Respiratory long-term facilitation following intermittent hypoxia requires reactive oxygen species formation

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Abstract

Acute intermittent hypoxia (AIH) elicits a form of respiratory plasticity known as long-term facilitation (LTF). LTF is a progressive and sustained increase in respiratory motor output as expressed in phrenic and hypoglossal (XII) nerve activity. Since reactive oxygen species (ROS) play important roles in several forms of neuroplasticity, and ROS production is increased by intermittent hypoxia, we tested the hypothesis that ROS are necessary for phrenic and hypoglossal LTF following AIH. Urethane anesthetized, paralyzed, vagotomized and pump ventilated Sprague Dawley rats were exposed to AIH (11% O_2, 3, 5 min episodes, 5 min intervals), and both phrenic and XII nerve activity were monitored for 60 min post-AIH. Although phrenic and XII LTF were observed in control rats, intravenous Manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), a superoxide anion scavenger, attenuated both phrenic and XII LTF in a dose dependent manner. Localized application of MnTMPyP (5.5mM; 10µl) to the intrathecal space of the cervical spinal cord (C_4) abolished phrenic, but not XII LTF. Thus, ROS are necessary for AIH-induced respiratory LTF, and the relevant ROS appear to be localized near respiratory motor nuclei since cervical MnTMPyP injections impaired phrenic (and not XII) LTF. Phrenic LTF is a novel form of ROS-dependent neuroplasticity since its ROS-dependence resides in the spinal cord.

Keywords

plasticity; spinal cord; control of breathing; superoxide anion

Acute intermittent hypoxia (AIH) elicits a form of respiratory plasticity known as long-term facilitation (LTF). LTF is a progressive increase in respiratory motor output that lasts hours following the final hypoxic episode (Fuller et al., 2000; Mitchell et al., 2001; Mitchell and Johnson, 2003; Feldman et al., 2003). Phrenic LTF requires serotonin-dependent protein synthesis in cervical spinal regions associated with the phrenic motor nucleus (Baker-Herman and Mitchell, 2002). In our working model (Mahamed and Mitchell, 2006; Baker-Herman et al., 2004), serotonin release during hypoxic episodes activates 5-HT$_{2A}$ receptors located on or

near phrenic motor neurons and initiates signaling cascades that maintain phrenic LTF. Postulated signaling events downstream from 5-HT$_{2A}$ receptor activation include protein kinase C activation (McGuire and Ling, 2004), new synthesis of brain derived neurotrophic factor (BDNF), and subsequent activation of its high affinity receptor tyrosine kinase, TrkB (Baker-Herman et al., 2004). Relevant events downstream from TrkB activation are not clear at this time, but most likely involve activation of MAP kinases (e.g. ERK) or protein kinase B (Akt). On the other hand, phrenic LTF is subject to inhibitory constraints since okadaic acid-sensitive protein phosphatases suppress phrenic LTF following sustained hypoxia (Wilkerson et al., 2007). These putative cellular mechanisms of LTF share many common features with other forms of neuroplasticity such as sensitization of the gill-withdrawal reflex in *Aplysia* (Brunelli et al., 1976; Purcell et al., 2003; and see review by Barbas et al., 2003) and activity-dependent hippocampal LTP in rats (Poo, 2001; Kovalchuk et al., 2002; Huang and Reichardt, 2003).

Reactive oxygen species (ROS), such as the superoxide anion ($^{\bullet-}O_2$) and hydrogen peroxide (H_2O_2) are necessary for activity-dependent synaptic plasticity in the mammalian hippocampus (Klann 1998; Klann et al. 1998). Consequently, mice over-expressing superoxide dismuatase have impaired hippocampal LTP (Thiels et al., 2000). Although the precise role of ROS in hippocampal LTP remains unknown, ROS affect the activity of both protein kinases and protein phosphatases (Klann et al. 1998; Klann and Thiels, 1999; Otani et al. 1993; Knapp and Klann, 2002). Indeed, protein kinase C, extracellular regulated kinase 2 (ERK2) and tyrosine kinase activities are enhanced, whereas protein phosphatases including serine/threonine (e.g. PP2A) and tyrosine phosphatases are inhibited by $^{\bullet-}O_2$ and H_2O_2 (Klann and Thiels, 1999). ROS are necessary for long-term facilitation of carotid chemosensory neurons following AIH in rats preconditioned with chronic intermittent hypoxia (CIH) (Peng et al., 2003). Carotid sensory LTF is blocked by daily administration of the superoxide dismutase mimetic Manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), thus implicating ROS in CIH-induced metaplasticity of sensory receptors that play an important role in the control of breathing (Peng et al., 2003).

Despite many similarities in cell-signaling mechanisms underlying multiple forms of neuroplasticity, the role of ROS in AIH-induced phrenic or XII LTF has not been explored. In support of such a role, brain ROS levels are increased during and following hypoxia (Fabian et al., 1995, Dirnagl et al., 1995; Fabian et al., 2004), particularly following intermittent hypoxia/re-oxygenation (Masaoka et al., 2005; Xu et al., 2004; Yuan et al., 2004). Further, BDNF release is greater in response to intermittent *versus* sustained hypoxia in PC-12 cells, a differential effect inhibited by the ROS scavenger N-acetyl-1-cysteine (Wang et al., 2005). Thus, the primary goal of this study was to test the hypothesis that ROS are necessary for phrenic and XII LTF, and that the relevant ROS are localized to the respective respiratory motor nuclei.

Experimental procedures

Experiments were performed on 3–4 month old male Sprague Dawley rats (Charles River, colony PO4). All experiments were approved by The Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin-Madison.

Surgical preparation

Anesthesia was induced using isoflurane, and the rats were tracheotomized and pump ventilated (Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA). Rats were maintained on isoflurane (3.5%) in 50% O_2 (balance N_2) for the duration of the surgery, and then slowly converted to urethane anesthesia (1.6–1.8 g/kg) via a tail vein catheter. Body temperature was maintained with a temperature controlled surgical table. Rectal temperature

was monitored throughout the procedure using a calibrated temperature sensor (Fisher Scientific, USA). The depth of anesthesia was assessed periodically by testing blood pressure and phrenic nerve responses to toe pinch; supplemental doses of urethane were given as necessary. Inspired O_2 concentration was monitored throughout the experiment using an O_2 sensor (TED 60T, Teledyne Analytical Instruments, USA). One hour after beginning surgery, an intravenous infusion of a 1:11 solution of sodium bicarbonate (8.4%) and Standard Lactated Ringer's solution was initiated to maintain acid-base balance.

