Neuroscience 121 (2003) 641–647

HYDROGEN PEROXIDE INCREASES THE ACTIVITY OF RAT SYMPATHETIC PREGANGLIONIC NEURONS *IN VIVO* AND *IN VITRO*

H. H. LIN,[a] C.-H. CHEN,[b] W.-K. HSIEH,[b] T. H. CHIU[b] AND C.-C. LAI[b]*

[a]*Department of Nursing, Tzu Chi College of Technology, 880 Section 2, Chien-Kuo Road, 970 Hualien, Taiwan*

[b]*Department of Pharmacology, Tzu Chi University, 701 Section 3, Chung-Yang Road, 970 Hualien, Taiwan*

Abstract—Reactive oxygen species (ROS) have been shown to modulate neuronal synaptic transmission and have also been implicated in cardiovascular diseases such as hypertension. The hypothesis that H_2O_2 acting on sympathetic preganglionic neurons (SPNs) affects spinal sympathetic outflow was tested in the present study. H_2O_2 was applied intrathecally via an implanted cannula to the T7–T9 segments of urethane-anesthetized rats. Blood pressure and heart rate were used as indices to evaluate the spinal sympathetic effects of H_2O_2 *in vivo*. Intrathecal H_2O_2 (100–1000 nmol) dose-dependently increased both the mean arterial pressure and heart rate. Reproducible pressor effects of H_2O_2 (1000 nmol) applied consecutively at intervals of 30 min were observed. The pressor effects of intrathecal H_2O_2 (1000 nmol) were attenuated by pretreatment with intrathecal administration of catalase (500 units), or *N*-acetyl-cysteine (1000 nmol). The pressor effects of intrathecal H_2O_2 (1000 nmol) were also antagonized dose-dependently by prior intrathecal injection of AP-5 (DL-2-amino-5- phosphonovaleric acid, 10 and 30 nmol), or 6-cyano-7- nitroquinoxaline-2,3-dione, 10 and 30 nmol. *In vitro* electrophysiological study in spinal cord slices showed that superfusion of 1 mM H_2O_2 for 3 min, which had no effect on membrane potential, caused an increase in amplitude of excitatory postsynaptic potentials in SPNs, but had little effect on that of inhibitory postsynaptic potentials. Taken together, these results demonstrated that oxidative stress in spinal cord may cause an increase in spinal sympathetic tone by acting on SPNs, which may contribute to ROS-induced cardiovascular dysfunction.

Key words: sympathetic nervous system, cardiovascular diseases, spinal cord, reactive oxygen species, electrophysiology, intrathecal.

Reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radicals are potentially cytotoxic. In addition, considerable evidence suggests that these species are able to act as cellular signaling molecules to regulate biological function (Adler et al., 1999). Oxidative stress results when ROS are not adequately removed. In addition to endogenous sources, xenobiotics with the ability to produce electrophilic metabolites may increase cellular oxidative stress. Elevated oxidative stress demonstrated in many disease states and the alleviation of the symptoms by antioxidants suggest the involvement of ROS in these diseases. The potentially pathological role of oxidative stress in cardiovascular diseases has been well documented (Dhalla et al., 2000). Treatment with ascorbic acid may lower blood pressure in hypertensive patients (Duffy et al., 1999). Alteration of the functions in endothelial cells and vascular smooth muscle by oxidative stress may be an important mechanism involved in ROS-induced cardiovascular diseases (Cai and Harrison, 2000; Irani, 2000). On the other hand, based on the examination of microinjection of superoxide dismutase into the rostral ventrolateral medulla (RVLM) of pigs treated with organic nitrate to induce oxidative stress, a recent study indicated indirectly that oxidative stress may exert excitatory actions on neurons in RVLM, which may disturb central sympathetic outflow and the cardiovascular function controlled by RVLM (Zanzinger and Czachurski, 2000). The effects of ROS such as H_2O_2 on neuronal synaptic transmission have been studied extensively in hippocampus. Electrophysiological studies indicated that H_2O_2 caused a reversible inhibition of field synaptic potentials and population spikes recorded in CA1 pyramidal cell by Schaffer collateral stimulation (Pellmar, 1995; Fowler, 1997; Avshalumov et al., 2000). However, augmentation of population spikes and field excitatory postsynaptic potentials have also been reported (Katsuki et al., 1997). Neurochemical studies showed an increase in the release of glutamate by ROS in cultured cortical neurons and cerebellar granule neurons as well as in hippocampal slices (Pellegrini-Giampietro et al., 1988; Satoh et al., 1998; Mailly et al., 1999). However, in cerebrocortical synaptosome preparations, H_2O_2 was found to inhibit Ca^{2+}-dependent exocytosis of glutamate (Zoccarato et al., 1995). Thus, the effects of ROS on neuronal synaptic transmission or the release of glutamate were variable.

Central sympathetic activity is an important factor in the regulation of cardiovascular function. Sympathetic preganglionic neurons (SPNs), located in thoracolumbar spinal cord, are the only link between central sympathetic output and the peripheral ganglia. Glutamate, GABA, and glycine have been shown to be the fast synaptic transmitters mediating the activity of SPNs (Inokuchi et al., 1992a,b). Both non-*N*-methyl-D-aspartic acid (non-NMDA)

*Corresponding author. Tel: +88-63-856-5301x7204; fax: +88-63-846-1220.
E-mail address: cclai@mail.tcu.edu.tw (C.-C. Lai).

