Differences in tyrosine hydroxylase expression after short-term hypoxia, hypercapnia or hypercapnic hypoxia in rat carotid body

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Abstract

In the carotid body (CB), it has been reported that the expressions of tyrosine hydroxylase (TH) mRNA and TH protein are enhanced by exposure to hypoxia. However, it is not known whether CO_2 affects the expression of TH in the CB. We examined the expression of TH mRNA and the immunoreactivity for TH in the CB of rats exposed to hypoxia (10% O_2), hypercapnia (10% CO_2) and hypercapnic hypoxia (10% O_2 and 10% CO_2) for 2 to 24 hours. The expression of TH mRNA in the CB was markedly enhanced in rats exposed to hypoxia for 4 hours (6.6-fold), 6 hours (6.0-fold) and 8 hours (7.8-fold), and in rats exposed to hypercapnic hypoxia for 12 hours (4.8-fold). The most intense TH immunoreactivity was observed in the CB from rats exposed to hypoxia for 12 and 24 hours and to hypercapnic hypoxia for 24 hours. The expressions of TH mRNA and the immunoreactivity for TH were not altered in the CB of rats exposed to hypercapnia. It is suggested that CO_2 does not affect TH expression in the CB, and that it inhibits hypoxia-enhanced TH expression.

Key words: respiratory reflex; hypoxia; hypercapnia; carotid body; tyrosine hydroxylase; chemoreception

1. Introduction

The respiratory reflexes differ among hypoxia, hypercapnia and hypercapnic hypoxia (Garcia and Cherniack, 1967; Hirakawa et al., 1997; Yasuma et al., 2001). It is generally suggested that hypoxia leads to an increased respiratory frequency and hypercapnia leads to an increased tidal volume (Garcia and Cherniack, 1967). Furthermore, Hirakawa et al. (1997) reported that the hyperventilation induced by hypercapnic hypoxia was more intense than the hyperventilation induced by hypoxia.

Arterial chemoreceptors sense lowering of PaO_2 and elevation of $PaCO_2$ to induce respiratory reflexes. One of the arterial chemoreceptors, the carotid body (CB), contains glomus cells that have sensitivity to O_2, CO_2 and H^+ (Dasso et al., 2000; Roy et al., 2000; Nurse, 2005). Hyperventilation induced by hypoxia and/or hypercapnia is mediated by the glomus cells, which transmit information concerning PaO_2 and $PaCO_2$ to primary sensory afferent terminals by the release of excitatory neurotransmitters, such as acetylcholine and ATP (Zhang and Nurse, 2004; Nurse, 2005; Gourine, 2005; Fitzgerald et al., 2006). It has also been reported that dopamine, which is secreted by the glomus cells, has an inhibitory role in CB excitation (Docherty and McQueen, 1978; Lahiri et al., 1984; Wang and Bisgard, 2002; Nurse, 2005; Powell, 2007). Dopamine binds to D_2 receptors to inhibit L-type Ca^{2+} channels in rat isolated glomus cells and to decrease acetylcholine release from rabbit CBs (Benot and López-Barneo, 1990; Kim et al, 2004). Existence of dopamine D_2 receptors in CBs has been confirmed (Lazanov et al., 2009; Kåhlin et al., 2010). Moreover, *in vivo* experiments demonstrated that dopamine is released from glomus cells during

hypoxia (Fidone et al., 1982; Iturriaga and Alcayaga, 1998; Buerk et al., 1998; Wang and Bisgard, 2002; Powell, 2007), and dopamine content in the CB is increased by exposure to sustained hypoxia for 2-28 days (Hanbauer et al., 1981; Olson et al., 1983; Pequignot et al., 1987; Hui et al., 2003). In the CB, both short-term (within 24 hours) and long-term (for over a week) hypoxia enhances expression of the rate-limiting enzyme for catecholamine synthesis, tyrosine hydroxylase (TH), at mRNA and protein levels (Czyzyk-Kruzeska et al., 1992; Wang et al., 1998; Wang and Bisgard, 2002; Hui et al., 2003; Kato et al., 2010). Furthermore, Buerk et al. (1998) reported that dopamine was initially increased by hypercapnia and then decreased substantially below baseline. It is not known whether dopamine release and TH expression are altered to modulate respiratory drive under hypercapnic and hypercapnic hypoxic conditions. In addition to respiratory reflex, discharge of carotid sinus nerve (CSN), which is afferent nerve from CB, are different between hypoxic stimulation and hypercapnic stimulation in rats and cats (Lahiri and DeLaney, 1975; Fukuda et al., 1987). Because respiratory reflexes and CSN discharge differ among different gases, it is expected that the pattern of TH expression changes with different environmental gases.

In the present study, we examined changes in the expression of TH in the CB of rats exposed to hypoxia, hypercapnia and hypercapnic hypoxia at both mRNA and protein levels using quantitative real-time RT-PCR and immunofluorescence, respectively.

2. Materials and Methods

2.1. Gas exposure

Animal experimental protocols were approved by the Committee on the Use of Live Animals in Teaching and Research of Iwate University.