Following tracheotomy, rats were vagotomized and a catheter was inserted into the right femoral artery to enable measurements of arterial blood pressure (Gould, P23, USA). Arterial blood samples were drawn periodically and analysed for O_2 (PO_2) and CO_2 (PCO_2) partial pressures, pH and base excess using a blood gas analyser (ABL 500, Radiometer, Copenhagen, Denmark). The left phrenic and XII nerves were dissected using a dorsal approach, cut distally and desheathed. The nerves were submerged in mineral oil and placed on bipolar silver recording electrodes. The rats were paralyzed with pancuronium bromide once nerve signals appeared. Rats were allowed ~50min for electroneurograms and blood pressure to stabilize. Nerve activity was amplified (gain, 10,000; A-M systems, Everett, WA), bandpass-filtered (100 Hz to 10 kHz), and integrated (CWE 821 filter; Paynter, Ardmore, PA; time constant, 50 msec). The signal was digitized, recorded, and analyzed using a WINDAQ data acquisition system (DATAQ Instruments, Akron, OH).

To establish baseline nerve activity, rats were ventilated with hyperoxia ($FIO_2 \sim 0.50$; $PaO_2 >$ 180 mmHg), with CO_2 added to the inspired gas. After the 50min stabilization period, the apneic threshold was determined by progressively lowering the inspired CO_2. Steady state baseline nerve activity was established with Pa_{CO_2} set 2–3 mmHg above the CO_2 recruitment threshold (Bach and Mitchell, 1996). The drug or vehicle was injected 15 min after establishing baseline conditions. A further 20 min of baseline was recorded before starting the intermittent hypoxia protocol. In two animals, the apneic threshold was repeated after drug was injected; there was no apparent change due to drug administration.

End-tidal CO_2 was monitored throughout the experiment using a flow-through capnograph (Novametrix, Wallingford, CT). Arterial blood gases and blood pressure were monitored and corrected as necessary to ensure that post-hypoxia values were maintained near baseline levels. $PaCO_2$ was adjusted by manipulating inspired CO_2; negative base excess values more severe than −3mEq/L were corrected with intravenous sodium bicarbonate, and progressive reductions in blood pressure were offset with intravenous injection of lactated Ringer's solution. Data from rats were included in the analysis only if they complied with the following criteria: (1) Pa_{O_2} during hypoxia was between 35 and 45 mmHg; (2) Pa_{O_2} during the hyperoxic baseline and recovery periods was >180 mmHg; and (3) Pa_{CO_2} remained within 1 mmHg of baseline throughout the post-hypoxia protocol. Phrenic and XII nerve activity were recorded continuously. Blood samples (0.3 ml in a heparinized glass syringe) were drawn before, during the first hypoxic episode and 15, 30, and 60 min after the last hypoxic episode to ensure that blood gases met the criteria outlined above. Measurements of phrenic and XII nerve burst frequency and amplitude were evaluated for one minute immediately prior to each blood sample. At the end of the experiment, rats were humanely killed by exsanguination in accordance with ethical standards of the institution.

Protocol

All rats received AIH, which consisted of three, 5 min episodes of isocapnic hypoxia (F_{IO_2}, 0.11) with each episode separated by 5 min of moderate hyperoxia (see Fig. 1). To investigate the role of ROS in LTF, the superoxide anion scavenger, MnTMPyP (Sigma, USA) was injected intravenously at doses of either 5 (412 ± 9g; N = 8) or 10 mg kg^{-1} (379 ± 16g; N = 8). For each experiment, 10mg of MnTMPyP was dissolved in either 1 (10 mg kg^{-1}) or 2 ml of

saline (5mg kg^{-1} BW); the volume injected into each treatment group equated to just 1% of the body weight of the rat. In a third group (402 ±16g; N=7) rats were injected with vehicle only (saline).

To localize the relevant ROS to the cervical spinal cord, a C_2 laminectomy and durotomy were performed to allow localized injections of MnTMPyP within the vicinity of the phrenic motor nucleus (C_4); this procedure has been described elsewhere (Baker-Herman and Mitchell, 2002). In brief, after C_2 laminectomy, a small hole was made in the dura, and a silicone catheter (outer diameter, 0.6mm; Access Technologies) was advanced about 2mm caudally. The catheter was primed with 5.5mM MnTMPyP prior to positioning. Each rat (408 ± 13g; N=7) received a 10µl injection or artificial CSF (vehicle) (369 ±15g; N = 9) over ~5 min beginning 20 min prior to an AIH protocol.

Statistical analysis

Peak amplitude and frequency (bursts per minute) of phrenic and XII nerve activity were averaged in 1 min bins at each recorded data point (baseline, at the end of the first hypoxic exposure, and 15, 30, and 60 min post-AIH). Minute phrenic nerve activity (expressed as a % change from baseline) was determined by multiplying breath frequency and integrated nerve burst amplitude. At all time points, changes in nerve burst amplitude are normalized as a percentage of the baseline value, whereas burst frequency is expressed as absolute values. For both i.v. and i.t. treated rats, we compared phrenic and XII nerve responses of rats receiving vehicle (saline or aCSF) *versus* drug injections. Statistical comparisons were made for time and drug treatment effects using two-way, repeated measures ANOVA. The Bonferroni *post hoc* test was used to identify statistically significant individual comparisons. Differences were considered significant at $p < 0.05$. All values are expressed as mean ± 1 SEM.

Results

Neither $PaCO_2$ nor pH were different from baseline values or between groups in any condition except during hypoxia in rats treated with 10mg kg^{-1} MnTMPyP. Despite maintenance of hypocapnia during AIH (">Table 1), a slight acidosis occurred during hypoxia in rats treated with 10mg kg^{-1} MnTMPyP; however, pH and base excess were restored to baseline values by 15 min post-AIH. As expected, both phrenic and XII nerve burst amplitude (Fig. 2A) and burst frequency (Fig. 2B) were significantly elevated above baseline levels during the first episode of isocapnic hypoxia ($PaO_2 = 38\pm2$ mmHg). Significant hypotension was observed during hypoxia in all groups similar to previous studies on urethane anesthetized rats.

