Histol Histopathol (2012) 27: 897-903
DOI: 10.14670/HH-27.897

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Arterial carbon dioxide partial pressure influences CYP4A distribution in the rat brain

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Summary. $PaCO_2$ is an important factor in the regulation of cerebral circulation, and it is often used to reduce intracranial pressure through hyperventilation during neurosurgery. Changes in concentration can cause changes in CBF (cerebral blood flow). 20-HETE is a product of CYP4A-mediated AA (arachidonic acid) metabolism and is a powerful endogenous vasoconstrictor; however, its effect on cerebral vasoconstriction in cats, dogs and rats remains to be confirmed. It is known that changes in $PaCO_2$ can influence the expression of CYP4A in the rat brain, demonstrating the important role of 20-HETE in the mechanism of CO_2-mediated cerebrovascular reactivity. Thirty healthy adult male Wistar rats that weighed between 200 g and 250 g were randomly divided into three groups (A, B, and C; n=10): group A, normocapnia ($PaCO_2$ was maintained at approximately 40-45 mmHg); group B, hypocapnia ($PaCO_2$ was maintained at approximately 20-25 mmHg); and group C, hypercapnia ($PaCO_2$ was maintained at approximately 60-65 mmHg). Physiological parameters, including HR (heart rate), MBP (mean blood pressure), PH and $PaCO_2$ were recorded every 30 min, and there were no significant hemodynamic or body temperature differences. The head was removed after 3.5 h to investigate brain CYP4A by immunohistochemistry. Relative to group A, group B exhibited the following changes: an increased pH, decreased $PaCO_2$, and increased brain CYP4A protein expression (P<0.05). In contrast, group C exhibited decreased PH, increased $PaCO_2$ and decreased CYP4A protein expression (P<0.05). CO_2 can decrease the expression of brain CYP4A during hypercapnia and increase its expression during hypocapnia.

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Key words: $PaCO_2$, CYP4A, 20-HETE, Brain tissue

Introduction

$PaCO_2$ is an important factor in the regulation of cerebral circulation (Patel and Drummond, 2006), and it is often used to reduce intracranial pressure through hyperventilation during neurosurgery. Changes in $PaCO_2$ can cause changes in CBF, primarily via myogenic factors, metabolic factors, neural factors and the regulation of endothelial factors. The effects of $PaCO_2$ also depend on cerebral perfusion pressure (CPP) and anesthetics. Studies of the mechanisms of $PaCO_2$ have shown that changing the pH of the cerebral arteries causes cerebrovascular tension (Patel and Drummond, 2006). There are five suggestions of probable mechanisms: 1) NO (Muthalif et al., 2001): NO plays an important role in CO_2-mediated cerebral vasodilation, but the inhibition of nitric oxide synthase (NOS) results in hypercapnia, which still holds about 10-70 % of cerebral vasodilation. Severe hypercapnia is not simply a sign of vasodilation. 2) Prostaglandin compounds: Prostaglandin is a cyclooxygenase-induced metabolite of AA. Further modification by other enzymes converts prostaglandin into either a vasoconstrictor or vasodilator. It has been shown in humans and animals that the cyclooxygenase inhibitor indomethacin can reduce the pressure level at which hypercapnia leads to cerebral vasodilation. However, in humans, cyclooxygenase does not cause cerebral vasodilation by itself during severe hypercapnia. 3) Cyclic nucleotide (You et al., 1994; Liwei et al., 2002): Graded cyclic nucleotide concentrations provide the signal using the pH, which guides changes in vascular smooth muscle tone. NO activation of guanylyl cyclase within VSMCs (vascular smooth muscle cells) increases cGMP concentration, whereas the vasodilatory prostaglandins PGE2 and PGI2 activate adenylyl cyclase to increase cAMP. During