Abbreviations: AP-5, DL-2-amino-5-phosphonovaleric acid; BPM, beats per minute; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EPSP, excitatory postsynaptic potential; HR, heart rate; IPSP, inhibitory postsynaptic potential; I.T., intrathecal; MAP, mean arterial pressure; NAC, *N*-acetyl-L-cysteine; NMDA, *N*-methyl-D-aspartic acid; ROS, reactive oxygen species; RVLM, rostral ventrolateral medulla; SPN, sympathetic preganglionic neuron; X, xanthine; XO, xanthine oxidase.

0306-4522/03$30.00+0.00
doi:10.1016/S0306-4522(03)00517-7

and NMDA receptors in SPNs mediated the excitatory action of glutamate (Inokuchi et al., 1992b). In contrast to brain, spinal cord is not protected by blood-tissue barriers. Thus, neurons in the spinal cord may be more liable to the action of exogenous agents with the ability to increase oxidative stress. In addition, many conditions such as ischemia-reperfusion, trauma, and infection may cause an increase in oxidative stress (Halliwell and Gutteridge, 1999). Spinal cord injury has been shown to increase the production of superoxide and H_2O_2, which may be involved in secondary spinal cord damage (Liu et al., 1998, 1999). Whether oxidative stress may affect the activity of SPNs and the underlying sympathetic activity remains unclear. Because the modulation of neuronal electrophysiological properties by ROS and the crucial role of oxidative stress in cardiovascular diseases such as hypertension have been suggested by previous studies, we hypothesized that ROS may affect the activities of SPNs and the underlying sympathetic outflow. To test the hypothesis, the present study was undertaken to (1) examine the effect of H_2O_2 applied intrathecally on spinal sympathetic outflow, using blood pressure and heart rate (HR) as indices, and (2) evaluate the electrophysiological effects of H_2O_2 on SPNs *in vitro*.

EXPERIMENTAL PROCEDURES

Animals

A breeding colony of Sprague–Dawley rats purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) was established at the Laboratory Animal Center, Tzu Chi University. Animals were housed two per cage in a room maintained at 22±1 °C with an alternating 12-h light/dark cycle. Food and water were available *ad libitum*. Rats at desired body weight and age were selected from the colony for use in the present study. All procedures were carried out in accordance with the guidelines of Institutional Animal Care and Use Committee of Tzu Chi University. Efforts were made to minimize both the suffering and the number of animals used.

In vivo experiments

Procedures for intrathecal (I.T.) administration to anesthetized rats were similar to those described earlier (Lai et al., 2000, 2002). Adult male rats weighing 250–350 g were used in this series of experiments. Under urethane anesthesia (1.2–1.5 g kg^{-1}, i.p.), the left femoral artery was cannulated with a polyethylene tubing (PE 50) and connected to a pressure transducer with its output to a Grass pen recorder (Model 7400) for recording of blood pressure. The mean arterial pressure (MAP) was calculated using $[(S-D)/3+D]$, where S indicates systolic blood pressure and D indicates diastolic blood pressure. The beat-by-beat heart rate (HR) were provided by built-in tachygraph function of the recorder from electrocardiogram (ECG) signals (three lead). The right femoral vein was cannulated for i.v. injection of hexamethonium. Rats were mounted in a stereotaxic header and implanted with a spinal catheter for I.T. injection. The spinal catheter is made of a length of PE-10 tubing. A slit was made in the atlanto-occipital membrane and the catheter was inserted down into the spinal subarachnoid space so that the tip was placed in the vicinity of T7–T9 segment; the position of the tubing was visually verified at the end of the experiment. Hydrogen peroxide and other agents at known concentrations were dissolved in saline and injected intrathecally at a volume of 10 μl, which was followed by 10 μl saline to wash in the agents. Results were reported as change in MAP and HR or percentage of maximal change in MAP and HR relative to the basal value prior to I.T. injection of various agents.

Whole-cell recording techniques

Immature (8–12 days old) rats were used in this series of experiments. Procedures used in obtaining 500 μm transverse spinal cord slices of T6–T10 segments were similar to those described previously (Lai et al., 2000, 2002). The spinal cord slice placed in recording chamber (RC-22, Warner Instrument Corporation, Hamden, CT, USA) was continuously perfused with a Krebs solution of the following composition (in mM) 117 NaCl, 2.0 KCl, 1.2 KH_2PO_4, 2.3 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, and 10 glucose; the solution was saturated with 95% O_2 and 5% CO_2. The flow rate of Krebs solution was kept constant at 2–3 ml/min. Whole-cell recordings were made from antidromically identified SPNs under current-clamp mode with the use of an Axoclamp 2B. Patch electrodes, filled with a solution containing (in mM) 130 K^+ gluconate, 1 $MgCl_2$, 2 $CaCl_2$, 4 ATP, 10 EGTA and 10 HEPES, had a resistance of 3–5 MΩ. A bipolar stimulating electrode (NEX-100; Rhodes Medical Instruments, Inc., Woodland, CA, USA) was placed near the ventral root exit for antidromic identification of SPNs and a second electrode was placed on the white matter dorsolateral to intermediolateral cell column (IML) for activation of the lateral funiculus, which presumably provides descending input to the SPNs. The postsynaptic potentials were displayed and saved on Tektronix digital oscilloscope (TDS 360). The potentials were also sent to a data acquisition system (DigiPack 1200B and pClamp 7.0; Axon Instruments, Inc., Foster City, CA, USA) for continuous recording. The stimulus artifact was eliminated by activating the BLANK ACTIVATE input of Axoclamp 2B. Recordings were made at room temperature (22±1 °C).