In vehicle (saline) treated rats, AIH caused a progressive increase in phrenic and XII nerve burst amplitude characteristic of LTF (Fig. 1 and Fig 3). Burst amplitude had significantly increased above baseline at 30 and 60 min post-AIH. At 60 min post-AIH, phrenic and XII nerve burst amplitudes were 93 ± 22% and 70 ± 12% above baseline, respectively (Fig. 3A and 3B). Phrenic LTF was significantly reduced by i.v. injection of 5mg kg^{-1} MnTMPyP, whereas XII LTF was abolished at this same dose. At 10mg kg^{-1} MnTMPyP, both phrenic and XII LTF were abolished (Fig 3A and 3B, respectively).

AIH also elicited a modest frequency LTF in the saline treated group, as indicated by a significant increase in nerve burst frequency 60 min post-AIH (Fig. 4A). This frequency LTF was also reduced (5mg kg^{-1}) or abolished (10mg kg^{-1}) by MnTMPyP, implying a drug effect on either central rhythm generating networks or on sensory pathways that modify respiratory rhythm such as the carotid body chemoreceptors (Peng et al., 2003). Minute phrenic and XII nerve activity also exhibited significant LTF in saline treated, but not MnTMPyP treated rats (Fig. 4B and 4C). MnTMPyP had no effects on phrenic or XII nerve activity during baseline conditions (data not shown) or during hypoxia (Fig 2).

To test the hypothesis that MnTMPyP affects phrenic LTF by actions in the spinal cord, the drug was applied locally to the intrathecal space at C4 in an additional group of rats. Such an approach has been used previously to test the requirement for cervical spinal serotonin receptor activation in phrenic LTF (Baker-Herman and Mitchell, 2002). In these studies, XII nerve activity serves as an internal control for unintended drug distribution. Accordingly, intrathecal application of 5.5mM MnTMPyP at C4 abolished phrenic LTF (Fig. 5A). In these same animals, XII LTF was not different from vehicle control rats (Fig 5B), thereby demonstrating that effective MnTMPyP concentrations were restricted to the spinal cord.

Discussion

The results demonstrate that ROS (particularly $^\bullet{}^-O_2$) are necessary for respiratory LTF following AIH in anesthetized rats. Further, the ROS relevant to phrenic LTF can be localized to the cervical spinal regions associated with the phrenic motor nucleus. Similarly, the lack of effect of intrathecal MnTMPyP on XII LTF, combined with the potent effect of intravenous MnTMPyP, suggest that XII LTF is also dependent on ROS, but localized near the XII motor nucleus in this case.

The superoxide dismutase (SOD) mimetic, MnTMPyP, is a cell permeable $^\bullet{}^-O_2$ scavenger that reduced both phrenic and XII LTF following systemic administration. Thus, $^\bullet{}^-O_2$ formation is necessary for the induction and/or maintenance of LTF in both motor outputs, thus suggesting a common mechanism. To our knowledge, this is the first example of ROS-dependent plasticity in the brainstem/spinal cord, although there is considerable evidence for ROS-dependence in other forms of neuroplasticity. For example, MnTMPyP (Klann 1998) and catalase (Klann et al., 1998) both block activity-dependent hippocampal LTP in CA1, suggesting possible roles for both $^\bullet{}^-O_2$ and H_SO_2 in its underlying mechanism. In transgenic mice over-expressing extracellular SOD, hippocampal LTP is impaired, an effect reversed by inhibition of copper-zinc SOD with a copper chelator (Thiels et al., 2000). ROS are also necessary for the mechanism whereby pretreatment with chronic intermittent hypoxia induces the capacity to express carotid chemosensory LTF following AIH (Peng et al. 2003). Carotid sensory LTF is a prolonged increase in chemoafferent neuron activity following AIH, but is observed only in rats pretreated with chronic intermittent hypoxia (Peng et al., 2003). Since sensory LTF is not elicited in rats receiving daily injections of MnTMPyP (5mg kg^{-1}) during chronic intermittent hypoxia exposures (Peng et al., 2003), ROS are required in the mechanism whereby chronic intermittent hypoxia induces this mechanism. Carotid sensory LTF in CIH-treated rats might share similar ROS mechanisms to phrenic and XII LTF following AIH, although the time domains of these forms of plasticity are quite distinct.

Inhibition of phrenic and XII LTF via intravenous MnTMPyP does not exclude the possibility of a peripheral or non-spinal/brainstem site of action. The ROS-dependence of the small, AIH-induced frequency LTF (Fig. 4A) provides suggestive evidence for non-spinal mechanisms of action of MnTMPyP, although this result could equally be due to actions on intra-spinal synaptic pathways of sensory neurons projecting to the respiratory rhythm generating circuitry in the brainstem. Observations that MnTMPyP had no effects on baseline respiratory motor output, the CO_2-apneic threshold or the magnitude of the short-term hypoxic phrenic nerve response provide evidence that the actions of MnTMPyP are highly specific to LTF, and thus do not reflect general actions of the drug on spontaneous neural activity or changes in afferent inputs such as the carotid bodies. Furthermore, differential responses of XII and phrenic LTF at low intravenous doses of MnTMPyP (5mg/kg) suggest differential sensitivity of the respective motor nuclei to ROS.

Intrathecal MnTMPyP administration to cervical spinal regions that contain the phrenic motor nucleus confirm that the ROS necessary for phrenic LTF are located in or near the phrenic

motor nucleus and not at peripheral sites of action. This experimental approach has been used successfully to differentiate spinal *versus* brainstem or systemic effects in LTF (Baker-Herman and Mitchell, 2002). While an i.v. injection of MnTMPyP blocked both phrenic and XII LTF, it does not exclude the possibility that ROS scavenging affects phrenic and XII LTF through modifications occurring elsewhere, such as the carotid body or brainstem. Indeed, selective inhibition of phrenic, but not XII, LTF following i.t. injections of MnTMPyP suggests that the relevant ROS for phrenic LTF occur within spinal regions containing the phrenic motor nucleus *versus* non-specific effects via unintended drug distribution. Although AIH-induced phrenic LTF is distinguishable from carotid sensory LTF in CIH pre-treated rats, similar responses to MnTMPyP suggest at least some commonalities in their ROS-dependence.