Male Wistar rats (8-10 weeks old; 180-200 g) were used in the present study. Animals were divided into four groups that were exposed to different gases: 1) hypoxia (10% O_2 and <1% CO_2), 2) hypercapnia (20% O_2 and 10% CO_2), 3) hypercapnic hypoxia (10% O_2 and 10% CO_2), 4) control (20% O_2 and <1% CO_2). Each rat was placed in a cage (16 x 27 x 13 cm) inside an acrylic chamber (50 x 40 x 50 cm). Three holes, located at the top of a sidewall of the chamber, were connected to three types of gas cylinders (O_2, CO_2 and N_2). O_2 and CO_2 levels within the chamber were monitored with a gas analyzer and each gas level was maintained automatically at a constant level. In the hypoxic experiment, O_2 concentration was maintained at 9.8-10.2% by N_2 influx. In the hypercapnic experiment, CO_2 and O_2 concentrations were maintained at 9.6-10.4% by CO_2 influx and at 20-20.5% by O_2 influx, respectively. In the hypercapnic hypoxic experiment, O_2 and CO_2 concentrations were maintained at 9.6-10.4% by N_2 influx and at 9.6-10.4% by CO_2 influx, respectively. All of the rats were allowed 15-30 minutes to adjust to their environment in the acrylic chamber ventilated by air. In the control experiment, O_2 concentration was maintained at 20-20.5% by O_2 influx and CO_2 concentration was maintained at <1% by N_2 influx. The temperature within the chamber was maintained at 25 ˚C. In quantitative real-time RT-PCR studies, rats were exposed to each gas condition for 2, 4, 6, 8,

12 and 24 hours (six rats per time point). The control experiment was performed to analyze the effect of stress associated with confinement in the chamber. For immunofluorescence, we used rats exposed to hypoxia, hypercapnia and hypercapnic hypoxia for 6, 12 and 24 hours (four rats per time point).

2.2. Quantitative real-time RT-PCR

For quantitative real-time RT-PCR analysis, the gas-exposed rats were anesthetized using ether gas. Then, the animals were sacrificed by exsanguination from abdominal aorta, and CB was dissected out and quickly frozen in liquid N_2. Total RNA from CB was extracted using a magnetic bead method (MELT total RNA extraction kit, Ambion, Austin, TX), and the quantities of TH mRNA were analyzed by SYBR green-based real-time RT-PCR method using StepOne real-time thermal cycler with Power SYBR Green RNA-to-C_T^{TM} 1-Step Kit (Applied Biosystems, Foster City, CA). All total RNA samples were checked at these concentrations and qualities by Bioanalyzer (Agilent Technologies, Waldbronn, DE). Bioanalyzer generates the RNA Integrity Number (RIN), a quantitative estimate, and can calculate ribosomal ratios of the total RNA sample. RIN provides a numerical assessment of the integrity of RNA. We used only samples for which an RIN value >7 was indicated. Primers for quantitative real-time RT-PCR analysis are listed in Table 1. Reverse transcription was performed for 30 min at 48 °C and initial PCR activation was incubated for 10 min at 95 °C. After reverse transcriptional, PCR amplifications were performed 40 cycles as follows: 15 sec at 95 °C for denaturation and 1 min at 60 °C for annealing. After PCR amplification, to assess cDNA quality we

observed melting curves of PCR products. All PCR experiments were externally calibrated using standards prepared from serially diluted and PCR products of known concentrations (Fig. 1). Statistical comparisons between control and experimental values were performed using the Kruskal-Wallis test with post-hoc test (Bonferroni test).

2.3. Immunohistochemistry

We examined the change in the fluorescent intensity of TH immunoreactivity in the glomus cells in each experimental group with an indirect immunofluorescnece technique. We also examined the change in the immunoreactivity of TH in the glomus cells of non-treatment rats as non-treatment control.

The animals were anesthetized using pentobarbital (15 mg/kg; intraperitoneal injection) and transcardially perfused with Ringer's solution (200 ml) followed by 4% paraformaldehyde and 0.5% picric acid in 0.1 M phosphate buffer (pH 7.4, 200 ml). The bifurcations of carotid arteries containing CB were then removed under a dissecting microscope and immersed in the same fixative for an additional 6-8 hours at 4 ˚C. After three washes in PBS for 10 minutes, the tissue samples were transferred to 30% sucrose in PBS at 4 ˚C for 24 hours. The CB was sectioned at a thickness of 10 μm and mounted on glass slides coated with chrome alum-gelatin. The sections were incubated with mouse monoclonal antibody against TH (1:2,000, Chemicon International, Temecula, CA) and then incubated with Alexa488-labeled donkey anti-mouse IgG (1:200, Invitrogen, Carlsbad, CA) for 2 hours at 25 ˚C. For morphometrical analysis, the

micrographs of the TH immunofluorescence were converted into grayscale images (256 shades of gray) for measurement of the TH fluorescent intensity. Then, the average of grayscale intensity (range 0-255) of pixels in the cluster of the glomus cells was measured using NIH Scion Image program (Scion Corp., Frederick, MD) on a personal computer. At least 20 clusters of the glomus cells were randomly measured for each experimental group.

3. Results

3.1. Quantitative real-time RT-PCR

3.1.1. Expression of TH mRNA in the CB of rats exposed to hypoxia

The time-dependent change in the expression of TH mRNA in the hypoxic exposure experiment is shown in Fig. 2A. TH mRNA was expressed in the CB of rats exposed to hypoxia for 0 hour (non-treatment; TH mRNA/ 18S rRNA, 0.0017 ± 0.0003), 2 hours (0.0037 ± 0.0020), 4 hours (0.0148 ± 0.0071), 6 hours (0.0136 ± 0.0037), 8 hours (0.0175 ± 0.0056), 12 hours (0.0101 ± 0.0041) and 24 hours (0.0049 ± 0.0015). The expressions of TH mRNA were significantly enhanced in the CB of rats exposed to hypoxia for 4 hours (approximately 6.6-fold), 6 hours (approximately 6.0-fold) and 8 hours (approximately 7.8-fold) compared with those of rats exposed for 0, 2 and 24 hours (Fig. 2A). A significant increase in TH mRNA was observed in the CB of rats exposed to hypoxia for 8 hours above that of 12 hours (approximately 4.4-fold; Fig. 2A). In the rat CB, the expression of TH mRNA was significantly elevated after 12 hours of exposure to hypoxia above that of 24 hours of exposure (Fig. 2A). The expression of TH mRNA was significantly increased in the CB of rats exposed to hypoxia for 8 hours compared with that of 2 hours (approximately 4.8-fold; Fig. 2A).