Chemicals and statistical data analysis

Hydrogen peroxide was purchased from Riedel-de Haen (Deisenhofen, Germany). CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) disodium and DL-2-amino-5-phosphonovaleric acid (AP-5) were obtained from Tocris Cookson Ltd. (Bristol, UK). Tetrodotoxin citrate was from Alomone Laboratories (Jerusalem, Israel). Catalase, *N*-acetyl-L-cysteine (NAC), and other chemicals for electrophysiology were purchased from Sigma Co. (St. Louis, MO, USA). For *in vivo* experiments, data were analyzed statistically using the repeated measure ANOVA (followed by Dunnett's post test) or paired Student's *t*-test with Prism version 3.0 2 for Windows, Graphpad Software (San Diego, CA, USA). $P<0.05$ was considered statistically significant. For *in vitro* electrophysiological experiments, data were analyzed using paired Student's *t*-test. Results were expressed as mean±S.E.M.

RESULTS

Effects of H_2O_2 on MAP and HR

The MAP and HR in urethane-anesthetized rats was 81.2±2.1 mm Hg and 338±5 beats per minute (BPM) under control conditions ($n=50$). Prior to I.T. injection of H_2O_2, saline and glutamate were applied intrathecally as a negative and positive control, respectively. I.T. saline did not elicit any significant changes in blood pressure and HR. In agreement with previous findings (Hong and Henry, 1992a), I.T. glutamate (1000 nmol, 100 mM/10 μl) caused a rapid rise in MAP and HR. I.T. injection of 30 nmol H_2O_2 did not cause significant changes in MAP and HR ($n=3$). Higher doses of H_2O_2 (100, 500, 1000 nmol) consistently increased the MAP and HR in a dose-dependent manner.

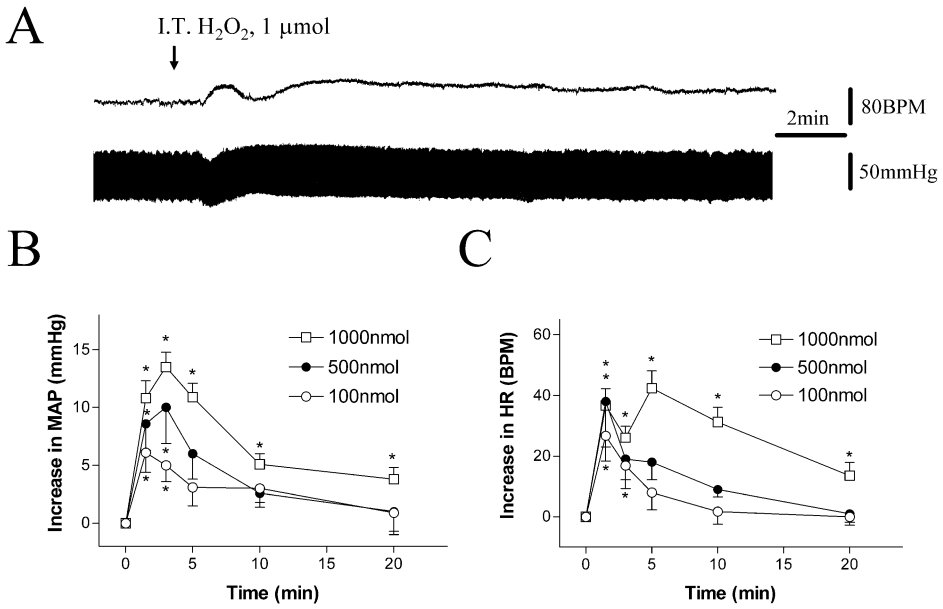

Fig. 1. Dose-response relationship of H_2O_2 given intrathecally on MAP and HR in urethane-anesthetized rats. A: Representative blood pressure and HR recordings after I.T. injection of H_2O_2 (1000 nmol/10 μl). Arrows mark time of I.T. injection. H_2O_2 caused a sustained rises in blood pressure and HR. B: Graph shows an increase in MAP (in mm Hg) over time (minutes) induced by three doses of H_2O_2 (100, 500, 1000 nmol) given intrathecally. C: Increase in HR in BPM over time (minutes) induced by three doses of H_2O_2 (100, 500, 1000 nmol) given intrathecally. Values are mean±S.E.M. from nine, seven, and 40 animals injected intrathecally with 100, 500, 1000 nmol of H_2O_2, respectively. * Indicates statistically significant.

A representative experiment in which I.T. injection of H_2O_2 (1000 nmol) caused pressor and tachycardic responses is illustrated in Fig. 1A. The time course of the mean increases in MAP and HR following H_2O_2 administration is shown in Fig. 1B and Fig. 1C. The maximal responses were reached at 1.5–5 min following the injection and lasted for over 10 min. Fig. 2 shows the percentage of maximal increase in MAP and HR elicited by H_2O_2 and glutamate. The magnitude of maximal increases in MAP and HR caused by I.T. H_2O_2 (1000 nmol) was slightly lower than those caused by the same dose of I.T. glutamate.