Our data imply that superoxide anion formation in respiratory motor nuclei may be increased during or following AIH. During AIH protocols, ROS may be generated by increased neuronal activity, by ligand binding to receptors that generate ROS when activated (e.g. $5-HT_2$ and TrkB receptors), by the hypoxia itself, or by the swings in oxygen tension. Since hippocampal LTP requires activity-dependent ROS production (Klann, 1998; Knapp and Klann, 2002), increased motor neuron activity during hypoxia may induce ROS formation, possibly from the mitochondria; Peng and colleagues (2003) proposed that complex I of the mitochondrial respiratory chain is a significant source of ROS generation in carotid sensory LTF. If so, activity-dependent source of ROS is not sufficient to induce LTF since multiple receptor antagonists (e.g. the serotonin receptor antagonist methysergide) block LTF but have no effect on the magnitude of phrenic or XII nerve activity during hypoxia (Bach and Mitchell, 1996). Furthermore, whereas AIH induces respiratory LTF, the same cumulative duration of sustained hypoxia (25 min; Baker and Mitchell, 2000) or hypercapnia (Baker et al., 2001) does not, further supporting the hypothesis that activity-dependent ROS formation is not sufficient to induce LTF. Another promising candidate for the necessary ROS formation is NADPH oxidase activity, a major source of superoxide during hypoxia (Henderson and Chappell, 1996) and intermittent hypoxia (Hitomi et al., 2003; Zhan et al., 2005). Indeed, Peng and colleagues (2006) recently reported that NADPH oxidase contributes ROS necessary for carotid sensory LTF in PC12 cells following repetitive serotonin administration. On the other hand, we cannot exclude the possibility that the ROS necessary for AIH-induced LTF result from constitutive ROS production not specifically related to AIH; in this sense, ROS may be necessary but not sufficient for AIH-induced LTF.

Using a superoxide sensitive electrode in the cortex, Fabian and colleagues (2004) demonstrate that extra-cellular superoxide levels are unaffected by 20 minutes of hypoxia (10% inspired O_2), but increase for a prolonged period (>20 min) following a transition from hypoxia to hyperoxia (100% inspired O_2). If a similar pulse of superoxide production occurs at each of the three consecutive phases of re-oxygenation in our protocol, a cumulative effect similar to RC-integration might produce sufficient ROS to induce LTF during or following AIH, whereas sustained hypoxia might not. Greater ROS production during intermittent (versus sustained) hypoxia may account, at least part, for the pattern-sensitivity of respiratory LTF (Baker and Mitchell, 2000).

In recent years, fairly detailed cellular/synaptic mechanisms have been proposed to explain respiratory LTF (Mitchell et al., 2001; Feldman et al., 2003; Baker-Herman et al., 2004; Mahamed and Mitchell, 2006), but a possible role of ROS in respiratory LTF has not been previously explored. ROS may regulate respiratory LTF via actions on the kinase/phosphatase balance (Mitchell and Johnson, 2003), including both increased activation of kinases that enable LTF, and by inhibiting phosphatases that constrain LTF (Wilkerson et al., 2007). For example, intrathecal okadaic acid administration, a serine/threonine phosphatase inhibitor, reveals phrenic LTF following sustained hypoxia. Although the mechanisms leading to differential regulation of phosphatase activity during intermittent versus sustained hypoxia

were not identified in these studies, ROS generated during and/or following AIH may activate relevant protein kinases (e.g. PKC) and/or inhibit relevant okadaic acid-sensitive protein phosphatases (e.g. PP2). As a provisional test of the latter possibility, we demonstrated that AIH-induced phrenic LTF is restored in rats treated with 10 mg/kg MnTMPyP (i.v.) when okadaic acid is administered intrathecally (MacFarlane and Mitchell, unpublished observation). Although not conclusive, these observations suggest that the failure of ROS-mediated phosphatase inhibition during AIH underlies the loss of LTF in rats treated with MnTMPyP, and that LTF is restored when another means is used to suppress those phosphatases (e.g. with okadaic acid).

Other possible mechanisms whereby ROS modulate LTF must be considered, including the regulation of intracellular calcium. AIH induces transient calcium spikes, causing auto-phosphorylation and sustained activation of calcium sensitive kinases such as ERK 1/2 (Putney, 1998). Indeed, ryanodine receptor inhibition blocks forms of hippocampal LTP (Martin and Buno 2003) and redox state influences intracellular Ca^{2+} release, thereby promoting synaptic plasticity via increased ERK phosphorylation (Hidalgo et al. 2005). Since ERK is one postulated "downstream" kinase from TrkB, indirect effects of calcium must be evaluated in future studies.

Conclusions

ROS formation during AIH is necessary for cellular events giving rise to brainstem-spinal cord mechanisms of respiratory LTF. Superoxide anions are likely to be the primary ROS species involved, primarily acting at sites localized in or near the respective motor nuclei (ie. phrenic and XII). One likely role of the superoxide anion is to regulate cellular phosphorylation state, thereby shifting the balance towards LTF-enabling processes. Overall, the results of this study significantly add to our understanding of the important signaling role played by spinal ROS formation in phrenic LTF. Further work is essential to determine the source of ROS and clarify its precise cellular and molecular targets.