3.1.2. Expression of TH mRNA in the CB of rats exposed to hypercapnia

The expressions of TH mRNA in the hypercapnic exposure experiment are shown in Fig. 2B. We observed expressions of TH mRNA in the CB of rats exposed to hypercapnia for 2 hours (0.0021 ± 0.0010), 4 hours (0.0030 ±

0.0006), 6 hours (0.0030 ± 0.0008), 8 hours (0.0032 ± 0.0010), 12 hours (0.0027 ± 0.0017) and 24 hours (0.0032 ± 0.0008). In the CB of rats exposed to hypercapnia, the expressions of TH mRNA did not significantly change between any time points (Fig. 2B).

3.1.3. Expression of TH mRNA in the CB of rats exposed to hypercapnic hypoxia

The change of the expression of TH mRNA by hypercapnic hypoxic exposure is shown in Fig. 2C. TH mRNA was also expressed in the CB of rats exposed to hypercapnic hypoxia for 2 hours (0.0032 ± 0.0015), 4 hours (0.0069 ± 0.0029), 6 hours (0.0067 ± 0.0040), 8 hours (0.0072 ± 0.0036), 12 hours (0.0124 ± 0.0052) and 24 hours (0.0052 ± 0.0015). The peak of the upregulation was observed at 12 hours of exposure in the CB of rats exposed to hypercapnic hypoxia (Fig. 2C). The expression of TH mRNA was significantly enhanced in the CB of rats exposed to hypercapnic hypoxia for 12 hours (approximately 4.8-fold) compared with those of 0, 2 and 24 hours (Fig. 2C).

3.1.4. Expression of TH mRNA in the CB of chamber control rats

In the control experiments using a chamber with normoxic/normocapnic gas, there was no change in the expressions of TH mRNA in any time point (exposure for 2 hours, 0.0028 ± 0.0005; 4 hours, 0.0029 ± 0.0017; 6 hours, 0.0042 ± 0.0017; 8 hours, 0.0037 ± 0.0011; 12 hours, 0.0035 ± 0.0013; 24 hours, 0.0035 ± 0.0015; Fig. 2D).

3.1.5. Comparison of expressions of TH mRNA among the three gas experiments

Fig. 3 shows the expressions of TH mRNA in the CB of rats exposed to hypoxia, hypercapnia, hypercapnic hypoxia and normoxic/normocapnic gas at each exposure time point. At 4, 6 and 8 hours, the expressions of TH mRNA were significantly enhanced in the CB of rats exposed to hypoxia compared with those of rats exposed to hypercapnia, rats exposed to hypercapnic hypoxia and chamber control rats (Fig. 3). Furthermore, at 12 hours, the expressions of TH mRNA were significantly enhanced in the CB of rats exposed to hypoxia and rats exposed to hypercapnic hypoxia compared with those of rats exposed to hypercapnia and chamber control rats (Fig. 3).

3.2. Immunohistochemistry

3.2.1. TH immunoreactivities in the glomus cells of rats exposed to hypoxia, hypercapnia and hypercapnic hypoxia

TH immunoreactive glomus cells were observed in the CB of all rats (Fig. 4). In the CB of rats exposed to hypoxia, the grayscale intensity of TH immunofluorescence was 84.5 ± 12.7 (exposure for 0 hour, non-treatment), 86.8 ± 12.8 (6 hours), 122.0 ± 9.5 (12 hours) and 127.1 ± 8.8 (24 hours). The TH immunoreactivity was significantly enhanced in the CB of rats exposed to hypoxia for 12 hours (vs. control, approximately 1.4-fold; vs. 6 hours, approximately 1.4-fold) and 24 hours (vs. control, approximately 1.5-fold; vs. 6 hours, approximately 1.5-fold) compared with those of the control and those exposed for 6 hours (Fig. 5). The TH immunoreactivities did not change in the CB of rats exposed to hypercapnia (6 hours, 88.2 ± 13.4; 12 hours, 89.9 ± 24.5; 24 hours, 82.3 ± 12.3; Fig. 5). We found that, following 24 hours (126.4 ± 14.7) of

exposure to hypercapnic hypoxia, the TH immunoreactivity was significantly increased in the CB compared with that of the control (approximately 1.5-fold), and those exposed for 6 hours (83.6 ± 13.7, approximately 1.5-fold) and 12 hours (98.4 ± 14.3, approximately 1.5-fold; Fig. 5).

3.2.2. Comparison of TH immunoreactivities among the three gas experiments

At each exposure time point, we examined the TH immunoreactivity of the CB among hypoxia, hypercapnia and hypercapnic hypoxia groups (Fig. 5). In the CB, the TH immunoreactivity of rats exposed to hypoxia at 12 hours was significantly increased compared with that of rats exposed to hypercapnia or hypercapnic hypoxia (Fig. 5). Furthermore, at 24 hours, the TH immunoreactivity was significantly enhanced in the CB of rats exposed to hypoxia or hypercapnic hypoxia compared with that of rats exposed to hypercapnia (Fig. 5).

