significance compared with vehicle (Fig. 1).

Fig. 1. Changes in arterial blood pressure (ABP) and heart rate (HR) produced by H₂O₂ injected into the intermediate nucleus tractus solitarius (NTS) of anesthetized rats. A: ABP and HR traces from an animal randomly injected with vehicle or H₂O₂ (125, 250, 500, 1,000, and 1,500 pmol/40 nl) into the intermediate NTS. B: nonlinear regression curve showing changes in mean arterial pressure (ΔMAP). C: changes in heart rate (ΔHR) induced by H₂O₂ injected into the intermediate NTS. Insets show MAP and HR responses evoked by injections of L-glutamate (500 pmol) at the same site. D: photomicrographs showing the location of H₂O₂ injections sites into the intermediate NTS (arrows). AP, area postrema. Dashed vertical lines in A show the moment of injection into the NTS. Results in B and C are expressed as means ± SE. Vehicle, n = 6 and H₂O₂, n = 4–7.
To confirm that injections reached sites into intermediate NTS that produce cardiovascular effects similar to those previously reported (25), before the injections of H2O2, rats received injections of L-glutamate into the intermediate NTS. Consistent with results from previous studies, injection of L-glutamate (500 pmol/40 nl) produced hypotension (−41 ± 7 mmHg) and bradycardia (−42 ± 5 bpm).

The photomicrographs in Fig. 1D show a typical injection site into the intermediate NTS in a rat representative of the groups studied.

Cardiovascular Responses Produced by ATZ Injection in the NTS of Anesthetized Rats

The catalase inhibitor ATZ was injected into the NTS to evaluate changes in MAP and HR evoked by accumulation of H2O2 produced endogenously.

The injection of ATZ (100 nmol/40 nl) also produced hypotensive (−37 ± 10 mmHg vs. vehicle: −6 ± 2 mmHg, t-test, P < 0.05, n = 4) and bradycardic (−23 ± 6 bpm vs. vehicle: −5 ± 1 bpm, t-test, P < 0.05, n = 4) responses (Fig. 2). These responses had a slower onset compared with those resulting from H2O2 injection, reaching their peaks within 2 to 3 min after ATZ injection and lasting for 5 to 6 min before fully returning to baseline (Fig. 2).

Effects of Ionotropic Glutamate Receptors Blockade in the NTS on H2O2-Evoked Cardiovascular Responses in Anesthetized Rats

To assess the role of ionotropic glutamate receptors in mediating cardiovascular effects of H2O2 in the NTS, the effects of H2O2 into the intermediate NTS was tested 10 min before and 10 and 60 min after injection of the ionotropic glutamate receptor antagonist kynurenate into the same site.

Prior injection of kynurenate (7 nmol/40 nl) into the NTS reduced the bradycardic response to H2O2 (500 pmol) by about 48% (−31 ± 5 bpm before vs. −16 ± 2 bpm 10 min after; n = 7, paired t-test, P < 0.05) but had no effect on the hypotension (−39 ± 5 mmHg before vs. −36 ± 4 mmHg 10 min after; paired t-test, P > 0.5) (Fig. 3). A normal bradycardic response was produced by H2O2 into the NTS 60 min after kynurenate injection (−52 ± 14 bpm), an indication that the attenuation of the H2O2-evoked reduction in HR was not due to local tissue damage by the injected volume. H2O2-evoked changes in MAP remained unaltered 60 min after kynurenate (−35 ± 7 mmHg).

Cardiovascular Effects of H2O2 Alone or Combined with the Blockade of Glutamate Receptors in the NTS of Conscious Rats

Baseline MAP and HR of freely moving conscious rats were 106 ± 2 mmHg and 362 ± 13 bpm, respectively. Injection of H2O2 (50 nmol/100 nl) into the intermediate NTS of conscious rats evoked an intense bradycardia (−208 ± 19 bpm, n = 5) and an initial hypotension (−55 ± 7 mmHg, n = 5) that reached the peak within 5 s and returned to baseline within 30 s (Fig. 3).

Prior (5 min) injection of kynurenate (7 nmol/100 nl) into the NTS completely blocked HR and MAP responses evoked by H2O2 in the same site (−13 ± 4 bpm and −2 ± 2 mmHg, respectively) (Fig. 3).

The pressor and bradycardic responses to H2O2 injected into the NTS 60 min after kynurenate (−60 ± 4 mmHg and −169 ± 14 bpm, respectively) were not different from the responses before kynurenate (−55 ± 7 mmHg and −208 ± 19 bpm, respectively) (Fig. 3). Injections of L-ascorbate (1 nmol/100 nl) into the intermediate NTS of conscious rats also produced hypotension (−52 ± 5 mmHg) and a bradycardic response (−206 ± 27 bpm), as did the H2O2 injection.

Effects of L-Ascorbate in the NTS on Cardiovascular Responses to H2O2 in Anesthetized Rats

To test whether effects of H2O2 into the NTS on MAP and HR could be prevented by antioxidant treatment, responses to injection of H2O2 (500 pmol/40 nl) were recorded 15 min before and 20 min after injection of pH neutral L-ascorbate (600 pmol/80 nl) into the same site.