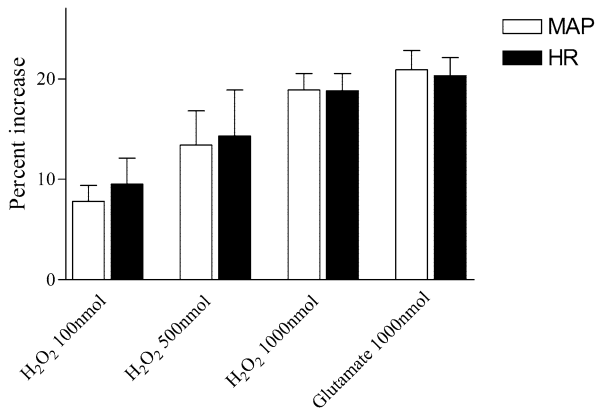

Fig. 2. Histograms showing the maximal increase in MAP and HR induced by I.T. H_2O_2 and glutamate. The basal values of MAP and HR prior to I.T. injection of H_2O_2 were used as control (i.e. 100%). Values are mean+S.E.M. from nine, seven, 40, and 50 animals injected intrathecally with 100, 500, 1000 nmol of H_2O_2 and 1000 nmol of glutamate, respectively. The increases were statistically significant ($P<0.05$).

I.v. injection of hexamethonium (20 mg/kg) via the cannulated femoral vein, which caused a rapid and large fall in the blood pressure with no significant change in the HR, significantly attenuated the pressor and tachycardic effects of a second I.T. injection of H_2O_2 (1000 nmol), which was administered immediately after the BP had stabilized following hexamethonium injection. The mean increases in MAP and HR at 5 min following I.T. H_2O_2 (1000 nmol) were 13.3±3.4 mm Hg and 35±5 BPM and 8.5±2.4 mm Hg and 15±5 BPM ($P<0.05$, $n=8$), respectively, before and after i.v. injection of hexamethonium. Repeated applications of H_2O_2 at intervals of 30 min induced reproducible increases in MAP. The pressor effects were repeatable, being maintained following up to three consecutive applications. Loss of the pressor effects of H_2O_2 in the fourth exposure was observed in three of six rats examined. A representative experiment in which four consecutive applications of H_2O_2 (1000 nmol) at intervals of 30 min induced similar degree of pressor effects is illustrated in Fig. 3.

Effects of catalase and NAC

I.T. injection of catalase (500 unit), an antioxidant enzyme that decomposes H_2O_2, or NAC, a scavenger of oxygen radicals, did not significantly change the MAP, but partially antagonized the pressor effects of a second I.T. injection of H_2O_2 (1000 nmol). The effects of H_2O_2 on MAP were not altered by prior I.T. administration of saline. A representative experiment in which pressor effects caused by I.T. H_2O_2 were inhibited by prior I.T. administration of catalase is demonstrated in Fig. 4A. The mean percentage changes

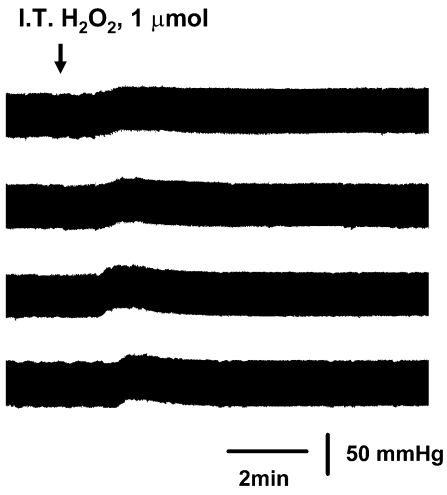

Fig. 3. Representative recordings of blood pressure showing the reproducible pressor effects induced by repeated applications of H_2O_2. Arrow marks time of I.T. injections. Hydrogen peroxide (1000 nmol) was applied at intervals of 30 min. Four traces from top to bottom in sequence represent the pressor effects induced by I.T. H_2O_2 in four consecutive injections.

in MAP caused by H_2O_2 before and after I.T. saline, catalase or NAC are shown in Fig. 4B.

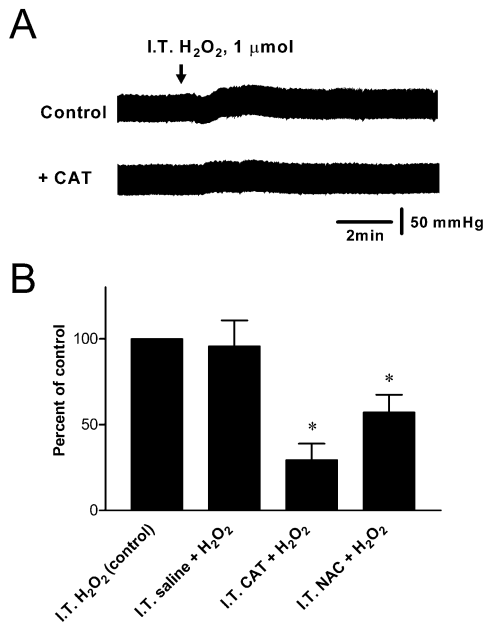

Fig. 4. Inhibition of the pressor effects of I.T. injection of H_2O_2 by prior I.T. injection of catalase (CAT) or NAC in anesthetized rats. A: Representative recordings of blood pressure following I.T. H_2O_2 (1000 nmol) and the blockage of the effects by prior I.T. administration of catalase (500 unit). B: Graphs show percentage changes in MAP following I.T. injection of H_2O_2 (1000 nmol) and the changes induced by a second injection of H_2O_2 in rats pretreated with saline (10 μl), catalase (500 unit/10 μl) or NAC (1000 nmol/10 μl) given intrathecally. The second injection of H_2O_2 was administered 4–5 min after saline, catalase or NAC injection. The maximal increases in MAP induced by first I.T. injection of H_2O_2 were taken as control (i.e. 100%). The increases in MAP were 16±4.3 (n=5), 15.9±3.4 (n=5), and 14.3±1.6 (n=6), respectively, before the application of saline, catalase, and NAC. Values are mean+S.E.M. * Indicates statistically significant.