4. Discussion

4.1. Hypoxic chemoreception in the CB

Our quantitative RT-PCR data, which indicated TH mRNA expression was enhanced by short-term hypoxia, confirms a report by Czyzyk-Krzeska et al. (1992). It has been shown that the enhancement of the expression of TH mRNA by hypoxia is induced by hypoxia-inducible factor (HIF; Schnell et al., 2003; Zagorska and Dulak, 2004; Powell and Fu, 2008). In isolated glomus cell, Roy et al. (2007) reported that expression of HIF-1α was increased by hypoxia. In the present study, therefore, the expression of TH mRNA may be induced by HIF-1 in the CB of rat exposed to hypoxia. However, recent study indicated possibility that hypoxia-induced TH mRNA was mediated by adenosine A_2A receptor but not HIF-1 in PC12 cells (Gammella et al., 2010). In addition to HIF-1, it is possible that A_2A receptor is one of candidates to induce TH mRNA expression under hypoxia.

Although the marked enhancement of TH mRNA by hypoxia was transient, the enhancement of TH immunoreactivity was maintained after over 12 hours of exposure to hypoxia. The hypoxia-increased TH mRNA leads to an increase in TH protein (Feinsilver et al., 1987). The enhancement of TH immunoreactivity induced by hypoxia could be due to an increase in TH protein. Therefore, it is suggested that the expression of TH mRNA returns to the control level after a sufficient amount of TH protein is secured. Conde et al. (2006) reported that dopamine content was increased by exposure to hypoxia in rat CB. Welsh et al. (1978) suggested that dopamine suppressed ventilation through action of the CB.

In addition, inhibition of peripheral D_2 receptor by domperidone, which is specific peripheral D_2 antagonist, enhanced sensitivity to hypoxia in the CB of various animal species (Letiner and Roumy, 1986; Kressin et al., 1986; Tomares et al., 1994; Edwards et al., 2008). From our results and these reports, we suggest that TH induced by exposure to hypoxia may stimulate dopamine synthesis in order to prevent intense hyperventilation.

4.2. Hypercapnic chemoreception in the CB

Hypercapnia as well as hypoxia increased glomus cell activity (Zhang and Nurse, 2004), CSN discharge (Roy et al., 2000) and respiratory reflex (Garcia and Cherniack, 1967; Yasuma et al., 2001). In contrast to the physiological changes, catecholamine release was not induced by exposure to hypercapnia in the CB (Hellstörm et al., 1989; Iturriaga and Alcayaga, 1998). In the present study, hypercapnia did not affect the expression of TH mRNA and the immunoreactivity for TH. Therefore, CO_2 may not induce synthesis and release of dopamine in the CB. Generally, CO_2 is sensed at not only the CB but also the central chemosensitive neurons (Lahiri and Forster, 2003). Some reports showed that the respiratory reflex induced by hypercapnia was separated into two parts, that is, fast and slow responses by the CB and the central chemosensitive neurons, respectively (Smith et al., 2006; Dahan et al., 2007). In addition, Nakano et al. (2002) reported that domperidone increased ventilatory response to hypoxia and did not affect that to hypercapnia. Because the TH expression was not increased in the CB of rats exposed to hypercapnia, dopamine may be barely associated with the fast response to CO_2.

4.3. Hypercapnic hypoxic chemoreception in the CB

Our results from hypercapnic hypoxic experiments suggest that CO_2 suppress the hypoxia-induced expression of TH mRNA. It has been reported that the activity of HIF-1α, which is one of the enhancers of TH mRNA expression, was suppressed by acidosis in endothelial cells (Melchionna et al., 2010). It is speculated that HIF-1α in glomus cells are inhibited by acidosis to suppress expression of TH mRNA. In glomus cells of rats exposed to hypercapnia, He et al. (1991) reported that intracellular pH was decreased. In the experiments involving exposure to hypercapnic hypoxia, therefore, it is suggested that the TH mRNA expression was suppressed due to inhibition of HIF-1α by CO_2-induced intracellular acidosis in the CB of rats exposed to hypercapnic hypoxia for 4-8 hours. We observed that the enhancement of TH immunoreactivity induced by hypoxia was delayed by hypercapnia. This delay is probably related to the suppression of the enhancement of TH mRNA. TH regulates dopamine synthesis and dopamine inhibits the activation of glomus cells (Wang and Bisgard, 2002). Zhang and Nurse (2004) reported that action potential frequency was higher in the glomus cells of rats exposed to hypercapnic hypoxia than in cells exposed to hypoxia or hypercapnia. In addition, it has been reported that the increase in $[Ca^{2+}]_i$ of the glomus cells induced by hypercapnic hypoxia is the largest among the three gas stimulations, and $[Ca^{2+}]_i$ responses of the glomus cells to hypoxia and hypercapnia are multiplicative in most cases (Dasso et al., 2000; Roy et al., 2000). Thus, the inhibition of TH in an early stage of exposure to hypercapnic hypoxia may lead to increased chemoreflex in the CB. In the rats

exposed to hypercapnic hypoxia, it is suggested that the intense hyperventilation is caused by the CB in cooperation with central chemosensitive neurons. Taken together, it is suggested that CO_2 is important factor in the modulation of hypoxia-induced expression of TH mRNA. The expression of TH mRNA may reflect differences in the respiratory reflex among the three gas conditions. Ventilatory responses to hypoxia and/or hypercapnia were different among newborn and adult animal species (Mortola and Lanthier, 1995; Putnam et al., 2004). Thus, it must be noted that expressions of TH mRNA induced by hypoxia and/or hypercapnia may vary among animal species and change during development.

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Figure legends

Fig. 1. Standard curves of TH mRNA (A) and 18S rRNA (B). Standard curves were generated by plotting the C_T vs. the quantity of PCR product of known concentration.