hypercapnia, cGMP levels rise in rat VSMCs, but not in the VSMCs of the brain. It is not currently known whether cGMP is a vasodilatory neurotransmitter during hypercapnia, but even if cAMP "allows" for hypercapnic cerebral vasodilation, the necessary conditions are not yet known. 4) K^+ channels: the inhibition of VSMC K^+ channels plays an important role in the regulation of cerebral vascular tone. There are four types of cerebral VSMC K^+ channels: Kv, KATP, Kca, and KIR. When the pH falls, the KATP channels open and cells become hyperpolarized. Hypercapnia activates KATP channels during vascular smooth muscle hyperpolarization and decreases intracellular Ca^{2+} concentration (Kinoshita and Katusic, 1997) so that the small arteries of the brain dilate, but the main artery of the brain does not work. 5) Intracellular calcium (Guiming et al., 1999; Liwei et al., 2002) intracellular Ca^{2+} controls vascular smooth muscle tension. During alkalosis the brain's VSMC Ca^{2+} concentration increases, causing increased tension and vascular tone. Extracellular acidosis causes small blood vessels to dilate; however, this condition could also potentially increase the intracellular Ca^{2+} concentration and prevent dilation, suggesting that Ca^{2+} acidosis plays an important role in VSMCs. The connection of CO_2 to cerebral vascular tone is complex. After an initial change in PH, the final common effector is the intracellular Ca^{2+} concentration, but there are still many questions regarding the relationship between these factors.

20-HETE is a product of CYP4A-mediated AA metabolism and is a powerful endogenous vasoconstrictor. 20-HETE serves as a second intracellular messenger in vascular smooth muscle cell contractions of the heart, kidneys, brain, skeletal muscles and mesenteric artery. It plays a vital role (Harder et al., 1995, 1997; Wallis et al., 1996). Abnormal VSMC contraction may lead to cerebrovascular disease, kidney disease, coronary disease, and hypertension. The diameter of the rat middle cerebral artery decreases by 20% when the transmural pressure changes from 60 to 140 mmHg (Sun et al., 1999). DDMS, 15-HETE or 6 (Z), and 15 (Z)-20-HEDE can prevent these vascular pressure-induced contractions. 20-HETE levels increase 4-fold when the transmural pressure of the murine middle cerebral artery increases from 20 to 140 mmHg. These results suggest that the middle cerebral artery promotes the release of 20-HETE by an increase in transmural pressure and blocks the formation of 20-HETE and the contraction caused by vessel pressure is reduced; hence, these observations indicate that 20-HETE is essential to the regulation of CBF. It has been reported that endogenous 20-HETE causes cerebral vasoconstriction. As the level of 20-HETE increases, the transmural pressure of the middle cerebral artery rises and the middle cerebral artery contracts. Interestingly, the CYP4A inhibitors 17-ODYA (17-octadecynoic) and DDMS inhibit this phenomenon (Gebremedhin et al., 2000), suggesting that 20-HETE is the major mediator of cerebrovascular myogenic contraction. Many reports indicate that 20-HETE acts as a vasoconstrictor by inhibiting Ca^{2+}-activated K^+ channels (Kca), activating L-type Ca^{2+} channels, PKC and other pathways to increase the intracellular calcium content and constrict the small arteries.

The role of 20-HETE on ion channels, membrane potential and PKC are similar to that of cerebrovascular reactivity under hypocapnia. Moreover, 20-HETE plays an important role in cerebrovascular tone regulation. CO_2 can cause changes in cerebrovascular tension, and there are many similarities between 20-HETE and CO_2 on the cerebrovascular mechanism of action, and both regulate cerebrovascular tension due to changes of Ca^{2+} concentration in VSMCs (Harder et al., 1997; Lange et al., 1997), although the relationship between 20-HETE and CO_2 is not fully understood.