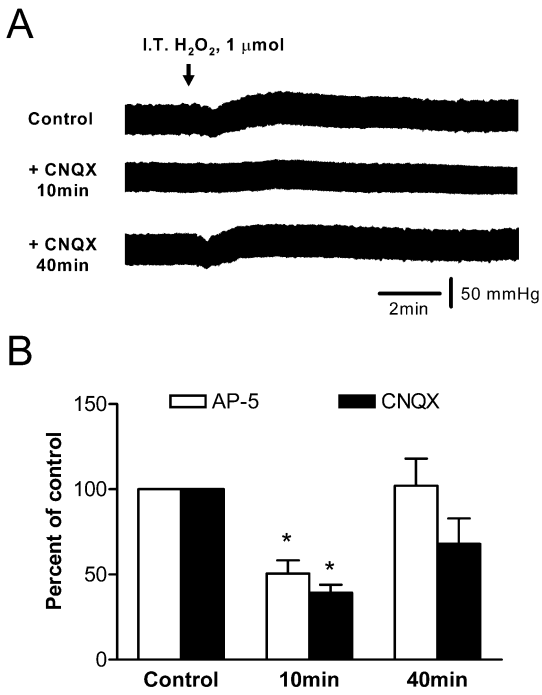

Fig. 5. Inhibition of the pressor effects of I.T. injection of H_2O_2 by prior I.T. injection of CNQX or AP-5, a non-NMDA receptor and NMDA receptor antagonists, respectively, in anesthetized rats. A: Representative recordings of blood pressure following I.T. injections of H_2O_2 (1000 nmol) 20 min before (as control), 10 min and 40 min after I.T. injection of CNQX (30 nmol/10 μl). B: Graphs show percentage changes in MAP following I.T. injection of H_2O_2 (1000 nmol) and the changes induced by second and third injections of H_2O_2 in rats pretreated with CNQX (30 nmol/10 μl) or AP-5 (30 nmol/10 μl) given intrathecally. The second and third injections of H_2O_2 were administered 10 and 40 min, respectively, after CNQX or AP-5 injection. The maximal increases in MAP induced by first I.T. injection of H_2O_2 were taken as control (i.e. 100%). Values are mean+S.E.M. of responses from five and seven animals pretreated with CNQX and AP-5, respectively. * Indicates statistically significant.

Effects of CNQX and AP-5

CNQX and AP-5 are selective non-NMDA and NMDA receptor antagonists, respectively. I.T. injections of different doses of CNQX (10 and 30 nmol) or AP-5 (10 and 30 nmol), which caused slight decreases or no changes in MAP, dose-dependently suppressed the pressor effects of a second I.T. injection of H_2O_2 (1000 nmol). In comparison to the pressor effects induced by first I.T. injection of H_2O_2 which were taken as control of 100%, the second H_2O_2-induced pressor effects decreased to 50.7±9.1% (n=5) and 39.3±4.6% (n=5) of the control 10 min after I.T. injections of 10 and 30 nmol of CNQX, respectively; the pressor effects fell to 77.7±6.4% (n=3) and 50.6±4.7% (n=7) of the control 10 min after I.T. injections of 10 and 30 nmol of AP-5, respectively. A representative experiment in which H_2O_2-induced pressor effect decreased by 70% 10 min after I.T. administration of CNQX and the effect was partially recovered 40 min after the administration is demonstrated in Fig. 5A. The mean percentage changes in MAP in animals injected with H_2O_2 before, 10 min and 40

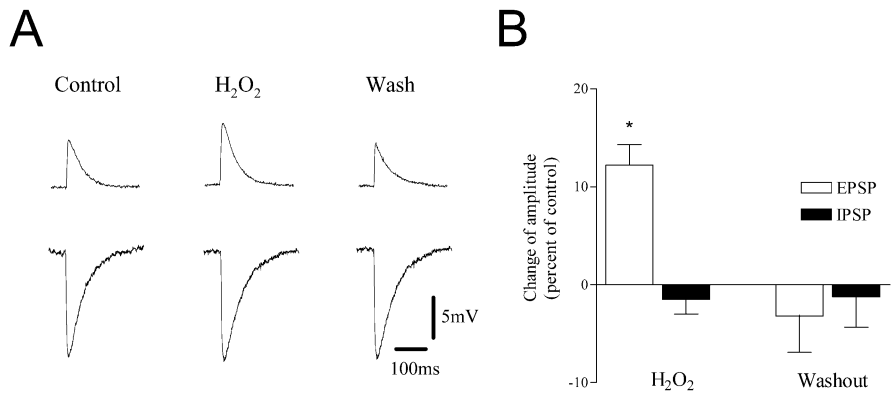

Fig. 6. Effects of 1 mM of H_2O_2 on EPSPs and IPSPs recorded in SPNs of spinal cord slices. Hydrogen peroxide was applied to the spinal cord by superfusion. A: Representative traces, averaged from eight EPSPs (upper) and IPSPs (lower), were taken before the application of H_2O_2 (left), 3 min after the addition of H_2O_2 to bath (middle), and 5 min after removal of H_2O_2 from bath (right). B: Percentage changes in peak amplitudes of EPSPs and IPSPs induced by superfusion of H_2O_2 for 3 min and 5 min after washout. The basal values of EPSPs and IPSPs prior to superfusion of H_2O_2 were taken as control (i.e. 100%). Values are mean±S.E.M. for changes in of EPSPs and IPSPs induced by H_2O_2 from 12 and 5 SPNs tested, respectively. * Indicates statistically significant.

min after I.T. administrations of CNQX and AP-5 are illustrated in Fig. 5B.