Fig. 2. Time course of expressions of TH mRNA in the CB of rats exposed to four gas conditions. (A) Expression of TH mRNA was marked enhanced by 4, 6 and 8 hours of exposure to hypoxia. (B) Expression of TH mRNA was not changed by exposure to hypercapnia. (C) Expression of TH mRNA was marked enhanced by 12 hours of exposure to hypercapnic hypoxia. (D) Expression of TH mRNA was not changed by exposure to control gas. [a] $p<0.05$ vs. 0, 2, 12 and 24 hours, [b] $p<0.05$ vs. 0, 2 and 24 hours, [c] $p<0.05$ vs. 0 and 24 hours.

Fig. 3. Difference of expression of TH mRNA induced by hypoxia (gray bar), hypercapnia (hatched bar), hypercapnic hypoxia (black bar) or chamber control (white bar) at each time point. Exposure to hypoxia enhanced expression of TH mRNA most intensely among four experiments at 4, 6 and 8 hours in the CB. At 12 hours, intense expression of TH mRNA was observed in the CB of rats exposed to hypoxia and hypercapnic hypoxia. [*1] $p<0.05$ vs. hypercapnia, hypercapnic hypoxia and chamber control, [*2] $p<0.05$ vs. hypercapnia and chamber control.

Fig. 4. Immunofluorescence for TH in glomus cells of control (no exposure; A), and rats exposed to hypoxia (B), hypercapnia (C) or hypercapnic hypoxia (D) for

24 hours. TH immunoreactivities were enhanced in the CB of rats exposed to hypoxia and hypercapnic hypoxia. TH immunoreactivity was not changed in the CB of rats exposed to hypercapnia.

Fig. 5. Time course of TH immunoreactivities induced by hypoxia (gray bar), hypercapnia (hatched bar), hypercapnic hypoxia (black bar) and control (non-treatment; white bar). TH immunoreactivity was markedly enhanced in the CB of rats exposed to hypoxia for 12 and 24 hours and hypercapnic hypoxia for 24 hours. [a] $p<0.05$ vs. control and 6 hours, [b] $p<0.05$ vs. 12 hours, [*1] $p<0.05$ vs. hypercapnia and hypercapnic hypoxia, [*2] $p<0.05$ vs. hypercapnia.

Table 1. Primers for quantitative real-time RT-PCR

mRNA (Accession number)	Primer sequences	Position	Product length (bp)
TH (NM_012740)	5'-GGAGCTGAAGGCTTATGGTG-3' (sense) 5'-CATTGAAGCTCTCGGACACA-3' (antisense)	1163-1182 1310-1329	167
VEGF (NM_031836)	5'-AATGATGAAGCCCTGGAGTG-3' (sense) 5'-ATGCTGCAGGAAGCTCATCT-3' (antisense)	259-278 353-372	114
18S rRNA (X03205)	5'-CCTGCGGCTTAATTTGACTC-3' (sense) 5'-AACTAAGAACGGCCATGCAC-3' (antisense)	1230-1249 1328-1347	118

Fig. 1

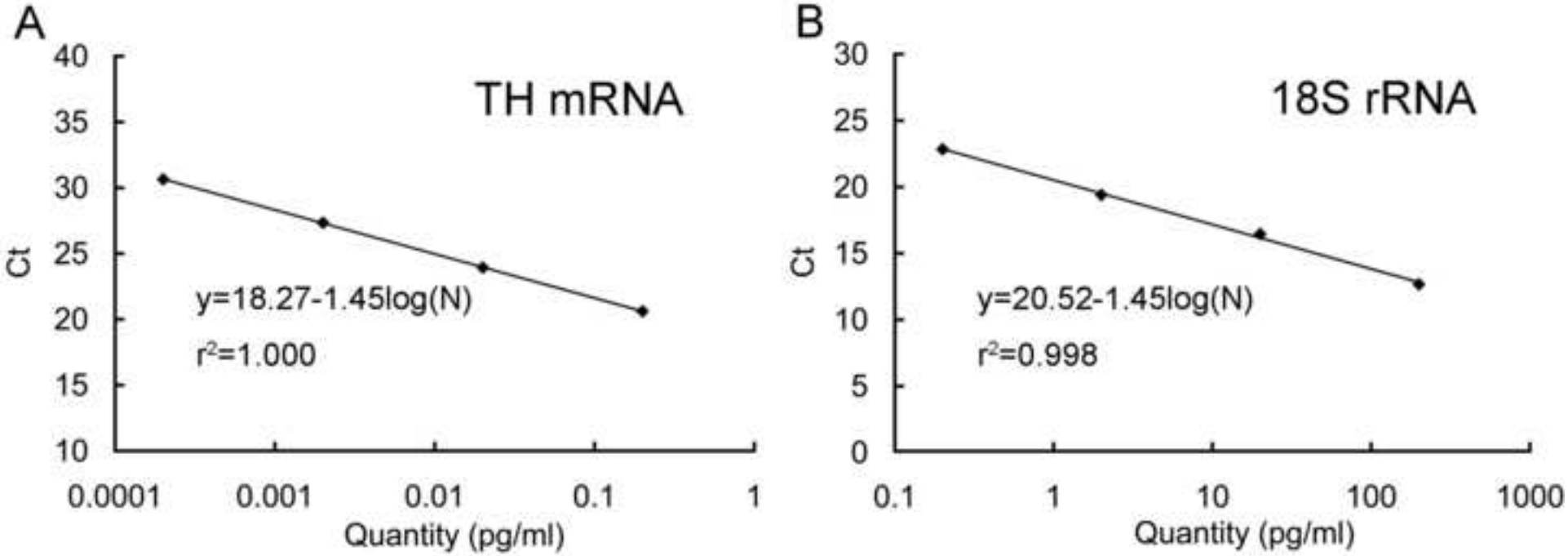

A
TH mRNA
Ct
40
35
30
25
20
15
10
y=18.27-1.45log(N)
r²=1.000
0.0001
0.001
0.01
0.1
1
Quantity (pg/ml)
B
18S rRNA
Ct
30
25
20
15
10
5
0
y=20.52-1.45log(N)
r²=0.998
0.1
1
10
100
1000
Quantity (pg/ml)