In this study, we found by immunohistochemistry that different levels of $PaCO_2$ influence the expression of CYP4A in the rat brain, which demonstrates the important role of 20-HETE as a regulating factor of the cerebrovascular effects of CO_2. To minimize the effect of cerebral blood flow on cerebral vasoconstriction, cerebral blood flow (as well as BP, HR, body temperature and PaO_2) should be stable during the experiment. In this study, BP and HR were not significantly different between the three groups. Throughout the experiment, an electric blanket was used to maintain a constant body temperature, and the rats inhaled 50% O_2 to ensure the maintenance of PaO_2 within the normal range. A low level of CO_2 promoted the expression of CYP4A in the brain and further confirmed that the level of CO_2 can be increased to induce the formation of 20-HETE, which caused vasoconstriction which lessened the CBF. High levels of CO_2 in the brain were observed to cause a decreased expression of CYP4A, as well as 20-HETE synthesis and increased CBF. We show in this study that 20-HETE plays a vital role in mediating the cerebrovascular effects of CO_2.

Materials and methods

BIOPAC 16-channel multi-channel physiological recorder (MP150, USA). Small animal ventilator (Harvard Apparatus 863, USA). Bayer blood gas analyzer (Rapidlab 248, Germany). METTLER electronic balance (AE240, Switzerland). Constant temperature water bath (DK-98-I, Tianjin Taisite Instrument Co., Ltd.). Desktop freezing microtome (Leica CM1100, Germany). Rabbit polyclonal antibody CYP4504A (ab3573, Abcam Co, USA). Rabbit two-step detection kit (PV-6001, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.).

Animals

Healthy adult male Wistar rats that weighed between 200 g and 250 g (Jilin University Experimental Animal Center Medical College) were randomly divided into three groups (A, B, and C; n=10 per group): A) normal

carbon dioxide (control), B) hypocapnia, and C) hypercapnia.

Mechanical ventilation

Each rat was anesthetized with an intraperitoneal injection of 3% sodium pentobarbital (50 mg/kg), and a catheter was inserted into the right femoral artery of each rat to monitor arterial blood pressure, heart rate and blood gas. A catheter that was inserted into the right femoral vein was used for drug delivery. The catheters were connected to a BIOPAC to monitor physiological functions. Thirty minutes into the operation, when the rats were stable, the trachea of each rat was exposed with a 14 BD catheter tracheotomy tube, and an intravenous injection of droperidol rocuronium (0.2 mg) was administered. Each animal was connected to a small animal mechanical ventilator. Group A inhaled 50 % FiO_2 + 50 % FiN_2 (the tidal volume on the ventilator was set to 6-10 mL/kg and $PaCO_2$ was maintained at 40-45 mmHg), group B inhaled 50 % FiO_2 + 50 % FiN_2 (the ventilator was adjusted to a $PaCO_2$ of 20-25 mmHg), and group C inhaled 50 % FiO_2 + 5 % $FiCO_2$ + 45 % FiN_2 (the ventilator was adjusted to a $PaCO_2$ of 60-65 mmHg). The suction/expiratory ratio was set to 1:1, and the respiratory rate was set to 45-55 breaths/min. After the rats were stable for 30 min, we measured several basic physiological indicators, including the heart rate (HR), mean arterial blood pressure (MBP), arterial PH, $PaCO_2$, PaO_2, HCO_3^-, BE, and SaO_2 every 15 min. The $PaCO_2$ value was adjusted based on the respiratory parameters. An electric blanket was used to maintain the body temperature of each rat at 38.5°C-39.5°C.

Perfusion and tissue preparation:

The rats were sacrificed after 3.5 h of mechanical ventilation. The chest wall was opened to expose the heart and the left ventricle to the aortic root was cannulated. We perfused the rats with 150 mL of normal saline and then with 300 mL of a 4 % paraformaldehyde phosphate buffer (PFA). The brains of the rats were then removed, placed in 4 % paraformaldehyde, and fixed for 24 h. After fixation, the brains were dehydrated at 4°C by sequential incubations in 10 % and 20 % sucrose phosphate buffers for 12 h, followed by a final incubation in a 30 % sucrose phosphate buffer until the brain tissue sank to the bottom. We then cryosectioned the brains (-20°C, 8-μm thick sections) in consecutive coronal slices, and the resultant slides were stored at 4°C for later use.