The treatment with L-ascorbate reduced depressor (−48 ± 5 mmHg before vs. −16 ± 3 mmHg 20 min after; n = 6, paired t-test, P < 0.05) and bradycardic (−55 ± 16 bpm before vs. −32 ± 13 bpm 20 min after, paired t-test, P < 0.05) responses to H2O2 injection into the NTS (Fig. 4). Injection of L-ascorbate produced a transient fall in MAP (−24 ± 6 mmHg

Fig. 2. Changes in arterial blood pressure and heart rate produced by 3-amino-1,2,4-triazole (ATZ) injected into the intermediate NTS of anesthetized rats. A: ABP and HR traces from an animal treated with ATZ (100 nmol/40 nl) into the intermediate NTS. Changes in mean arterial pressure (ΔMAP) (B) changes in mean arterial pressure (ΔMAP) (C) induced by ATZ or vehicle injected into the intermediate NTS. Dashed vertical lines in A show the moment of injection into the NTS. Results in B and C are expressed as means ± SE. *Significantly different from vehicle (P < 0.05; unpaired t-test); n = 4.
DISCUSSION

The present results show that changes in the redox state of the intermediate NTS produced by H\textsubscript{2}O\textsubscript{2} delivery or catalase inhibition by ATZ evoked transient hypotensive and bradycardic responses in conscious or anesthetized rats, likely as a result of activation of baroreflex circuitry within the NTS, which may change efferent autonomic activity to the cardiovascular system. Except for the hypotension in anesthetized rats, these responses were attenuated by blocking local ionotropic glutamate receptors with kynurenate. The hypotension and bradycardia produced by H\textsubscript{2}O\textsubscript{2} in the NTS were also reduced by local pretreatment with the antioxidant L-ascorbate, which suggests that cardiovascular effects depend on oxidative modifications produced by H\textsubscript{2}O\textsubscript{2} within the NTS.

Although cellular mechanisms were not addressed in the present study, the results suggest that increased levels of H\textsubscript{2}O\textsubscript{2} in the NTS have a net effect on the autonomic nervous system that is strong enough to change MAP and HR. The fact that injections of H\textsubscript{2}O\textsubscript{2} evoked transient responses with blood pressure and heart rate returning to preinjection levels in a relative short time (~1 to 2 min) suggests that whatever the underlying cellular mechanisms might be, they do not appear to cause either permanent oxidative modifications or cell damage. Moreover, responses to low doses of H\textsubscript{2}O\textsubscript{2} were also
Both bradycardic and hypotensive responses to H$_2$O$_2$ were attenuated by previous injection of L-ascorbate into the NTS. This indicates that effects of H$_2$O$_2$ are likely dependent on oxidative processes rather than nonspecific effects, such as an increase in O$_2$ levels secondary to H$_2$O$_2$ degradation. L-ascorbate is normally present in the cerebrospinal fluid at concentrations ranging from 0.2 to 0.4 mM (7, 22, 26) and can scavenge stable (H$_2$O$_2$), as well as more labile (O$_2$•$^-$ and HO$^\cdot$) ROS (26). Attenuation of H$_2$O$_2$-evoked responses following L-ascorbate injection may involve greater H$_2$O$_2$ scavenging or conversion of molecular targets oxidized by H$_2$O$_2$ to a reduced form, or both. However, L-ascorbate alone into the NTS also reduced MAP, suggesting that molecular mechanisms involved in the effects of L-ascorbate need further investigation.

Injection of H$_2$O$_2$ into the NTS produced a greater fall in HR in conscious freely moving rats than in anesthetized rats, whereas hypotensive responses were similar. Injection of L-glutamate at the same site also produced robust bradycardic and depressor responses. The pattern of blood pressure change in response to L-glutamate in conscious rats is not consistent with results from previous studies, which showed pressor responses to L-glutamate injections in the NTS of conscious rats (12, 20). Perhaps the reason for these differences is the more rostral position of injection sites in the present study that may activate mainly baroreflex-related neurons that produces hypotension and bradycardia.

Bradycardia and hypotension evoked by H$_2$O$_2$ into the NTS resemble cardiovascular responses to baroreflex activation or those induced by L-glutamate injected into the intermediate NTS in anesthetized rats (12, 19, 20, 33). L-glutamate is the main excitatory amino acid in the central nervous system and is the major excitatory transmitter released by visceral afferent input to NTS (12, 19, 20, 30, 32, 33). The bradycardic and hypotensive responses to H$_2$O$_2$ into the NTS in conscious rats and the bradycardic response to H$_2$O$_2$ into the NTS in anesthetized rats were abolished by pretreatment with kynurenate into the NTS, indicating that the effects of H$_2$O$_2$ in the NTS might depend on ionotropic glutamatergic receptor activation. The recovery of the H$_2$O$_2$-evoked responses after the usual time required for clearance of kynurenate from the tissue refutes the possibility that effects of kynurenate could be an indirect effect of tissue damage. However, H$_2$O$_2$-evoked hypertension in anesthetized rats was not affected by kynurenate injection into the NTS, which suggests that hypotension to H$_2$O$_2$ into the NTS in this situation is not dependent on the activation of ionotropic L-glutamate receptors. Clearly, this is a controversial result but is similar to reports in the literature showing that kynurenate was not effective at inhibiting the bradycardic or hypotensive response to L-glutamate in the NTS of anesthetized rats (19, 31). This suggests that kynurenate-sensitive L-glutamate receptors might not be the only L-glutamate receptors that participate in control of the blood pressure in the NTS, at least during anesthesia. Activation of metabotropic L-glutamate receptors in the NTS also induce hypotension in anesthetized animals (15, 19, 24). Therefore, the hypertensive responses to H$_2$O$_2$ in anesthetized rats may depend on activation of this class of receptors or another mechanism not investigated in the present study. Because kynurenate abolished bradycardia in response to H$_2$O$_2$ in the NTS, the remaining hypotension is probably the result of withdrawal of sympathetic tone to the vasculature. Further studies are necessary.
REFERENCES