Acknowledgements

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Abbreviations

5-HT, serotonin

aCSF, artifical cerebral spinal fluid

AIH, acute intermittent hypoxia

BDNF, brain derived neurotrophic factor

CIH, chronic intermittent hypoxia

ERK, extracellular regulated kinase

H_2O_2, hydrogen peroxide

i.v., intravenous

i.t., intrathecal

LTF, long term facilitation

$^{\bullet-}O_2$, superoxide anion

PaO2, arterial partial pressure of Oxygen

PaCO2, arterial partial pressure of carbon dioxide

ROS, reactive oxygen species

TrkB, tyrosine receptor kinase

XII, hypoglossal
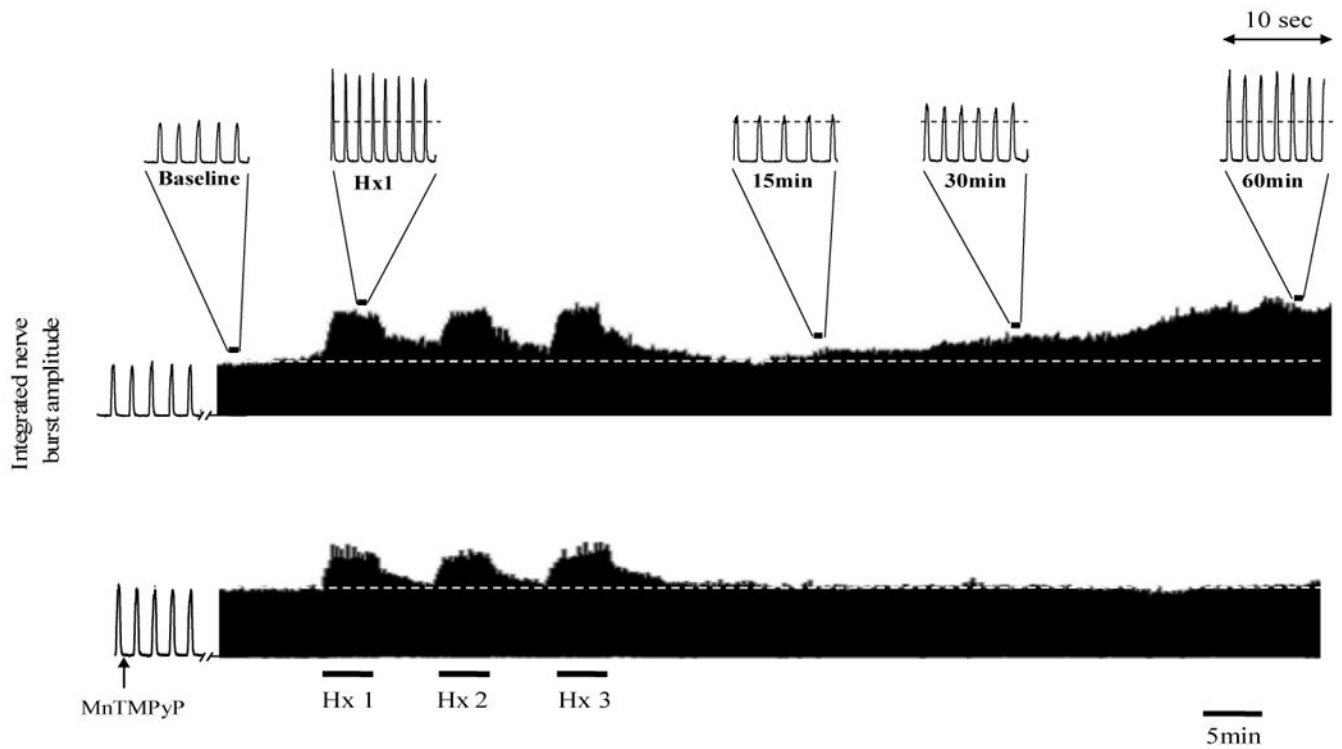
Figure 1.
Representative neurograms recorded from the phrenic nerve of an anesthetized rat before, during and after acute intermittent hypoxia (AIH) and with vehicle (top trace) or 10mg kg^{-1} MnTMPyP injected intravenously (lower