Table 1. The different time changes in HR (bpm, n=10, $X\pm S$).

group	0.5h	1.5h	2.5h	3.5h
A	371.10±5.63	373.20±5.79	374.40±4.97	375.00±5.46
B	370.40±5.68	371.80±5.16	374.50±4.74	376.50±4.65
C	369.80±6.44	371.30±6.36	372.80±6.34	374.70±5.25

CYP4A immunohistochemistry

The slides were initially washed in PBS (5 min x 3 times). Next, the slides were incubated for 1 h with a primary antibody (rabbit polyclonal CYP4504A that had been diluted 1:200 in PBS), washed in PBS (5 min x 3 times), incubated at room temperature for 30 min with a rabbit secondary antibody and washed in PBS (5 min x 3 times). Immunoreactive cells were observed after a diaminobenzidine (DAB) color reaction. The slides were washed with distilled water to stop the reaction and were then stained with hematoxylin, dehydrated in graded ethanols with xylene, and finally cemented in turpentine as a terminal step. The CYP4A primary antibody was replaced with PBS as a negative control.

Main outcome measures

CYP4A protein expression was quantified in frozen sections of rat brain.

Statistical analysis

All data were analyzed by SAS9.1 statistical analysis software, and measurements are presented as the mean ± standard deviation. An analysis of variance with repeated measures was used to compare the different stages between the three groups. A single-factor analysis of variance was used to compare the expression of CYP4A. In all cases $P<0.05$ was considered to be statistically significant.

Results

Comparison of three physiological endpoints

MBP and HR did not significantly differ between the three groups (Tables 1, 2; Figs. 1, 2). In comparison to group A, group B had a significantly increased PH and a significantly decreased $PaCO_2$ ($P<0.05$), whereas group

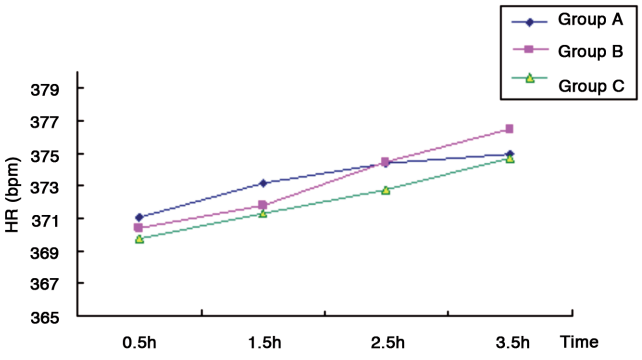

Fig. 1. Three changes of HR at different times.

C had a significantly lower PH and a significantly higher $PaCO_2$ (P<0.05; Tables 3, 4).

Comparison of CYP4A protein expression

Our immunohistochemistry results demonstrated that in comparison to group A, CYP4A expression was higher in group B and lower in group C (Table 5; Figs. 3-9).

Discussion

$PaCO_2$ is an important factor in the regulation of cerebral circulation. Changes in $PaCO_2$ can lead to cerebral vascular CO_2 reactivity, causing increased cerebral vascular resistance and changes in CBF. $PaCO_2$ can influence CBF, wherein for every 1 mmHg change

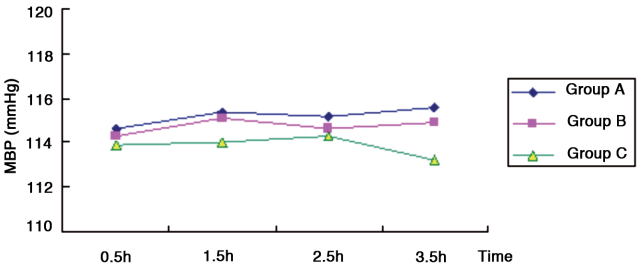

Fig. 2. Three changes of MBP at different times.

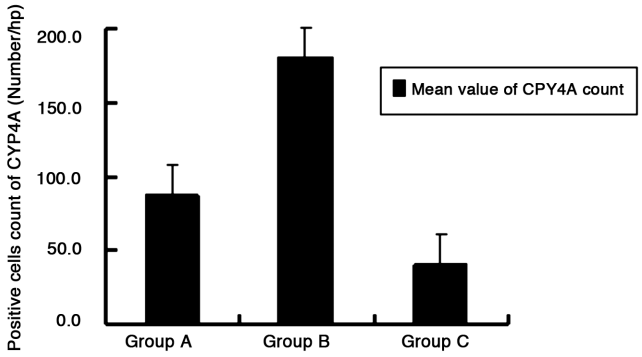

Fig. 3. The positive cells count of CYP4A.