Effects of H_2O_2 on peak amplitude of postsynaptic potentials

A single electrical stimulus (0.1–0.3 ms, 3–8 V) elicited an excitatory postsynaptic potential (EPSP) and/or inhibitory postsynaptic potential (IPSP) in SPNs (Wu and Dun, 1993). For studying the effects of H_2O_2 on EPSPs, strychnine (1 μM, a glycine receptor antagonist) and bicuculline (10 μM, a $GABA_A$ receptor antagonist) were added to the perfusing Krebs solution to isolate the EPSPs pharmacologically. The effects of H_2O_2 on IPSPs were examined in the presence of the NMDA receptor antagonist AP-5 (10 μM) and the non-NMDA receptor antagonist CNQX (10 μM). Superfusion of 0.3 mM of H_2O_2 for 3 min had no significant effects on EPSPs or IPSPs (n=3). Hydrogen peroxide at 1 mM increased the peak amplitude of EPSPs in most of the SPNs tested (n=12), but showed insignificant effect on that of IPSPs (n=5). The increase in EPSPs by H_2O_2 was reversible upon washing. A representative recording is shown in Fig. 6A. The mean percentage change of EPSP and IPSP following superfusion of H_2O_2 for 3 min and washing for 5 min is shown in Fig. 6B. Superfusion of H_2O_2 at a higher concentration (3 mM) for 3 min increased the amplitude of EPSPs in four of 10 SPNs tested by 15 to approximately 42% (29±6%, n=4), but decreased the EPSPs in the remaining SPNs by 16±1% (n=6); both effects were fully or partially reversible upon washing. The enhancement of the EPSPs by H_2O_2 (1 or 3 mM) in some of the SPNs was sufficient to elicit action potential firing. Superfusion of H_2O_2 slightly suppressed the amplitude of IPSPs in all SPNs tested by 10.6±4.5% (n=5). The membrane potentials and input resistances of SPNs were not significantly affected by H_2O_2 at concentration of 1 and 3 mM.

DISCUSSION

The present study is the first report to examine the direct action of H_2O_2, a ROS, on central sympathetic neurons both *in vivo* and *in vitro*. The results demonstrated an increase by H_2O_2 in spinal sympathetic outflow using MAP and HR as indices, and in excitatory synaptic transmission in SPNs. The purpose of injecting H_2O_2 into T7–T9 segments is to exert effect on SPNs innervating adrenal medulla. Injection of H_2O_2 to T7–T9 segments may activate the SPNs including, those innervating adrenal medulla. Because the pressor and tachycardic effects of I.T. H_2O_2 were attenuated by pretreatment with i.v. hexamethonium, a ganglionic blocker, and i.v. injection of H_2O_2 at doses equivalent to that of I.T. injection (1000 nmol) did not affect MAP and HR (results not shown), the pressor and tachycardic effects of H_2O_2 injected around T7–T9 segments may result mainly from the activation by H_2O_2 of SPNs innervating adrenal medulla and the subsequent release of catecholamine from adrenal medulla. The heart is controlled by SPNs mainly located in the T1–T3 segment. H_2O_2 injected to T7–T9 segments is unlikely to diffuse to T1–T3 segments and the HR may also be affected by baroreflex if the magnitude of blood pressure change is large enough. This may contribute to the complex waveform of HR recording following I.T. injection of higher dose of H_2O_2 (1000 nmol) in some of the rats tested (Fig. 1A, C). For around 50% of rats tested, I.T. injection of a higher dose of H_2O_2 (1000 nmol) elicited a small drop followed by a sustained increase in blood pressure and the initial drop in blood pressure was blocked by prior I.T. administration of catalase. The initial drop in blood pressure may result from the activation of SPNs by H_2O_2 and subsequently the enhancement of the activity of sympathetic postganglionic neurons and relaxation of some blood vessels. This effect may also be related to the inhibition by higher concentrations of H_2O_2 of EPSPs in some of SPNs examined in the *in vitro* electrophysiological study.

As explained previously, change in blood pressure is more directly related to changes in activity of SPNs at T7–T9 segments than that in HR. Therefore, in the experiments examining the antagonism of H_2O_2-induced pressor effects only MAP was taken as an index. The attenuation of the H_2O_2-induced pressor effects by I.T. pretreatment with catalase or NAC suggested that H_2O_2 exert an excitatory action by itself and/or by the production of oxygen radicals in the spinal cord. Hydrogen peroxide, metabolized by a catalase pathway, has been demonstrated to act as a source of O_2 in neonatal rat spinal cord maintained *in vitro* (Walton and Fulton, 1983). Furthermore, the increased generation of ROS and the release of L-glutamate by high O_2 have been shown in cultured hippocampal neurons (Ishikawa et al., 1999). Therefore, the possibility that H_2O_2 exerts its effects partly through the production of O_2 cannot be ruled out.