Fig. 2

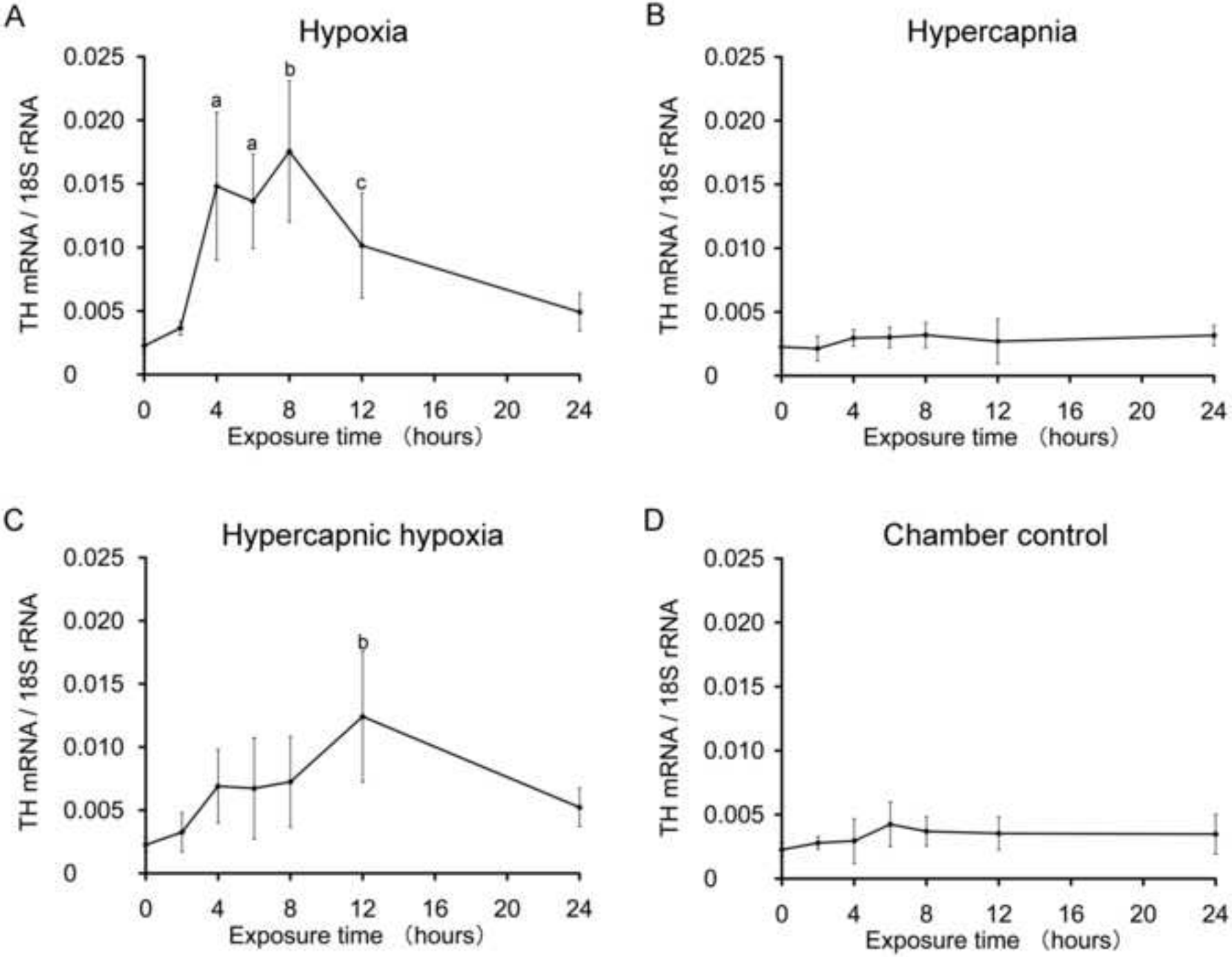

A
Hypoxia
TH mRNA / 18S rRNA
0.025
0.020
0.015
0.010
0.005
0
a
a
b
c
0
4
8
12
16
20
24
Exposure time (hours)
B
Hypercapnia
TH mRNA / 18S rRNA
0.025
0.020
0.015
0.010
0.005
0
0
4
8
12
16
20
24
Exposure time (hours)
C
Hypercapnic hypoxia
TH mRNA / 18S rRNA
0.025
0.020
0.015
0.010
0.005
0
b
0
4
8
12
16
20
24
Exposure time (hours)
D
Chamber control
TH mRNA / 18S rRNA
0.025
0.020
0.015
0.010
0.005
0
0
4
8
12
16
20
24
Exposure time (hours)