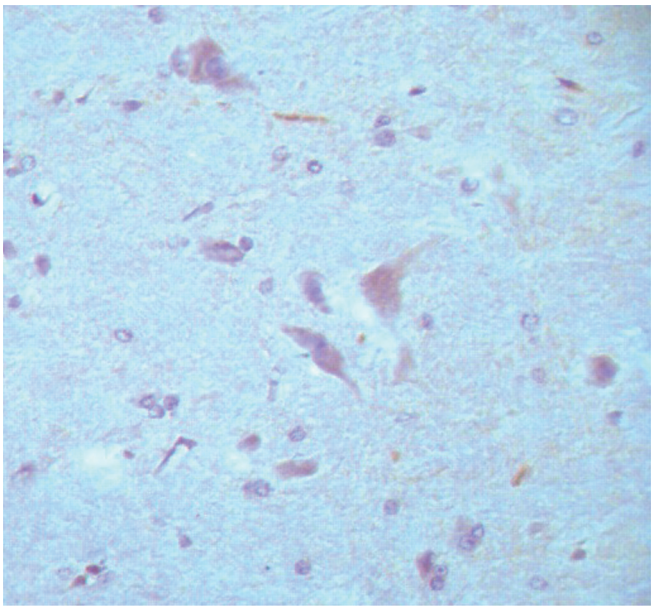

Fig. 4. CYP4A positive cells in normal group. x 400

Table 2. The different time changes in MBP (mmHg, n=10, $X\pm S$).

group	0.5h	1.5h	2.5h	3.5h
A	114.60±2.17	115.40±2.17	115.20±2.30	115.60±1.84
B	114.30±2.36	115.10±1.45	114.60±1.65	114.90±1.66
C	113.90±3.14	114.00±2.98	114.30±3.23	113.20±2.82

Table 3. The different time PH value (n=10, $X\pm S$).

group	0.5h	1.5h	2.5h	3.5h
A	7.40±0.02	7.39±0.02	7.40±0.02	7.41±0.01
B	7.41±0.03	7.49±0.01	7.52±0.01	*7.56±0.01
C	7.39±0.02	7.28±0.01	7.24±0.01	*7.21±0.01

* P<0.05 vs Normocapnia group.

Table 4. The different time PaCO2 (mmHg, n=10).

group	0.5h	1.5h	2.5h	3.5h
A	41.59±2.03	41.15±1.21	41.00±0.98	40.63±0.55
B	41.03±1.75	31.22±0.90	25.95±0.91	*20.95±0.50
C	41.31±1.72	59.65±1.11	61.97±0.89	*63.11±0.82

* P<0.05 vs Normocapnia group.

Table 5. Three groups of CYP4A positive count (n=10).

group	CYP4A(number/ visual field)
A	87.70±7.75
B	*181.30±14.82
C	*40.30±12.31

* P<0.05 vs Normocapnia group.

in $PaCO_2$ the CBF responds accordingly with a 1-2 ml/100 g•min change (Qiming and Xiujuan, 1997). CBF follows a changing $PaCO_2$ in a stepwise fashion. When $PaCO_2$ increases to 80 mmHg or higher, the CBF of animals will also greatly increase, leading to a higher incidence of cerebral edema and herniation. When $PaCO_2$ is reduced to 20-25 mmHg, CBF will also decrease by 40-50%, leading to a risk of brain seizures and cerebral ischemia. It is known that hypercapnia and hypocapnia are significant for patients undergoing rehabilitation and treatment. Therefore, elucidating their mechanism is important for enhancing patient quality of life.