I.T. administration of glutamate increased both MAP and HR as demonstrated in previous reports, indicating the involvement of glutamate receptors in the regulation of spinal sympathetic output (Hong and Henry, 1992a). Both NMDA and non-NMDA glutamate receptors were involved in mediating the effects of glutamate (Hong and Henry, 1992a,b). In the present study, the pressor effects of I.T. H_2O_2 were antagonized dose-dependently by I.T. pretreatment with AP-5 (NMDA receptor antagonist) or CNQX (non-NMDA glutamate receptor antagonist), indicating that the excitatory effects of H_2O_2 on SPNs may be associated with the activation of glutamatergic synapses. Glutamate acting on non-NMDA receptors contributes more to components of EPSCs evoked from lateral funiculus than that on NMDA receptors; EPSCs evoked in the dorsal horn were mainly carried by NMDA receptors (Krupp and Feltz, 1995). The contribution of the NMDA receptor-mediated component of EPSCs (EPSPs) in SPNs increased with depolarization or following high frequency stimulations (Krupp and Feltz, 1995; Spanswick et al., 1998). Blockade of non-NMDA receptors would contribute some loss of NMDA receptor activation due to failure to depolarize the cell sufficient to remove the voltage-dependent block of NMDA receptors by magnesium ions. This may explain the results that CNQX had a slightly greater inhibitory action on H_2O_2-induced pressor effects than AP-5.

A specific action of H_2O_2 on glutamatergic synapses was indicated since an enhancement was observed only on EPSPs, not on IPSPs. The action of H_2O_2 on EPSPs may result from an increased release of glutamate as observed in several neurochemical studies and/or an inhibition of the uptake of glutamate (Pellegrini-Giampietro et al., 1988; Volterra et al., 1994; Satoh et al., 1998; Mailly et al., 1999; Avshalumov and Rice, 2002). The increased release of glutamate and the activation of NMDA receptors have been demonstrated to participate in the neurotoxicity induced by xanthine (X) plus X oxidase (XO) or H_2O_2 (Satoh et al., 1998; Mailly et al., 1999). The marked decrease of high-affinity glutamate transport elicited by X+XO or H_2O_2 has also been shown in rat cortical astrocytic cultures (Volterra et al., 1994). In addition, a study showed that lipid signaling molecules may be involved in the augmentation of population spikes and field potentials by H_2O_2 in the hippocampal slices since the augmenting effect was largely attenuated by phospholipase A_2/C inhibitor (Katsuki et al., 1997). Additional factors may be involved in the actions of higher concentrations of H_2O_2 since inhibition, rather than enhancement of EPSPs, was observed in some of the SPNs tested. In fact, several studies have demonstrated the inhibition of field synaptic potentials and population spike by ROS in rat hippocampal slices (Pellmar, 1995; Fowler, 1997; Avshalumov et al., 2000). Presynaptic inhibition of Ca^{2+}-dependent transmitter release has been proposed to be the mechanism accounting for the effect of H_2O_2 (Pellmar, 1987). However, a recent study showed that the mechanism of H_2O_2-induced depression of evoked population spike in hippocampal slice may not involve presynaptic Ca^{2+} per se (Avshalumov et al., 2000). Whether the inhibition of IPSPs or EPSPs in some of the SPNs tested in the present study is related to the interruption of presynaptic Ca^{2+}-dependent mechanisms remains to be investigated and the exact molecular mechanisms for the effects of H_2O_2 on the synapse needs to be further clarified.

The electrophysiological results showed that 1 mM of H_2O_2 enhanced EPSPs without significant effect on IPSPs and higher concentrations of H_2O_2 (3 mM) increased the amplitude of EPSPs in some of SPNs tested, but slightly inhibited that of IPSPs in all of the SPNs examined. Thus, the overall effect of H_2O_2 on synaptic transmission in SPNs would be excitatory, which may contribute to the increase in MAP and HR observed following I.T. administration of H_2O_2 *in vivo*. Though the magnitude of percentage increases in cardiovascular effects of H_2O_2 *in vivo* and the enhancement of EPSPs by H_2O_2 *in vitro* was around 20%, these increases were consistently observed in many rats tested and the increases were statistically significant. In addition, the magnitude of percentage increases in MAP and HR by H_2O_2 at dose (1000 nmol) equivalent to glutamate was only slightly lower than that of glutamate in the *in vivo* study. It should be pointed out that the present experiments could not provide an exact concentration of H_2O_2 that acts on SPNs because of the tissue barriers that H_2O_2 has to diffuse across and the metabolism of H_2O_2 by tissues following I.T. injection and superfusion. However, it suggested that the final concentration of H_2O_2 that affects the activity of SPNs would be much less than the concentrations used in the experiment. Actually, the concentrations used in our *in vitro* electrophysiological study were similar to the effective concentrations reported for the neurons of rat hippocampal slices (Fowler, 1997; Katsuki et al., 1997). Thus, the present results indicate that the increase of oxidative stress in spinal cord may lead to an increase in MAP and HR by acting on SPNs. In conclusion, the present study demonstrated that H_2O_2 exerts a stimulatory effect on SPNs and the activation of SPNs increases spinal sympathetic outflow, which may contribute to ROS-induced cardiovascular diseases.

Acknowledgements—This study was supported by grants from National Science Council (NSC 89-2320-B-320-034; NSC 90-2320-B-277-001), Taiwan.

REFERENCES

Adler V, Yin Z, Tew KD, Ronai Z (1999) Role of redox potential and reactive oxygen species in stress signaling. Oncogene 18:6104–6111.

Avshalumov MV, Chen BT, Rice ME (2000) Mechanisms underlying H_2O_2-mediated inhibition of synaptic transmission in rat hippocampal slices. Brain Res 882:86–94.