Fig. 3

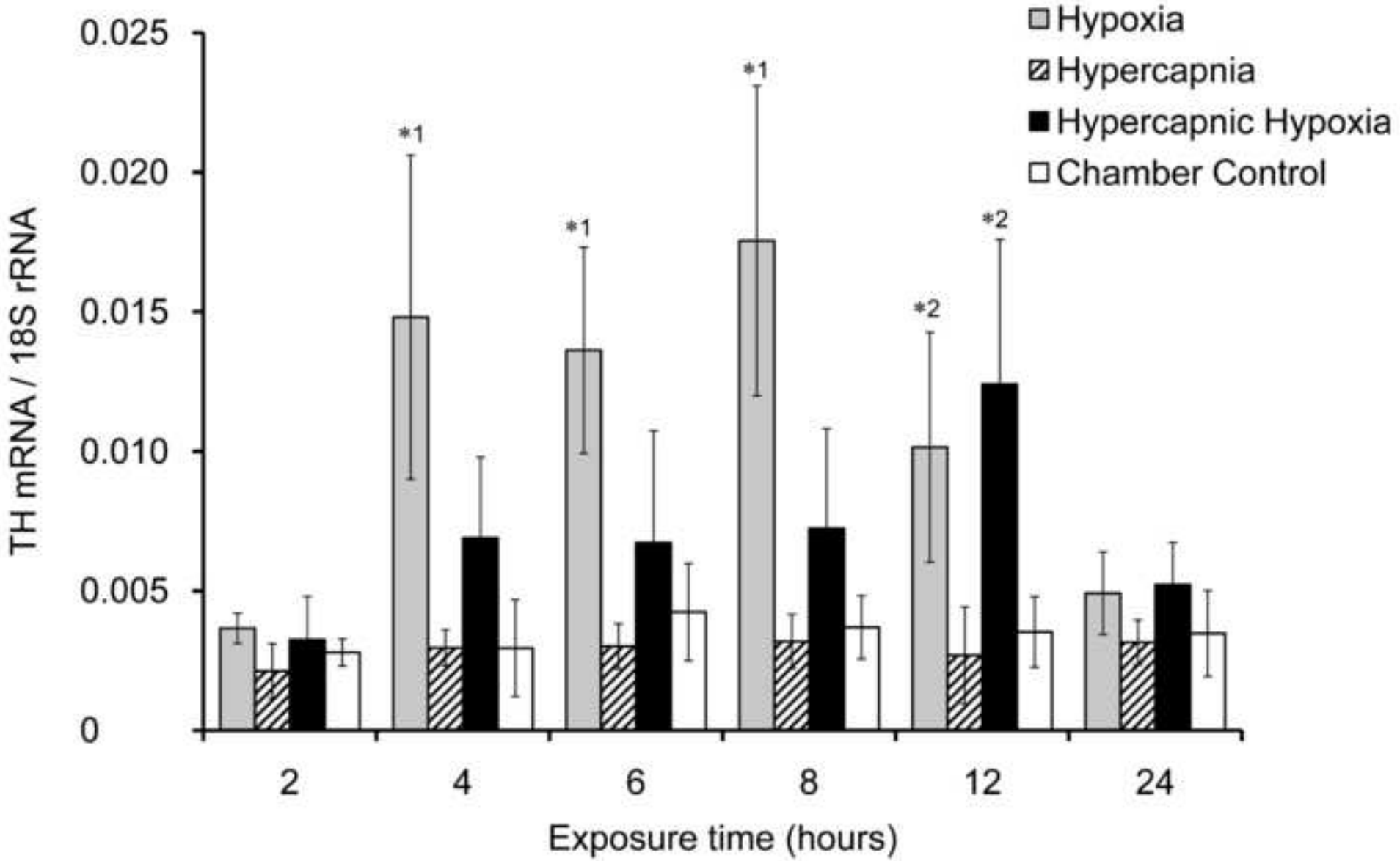

Hypoxia
Hypercapnia
Hypercapnic Hypoxia
Chamber Control
TH mRNA / 18S rRNA
0.025
0.020
0.015
0.010
0.005
0
*1
*1
*1
*2
*2
2
4
6
8
12
24
Exposure time (hours)