The mechanism of cerebrovascular CO_2 reactivity is not entirely clear, but may be related to the following factors: NO, prostaglandin compounds, cyclic nucleotide, K^+ channels, and intracellular calcium (You et al., 1994; Kinoshita and Katusic, 1997; Kajita et al., 1998; Guiming et al., 1999; Gebremedhin et al., 2000; Muthalif et al., 2001; Liwei et al., 2002). This experiment confirmed that 20-HETE is one of the mechanisms by which hypocapnia leads to cerebral vasoconstriction. In this study, we observed by

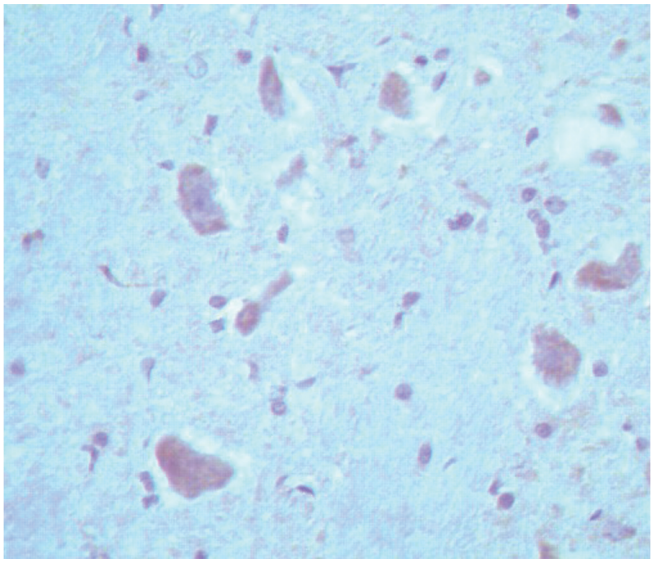

Fig. 5. CYP4A positive cells in normal group. x 400

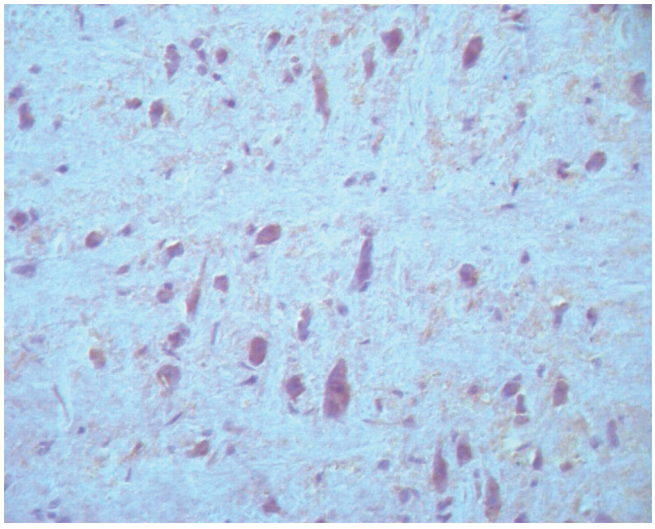

Fig. 6. CYP4A positive cells in hypocapnia group. x 400

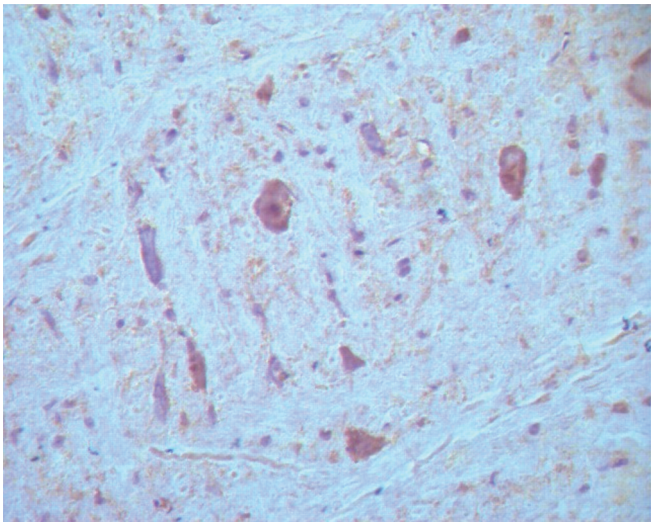

Fig. 7. CYP4A positive cells in hypocapnia group. x 400

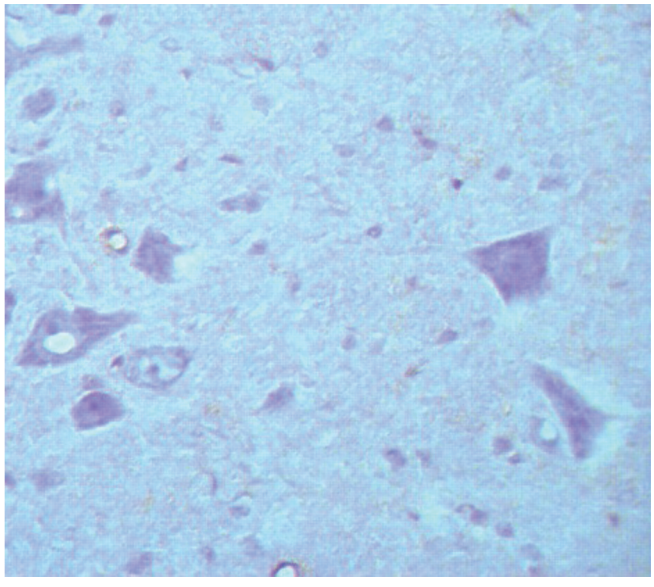

Fig. 8. CYP4A positive cells in hypercapnia group. x 400

immunohistochemistry that differences in $PaCO_2$ influence the expression of CYP4A in the rat brain, demonstrating the important role of 20-HETE as a factor in CO_2-mediated cerebrovascular reactivity.

Arachidonic acid (AA) is abundant in the body, and its metabolites have important physiological and pathological roles. It is known that AA is activated by receptor-dependent PLA2, which is released from the cell membrane. There are three ways in which AA is metabolized: first, the cyclooxygenase pathway (prostaglandin-mediated), of which PGI2 and thromboxane (TXA2) are the end products; second, the enzymatic lipid oxidation pathway, which forms HPETES, HETES and leukotrienes; and third, the CYP pathway. The CYP pathway can metabolize AA in one of three ways: First, Propylene oxidation, where 5-, 8-, 9-, 11-, 12-, 15-HETE are the