Avshalumov MV, Rice ME (2002) NMDA receptor activation mediates hydrogen peroxide-induced pathophysiology in rat hippocampal slices. J Neurophysiol 87:2896–2903.

Cai H, Harrison DG (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 87:840–844.

Dhalla NS, Temsah RM, Netticadan T (2000) Role of oxidative stress in cardiovascular diseases. J Hypertens 18:655–673.

Duffy SJ, Gokce N, Holbrook M, Huang A, Frei B, Keaney JF, Vita JA (1999) Treatment of hypertension with ascorbic acid. Lancet 354: 2048–2049.

Fowler JC (1997) Hydrogen peroxide opposes the hypoxic depression of evoked synaptic transmission in rat hippocampal slices. Brain Res 766:255–258.

Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine. New York: Oxford University Press.

Hong YG, Henry JL (1992a) Glutamate, NMDA and NMDA receptor antagonists: cardiovascular effects of intrathecal administration in the rat. Brain Res 569:38–45.

Hong Y, Henry JL (1992b) Intrathecal administration of non-NMDA receptor agonists increases arterial pressure and heart rate in the rat. Brain Res 591:62–68.

Inokuchi H, Yoshimura M, Trzebski A, Polosa C, Nishi S (1992a) Fast inhibitory postsynaptic potentials and responses to inhibitory amino acids of sympathetic preganglionic neurons in the adult cat. J Auton Nerv Syst 41:53–59.

Inokuchi H, Yoshimura M, Yamada S, Polosa C, Nishi S (1992b) Fast excitatory postsynaptic potentials and the responses to excitant amino acids of sympathetic preganglionic neurons in the slice of the cat spinal cord. Neuroscience 46:657–667.

Irani K (2000) Oxidant signaling in vascular cell growth, death, and survival: a review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. Circ Res 87:179–183.

Ishikawa Y, Satoh T, Enokido Y, Nishio C, Ikeuchi T, Hatanaka H (1999) Generation of reactive oxygen species, release of L-glutamate and activation of caspases are required for oxygen-induced apoptosis of embryonic hippocampal neurons in culture. Brain Res 824:71–80.

Katsuki H, Nakanishi C, Saito H, Matsuki N (1997) Biphasic effect of hydrogen peroxide on field potentials in rat hippocampal slices. Eur J Pharmacol 337:213–218.

Krupp J, Feltz P (1995) Excitatory postsynaptic currents and glutamate receptors in neonatal rat sympathetic preganglionic neurons in vitro. J Neurophysiol 73:1503–1512.

Lai C-C, Lin HH, Chen C-W, Chen S-H, Chiu TH (2002) Excitatory action of lead on rat sympathetic preganglionic neurons in vitro and in vivo. Life Sci 71:1035–1045.

Lai C-C, Wu SY, Chen C-T, Dun NJ (2000) Nociception inhibits rat sympathetic preganglionic neurons in situ and in vitro. Am J Physiol Regulatory Integrative Comp Physiol 278:R592–R597.

Liu D, Liu J, Wen J (1999) Elevation of hydrogen peroxide after spinal cord injury detected by using the Fenton reaction. Free Radic Biol Med 27:478–482.

Liu D, Sybert TE, Qian H, Liu J (1998) Superoxide production after spinal injury detected by microperfusion of cytochrome c. Free Radic Biol Med 25:298–304.

Mailly F, Marin P, Israel M, Glowinski J, Premont J (1999) Increase in external glutamate and NMDA receptor activation contribute to H_2O_2-induced neuronal apoptosis. J Neurochem 73:1181–1188.

Pellegrini-Giampietro DE, Cherici G, Alesiani M, Carla V, Moroni F (1988) Excitatory amino acid release from rat hippocampal slices as a consequence of free-radical formation. J Neurochem 51: 1960–1963.

Pellmar TC (1987) Peroxide alters neuronal excitability in the CA1 region of guinea-pig hippocampus in vitro. Neuroscience 23:447–456.

Pellmar TC (1995) Use of brain slices in the study of free-radical actions. J Neurosci Methods 59:93–98.

Satoh T, Numakawa T, Abiru Y, Yamagata T, Ishikawa Y, Enokido Y, Hatanaka H (1998) Production of reactive oxygen species and release of L-glutamate during superoxide anion-induced cell death of cerebellar granule neurons. J Neurochem 70:316–324.

Spanswick D, Renaud LP, Logan SD (1998) Bilaterally evoked monosynaptic EPSPs, NMDA receptors and potentiation in rat sympathetic preganglionic neurones in vitro. J Physiol 509:195–209.

Volterra A, Trotti D, Tromba C, Floridi S, Racagni G (1994) Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. J Neurosci 14:2924–2932.

Walton K, Fulton B (1983) Hydrogen peroxide as a source of molecular oxygen for in vitro mammalian CNS preparations. Brain Res 278: 387–393.

Wu SY, Dun NJ (1993) Excitatory amino acids depress synaptic currents in neonate rat sympathetic preganglionic neurons. J Neurophysiol 69:2030–2038.

Zanzinger J, Czachurski J (2000) Chronic oxidative stress in the RVLM modulates sympathetic control of circulation in pigs. Pflugers Arch 439:489–494.

Zoccarato F, Valente M, Alexandre A (1995) Hydrogen peroxide induces a long-lasting inhibition of the Ca^{2+}-dependent glutamate release in cerebrocortical synaptosomes without interfering with cytosolic Ca^{2+}. J Neurochem 64:2552–2558.

(Accepted 2 April 2003)