Fig. 4

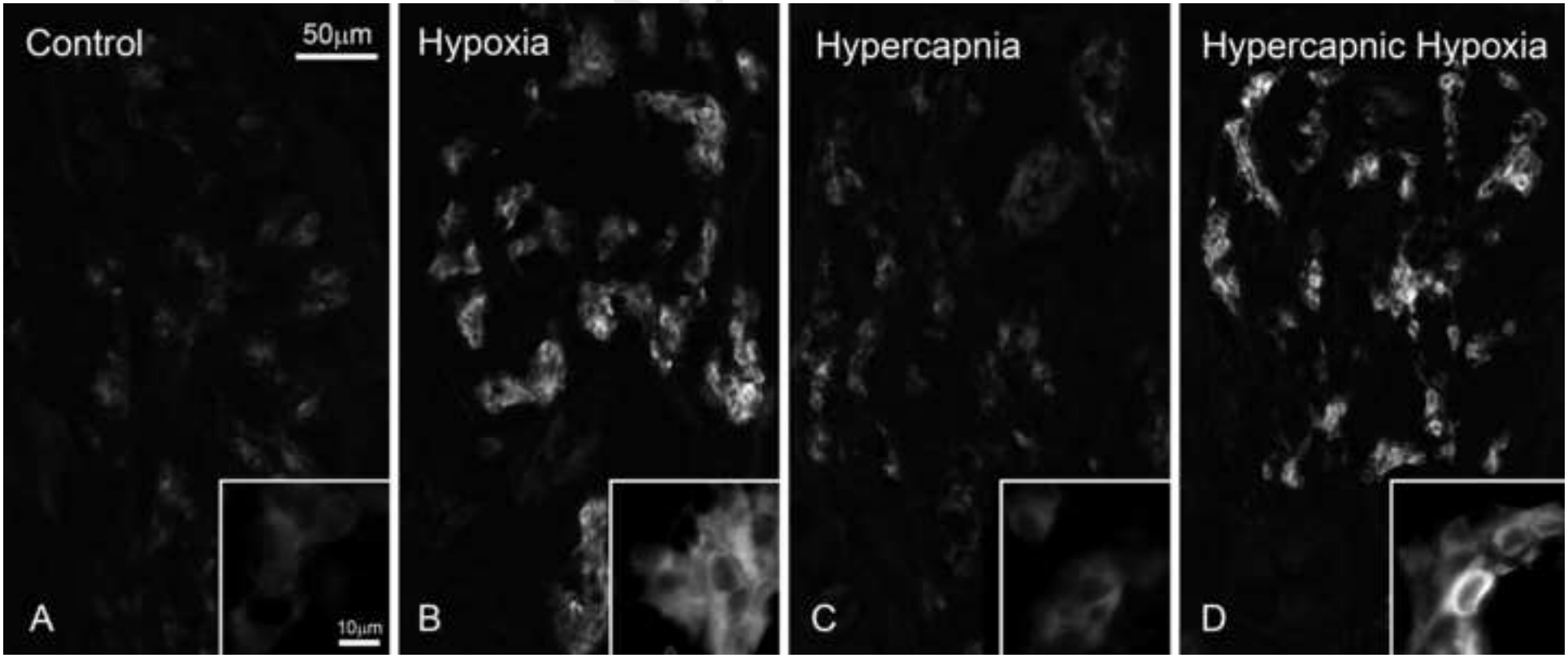
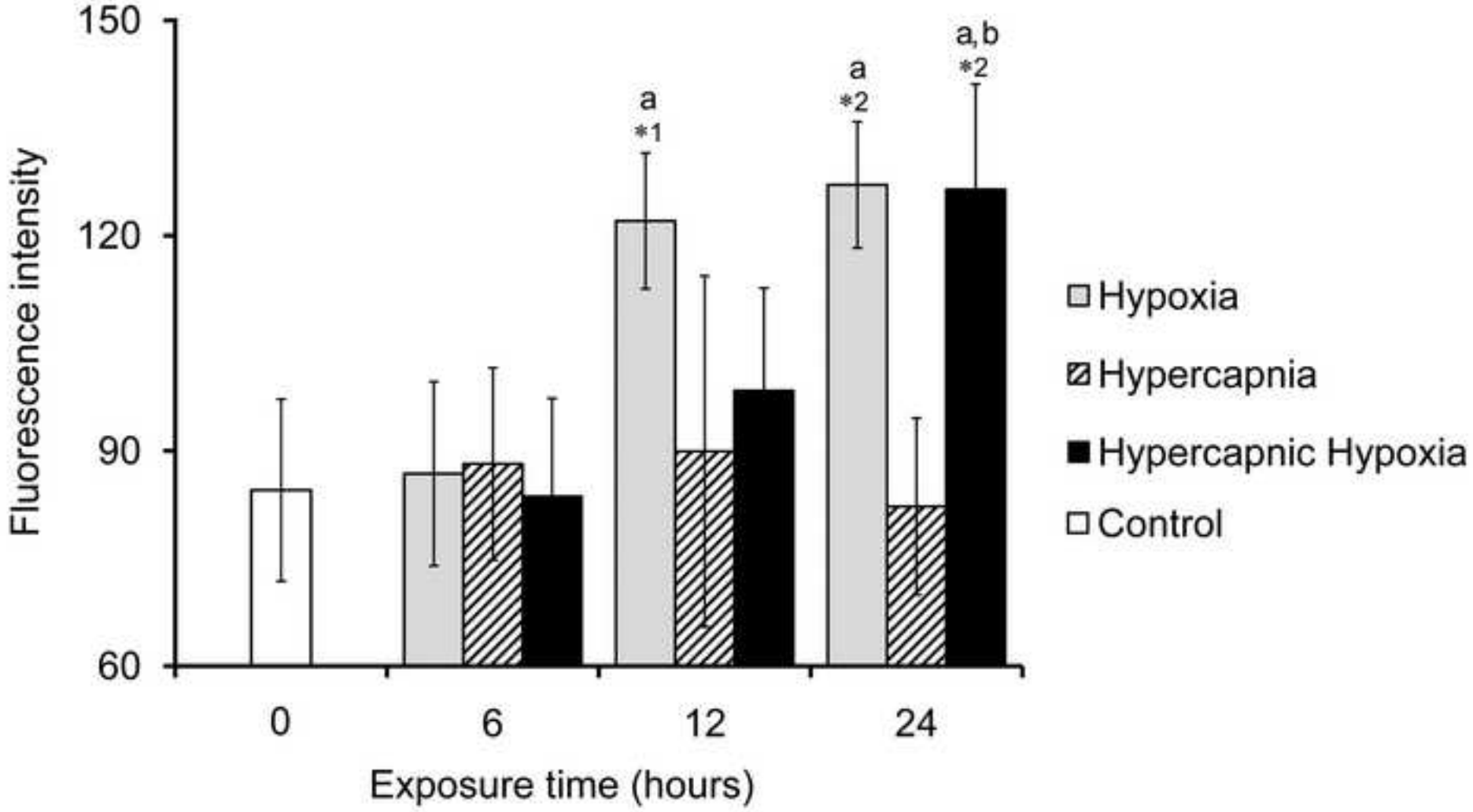

Control
50μm
A
10μm
Hypoxia
B
Hypercapnia
C
Hypercapnic Hypoxia
D

Fig. 5


150
120
90
60
Fluorescence intensity
a
*1
a
*2
a,b
*2
0
6
12
24
Exposure time (hours)
Hypoxia
Hypercapnia
Hypercapnic Hypoxia
Control