products; Second, oxidation, which produces 5 -, 6 -, 8 -, 9 -, 11 -, 12 - and 14 -, in addition to 15-EET and lower product DHETs; and third, Omega/omega-1 hydroxylation, which produce 20- and 19-HETE (Harder et al., 1995; Capdevila et al., 2000). According to its amino acid sequence, CYP450 is divided into multiple genes/gene subunit families, and CYP4A from the same gene family has similar functions. It has been found that the CYP450 4A-hydroxylase family has four subfamilies in rats: CYP4A1, 4A2, 4A3 and 4A8. CYP4A1, 4A2 and 4A3 may undergo Omega/omega-1 hydroxylation of AA, forming 19- and 20-HETE, wherein the primary physiological effector is 20-HETE.

There are many isoenzymes in the CYP4A family that catalyze the conversion of AA to 20-HETE in the brain. 20-HETE regulates cerebral blood flow by regulating vascular tension in the brain. This is an important autoregulatory component of cerebral blood flow (Wallis et al., 1996; Gebremedhin et al., 2000; Harder et al., 2011). 20-HETE is a powerful endogenous vasoconstrictor that causes cerebral vasoconstriction. Harders et al. found that the CYP4A inhibitors 17-OYDA and DDMS inhibited 20-HETE-induced cerebral vasoconstriction. 20-HETE is an important part of the signaling pathways of endothelin, AngII and other regulators (Oyekan et al., 1998; Good et al., 1999; Roman et al., 2006), and it plays an important role in blood pressure regulation. 20-HETE in the smooth muscle cells is considered to be a second messenger. Specifically, it is released during the stretch response, increases with transmural pressure, and is related to microvascular self-regulation, especially with regard to renal and cerebral artery myogenic regulation. 20-HETE is an important factor in local vascular tone.