trace). Also indicated are magnified bins (10 seconds duration) depicting the various time points for data analysis and blood sampling throughout the protocol Dashed line indicates baseline amplitude. Note after the third and final episode of hypoxia (Hx 3) the gradual increase in phrenic nerve burst amplitude above pre-AIH levels (i.e. LTF, top trace) compared to MnTMPyP injected rats in which LTF was blocked (lower trace).
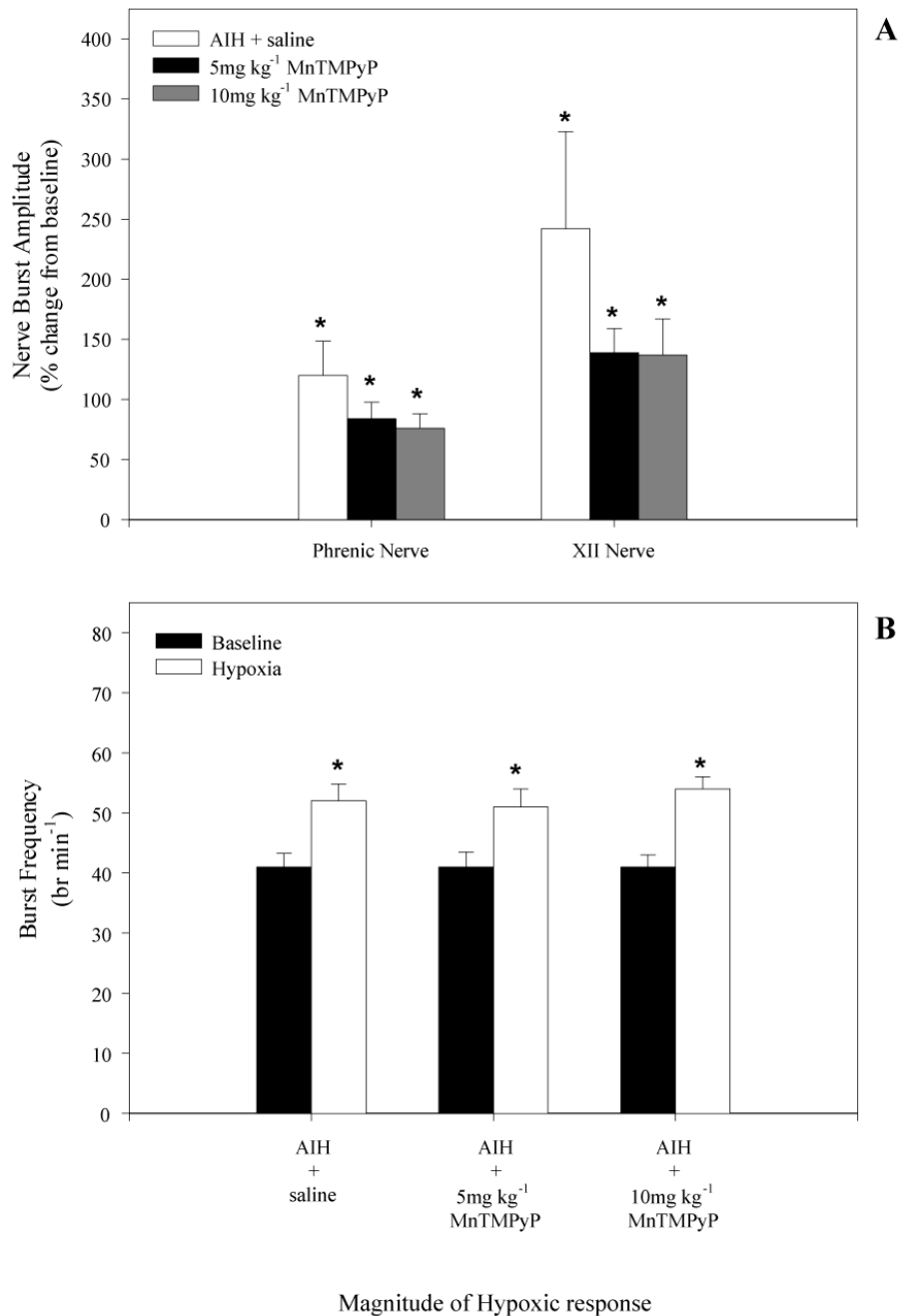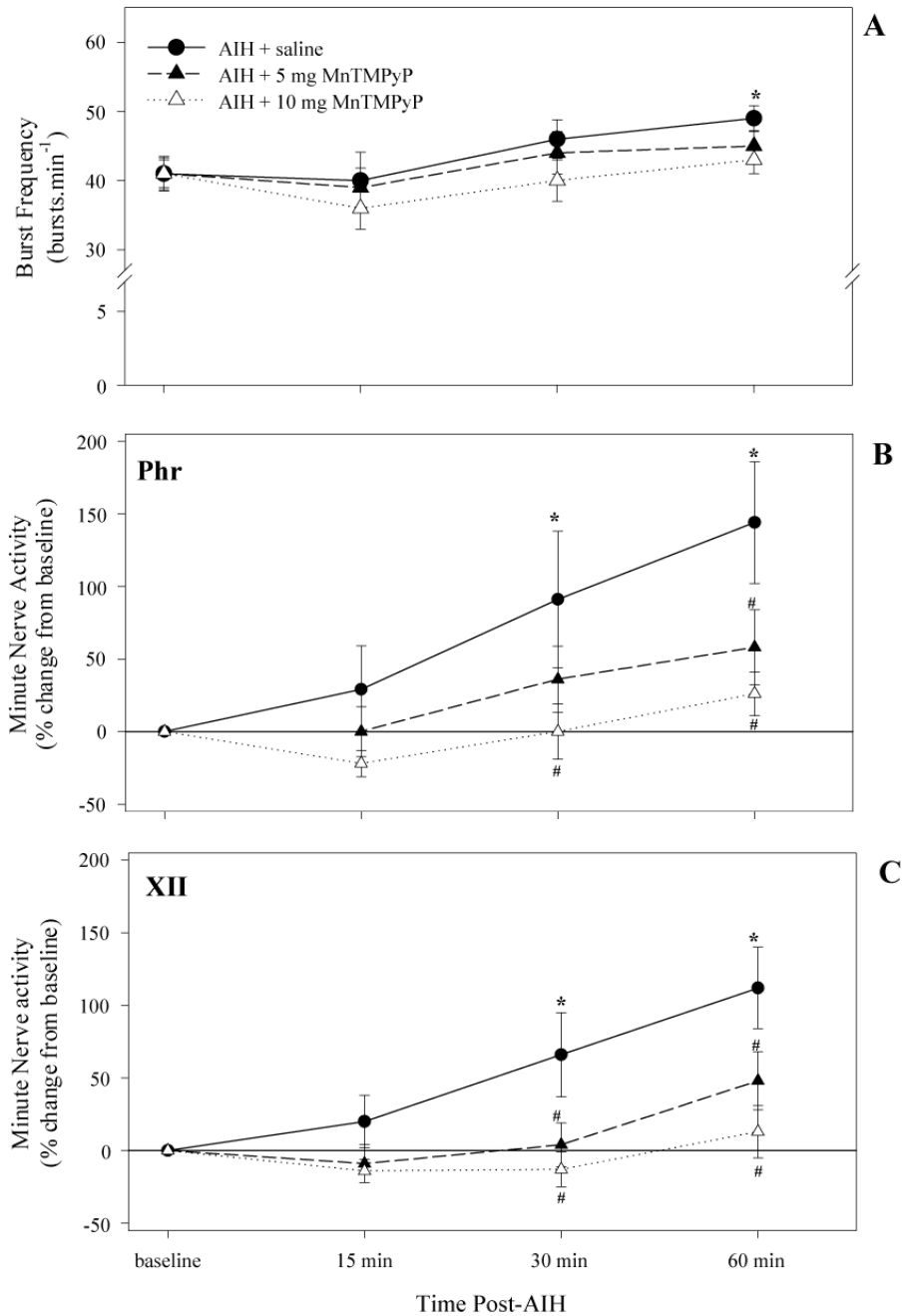
Figure 2.
(A) Phrenic and XII nerve burst amplitude during the first hypoxic episode in rats that received i.v. injections of either saline or MnTMPyP (5 and 10mg•kg^{-1}). Treatment groups are as indicated. Note, compared to saline injected rats, there was no effect of MnMTPyP injected i.v. on the magnitude of the hypoxic amplitude of phrenic or