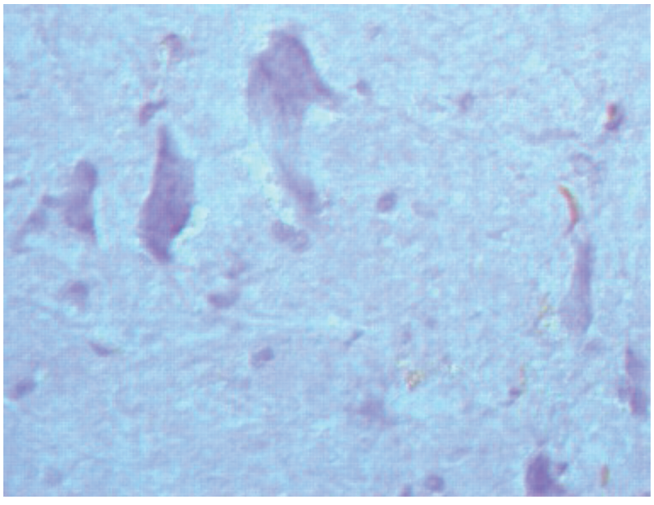

Fig. 9. CYP4A positive cells in hypercapnia group. x 400

In this study, CO_2 decreased the expression of CYP4A in the brain during hypercapnia such that 20-HETE generation was low, vasodilatation decreased, and cerebral blood flow increased. We found the opposite effects during hypocapnia. It has been reported that 20-HETE inhibitors, 15-HETE and 20-HEDE, can inhibit cerebrovascular contraction and can damage cerebral blood flow autoregulation in rats. CYP4Aω-hydroxylase inhibition with DDMS can also damage cerebral blood flow autoregulation. This shows that CYP4A and 20-HETE play important roles in cerebral blood flow autoregulation, which can be influenced by changes in CYP4A activity (Wallis et al., 1996). Studies also show that CYP4A protein overexpression or inhibition in small arteries can reduce or increase vascular reactivity and myogenic tension, respectively (Roman, 2002; Zhang et al., 2004), which is consistent with our findings.

20-HETE is a metabolite of AA via CYP4Aω hydroxylation. In this study, changes in brain CYP4A protein expression were observed as $PaCO_2$ varied; CYP4A expression increased with low $PaCO_2$ and decreased with high $PaCO_2$. CO_2 promotes CYP4A expression in the brain during low $PaCO_2$ so that 20-HETE increases, cerebral blood vessels contract, and CBF decreases. During high $PaCO_2$, CO_2 suppresses CYP4A, decreases 20-HETE, dilates the cerebral vasculature and increases CBF.

To remove any impact of CBF on cerebral vasoconstriction, CBF during the experiment should be stable, in addition to other factors that could affect CBF, such as BP, HR, body temperature and PaO_2. In our study, the BP and HR were consistent between the three groups, the experiments were performed with an electric blanket to keep the body temperatures of all of the animals constant, and the rats inhaled 50 % O_2 to ensure that PaO_2 would be maintained within the normal range, thus excluding any effects of CBF. However, due to the high sequence homology of CYP4A, our antibody may not have enough specificity, making our immunohistochemistry results difficult to interpret. Follow-up experiments using RT-PCR will further enhance our CYP4A expression analysis.

Overall, we found that during hypercapnia CO_2 inhibits the expression of CYP4A in the brain, decreases

20-HETE production, expands the cerebrovasculature and increases CBF. During hypocapnia, CO_2 promotes the expression of CYP4A in the brain, increases 20-HETE production, constricts the cerebrovasculature and decreases CBF. We have demonstrated that 20-HETE plays an important role in the mechanism of CO_2-mediated cerebrovascular reactivity. Our study may be used as a reference for clinical research.

Acknowledgements. We thank Dr. Daling Zhu of Harbin Medical University for technical assistance.

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Accepted February 1, 2012