XII nerves. **(B)** Baseline breath frequency and hypoxic frequency response in saline, 5, and 10 mg•kg^{-1} treated rats. Note, there was no difference in baseline frequency between saline or MnTMPyP injected rats. MnTMPyP also had no effect on the magnitude of the hypoxic frequency response compared to saline treated rats. *indicates significant difference from baseline value (P<0.05).
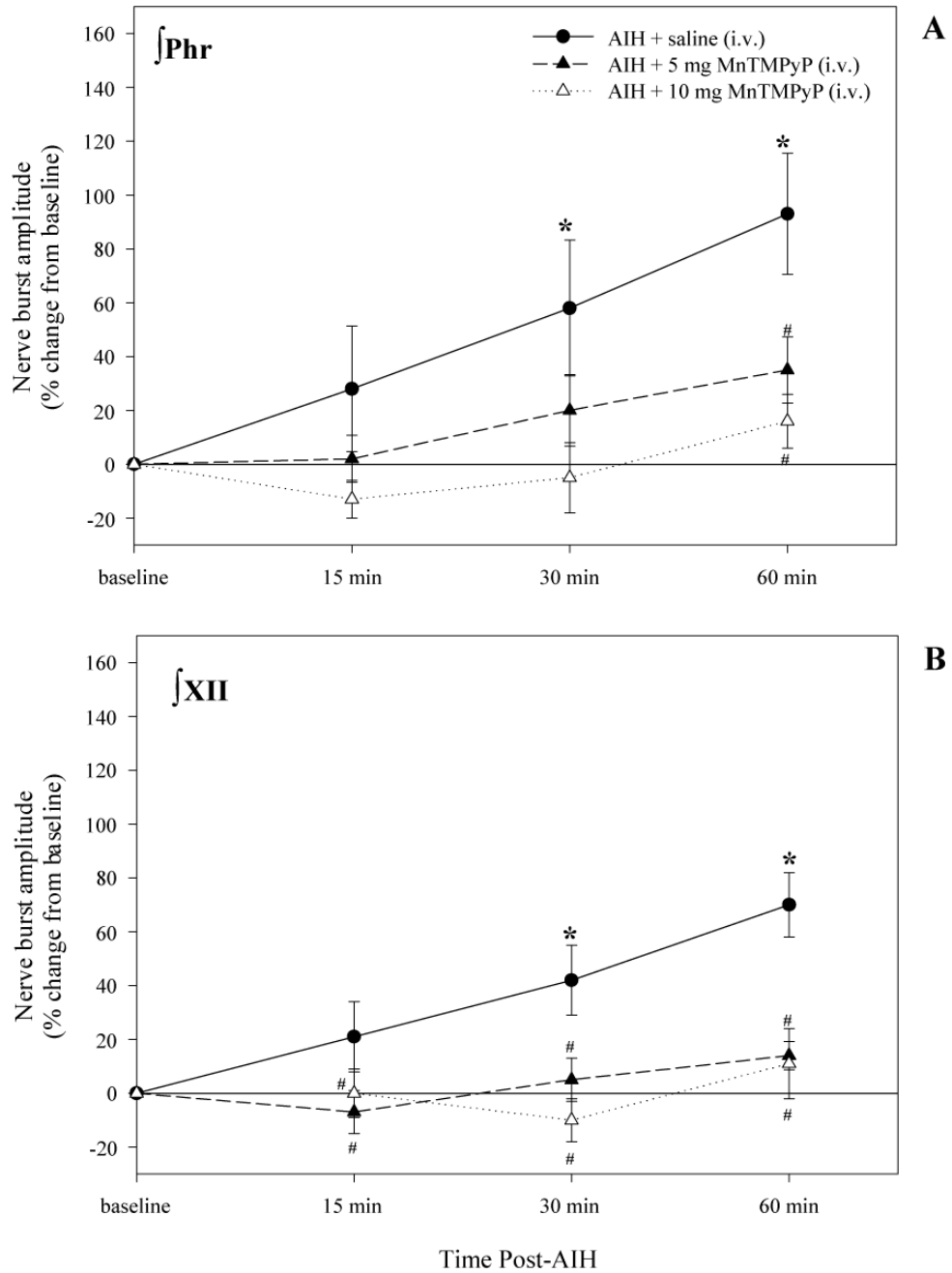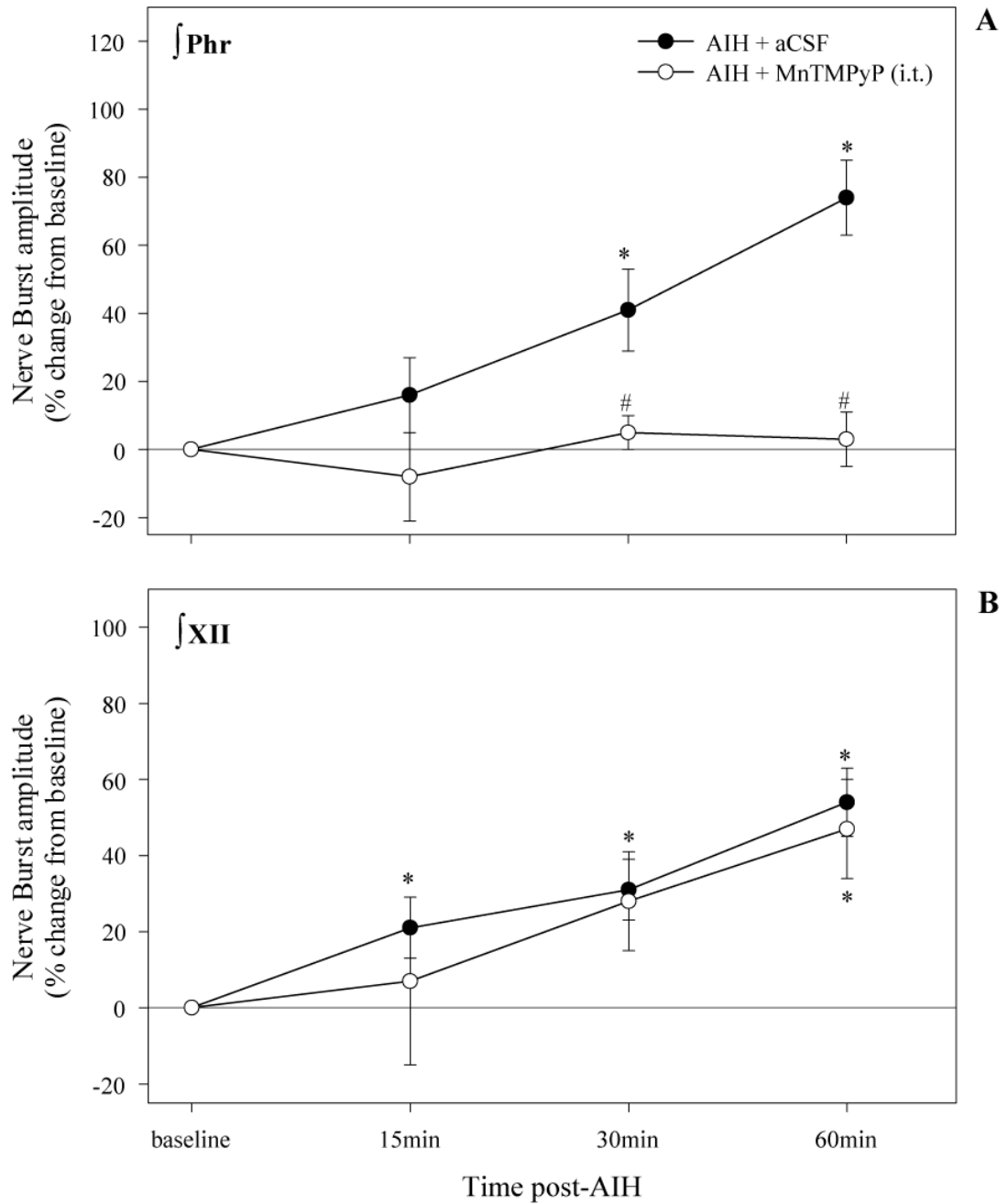
Figure 3.
Changes in phrenic **(A)** and XII **(B)** nerve burst amplitude at 15, 30, and 60 minutes post-AIH for each treatment group. Values are means ± 1 SEM and are expressed as % change from baseline amplitude (i.e. 0%). *indicates significant difference from baseline value (P<0.05); #indicates significant difference from AIH treated groups at each experimental time point (P<0.05).

Figure 4.
Frequency of respiratory motor output **(A)** and changes in minute neural activity (quantified as nerve burst amplitude (mV) $\times$ frequency (br min^{-1}) as recorded from the phrenic **(B)** and XII **(C)** nerves at 15, 30, and 60 minutes post-AIH for each treatment group. Treatment groups are as indicated. Values are means $\pm$ 1 SEM and are expressed as a % change from baseline. *indicates significant difference from baseline value; #indicates a significant difference from vehicle injected rats at the corresponding time point. Values are means $\pm$ 1 SEM.

Figure 5.
Phrenic and XII LTF in rats treated with an intrathecal injection of either aCSF (vehicle) or 5.5mM of the SOD mimetic, MnTMPyP. Note that application of the SOD mimetic to a localized region within the phrenic motor nuclei inhibited phrenic, but not XII LTF. Values are means ± 1 SEM. *signifies a significant difference from baseline; #indicates a significant difference from vehicle injected rats at the corresponding time point.

Table 1

Body temperature (Tb) and blood gas values for i.v. treated groups. Values are means ± 1SEM.

	Group	Tb	PaO$_2$	PaCO$_2$	pH	MAP
baseline	AIH + saline	37.3 ± 0.1	266.5 ± 6.5	44.9 ± 0.8	7.399 ± 0.013	111.1 ± 5.9
	AIH + 5mg kg^{-1} MnTMPyP	37.4 ± 0.1	258.4 ± 10.3	44.1 ± 1.2	7.379 ± 0.012	105.5 ± 4.7
	AIH + 10mg kg^{-1} MnTMPyP	37.3 ± 0.1	276.9 ± 8.0	46.4 ± 0.7	7.374 ± 0.005[#]	103.3 ± 6.6
Hypoxia	AIH + saline	37.3 ± 0.1	37.7 ± 1.5*	44.2 ± 1.0	7.397 ± 0.014	77.1 ± 8.5*
	AIH + 5mg kg^{-1} MnTMPyP	37.3 ± 0.1	40.2 ± 1.5*	44.1 ± 1.1	7.371 ± 0.011	69.7 ± 5.4*
	AIH + 10mg kg^{-1} MnTMPyP	37.2 ± 0.1*	38.7 ± 1.7*	46.8 ± 1.0	7.354 ± 0.008[#]	65.9 ± 7.0*
15 mins	AIH + saline	37.3 ± 0.1	209.9 ± 14.8*	44.7 ± 1.0	7.397 ± 0.018	112.3 ± 5.3
	AIH + 5mg kg^{-1} MnTMPyP	37.3 ± 0.1	196.6 ± 16.8*	44.3 ± 1.4	7.373 ± 0.011	105.9 ± 6.4
	AIH + 10mg kg^{-1} MnTMPyP	37.2 ± 0.1	200.1 ± 8.1*	46.2 ± 1.1	7.372 ± 0.007	112.8 ± 4.7
30 mins	AIH + saline	37.3 ± 0.1	250.7 ± 9.6	45.0 ± 1.0	7.402 ± 0.015	104.2 ± 4.9
	AIH + 5mg kg^{-1} MnTMPyP	37.3 ± 0.0*	238.6 ± 11.1	44.6 ± 1.4	7.376 ± 0.013	98.1 ± 7.9
	AIH + 10mg kg^{-1} MnTMPyP	37.2 ± 0.1*	254.9 ± 7.0	45.9 ± 0.9	7.383 ± 0.009	104.4 ± 5.2
60 mins	AIH + saline	37.4 ± 0.1	257.1 ± 10.1	45.2 ± 0.8	7.406 ± 0.015	97.5 ± 4.8*
	AIH + 5mg kg^{-1} MnTMPyP	37.3 ± 0.1	246.5 ± 8.1	44.0 ± 1.2	7.389 ± 0.014	93.7 ± 6.3*
	AIH + 10mg kg^{-1} MnTMPyP	37.2 ± 0.1*	266.0 ± 8.0	46.1 ± 0.5	7.388 ± 0.008*	99.4 ± 4.5

*
significant difference from baseline;

[#] significant difference from control group (AIH+saline); $P < 0.05$. Tb, body temperature; PaO2 and PaCO2, arterial pressure of oxygen and carbon dioxide, respectively; MAP, mean arterial pressure.