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Endogenous generation of sulfur dioxide in rat tissues

Liman Luo [a], Stella Chen [b], Hongfang Jin [a], Chaoshu Tang [c,d], Junbao Du [a,d,*]

[a] Department of Pediatrics, Peking University First Hospital, Beijing 100034, China
[b] Department of Biological Sciences, University of California, San Diego, La Jolla, CA 92093, United States
[c] Department of Physiology and Pathophysiology, Peking University Health Science Center, Beijing 100191, China
[d] Key Laboratory of Molecular Cardiology, Ministry of Education, Beijing 100191, China

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ABSTRACT

While sulfur dioxide (SO_2) has been previously known for its toxicological effects, it is now known to be produced endogenously in mammals from sulfur-containing amino acid L-cysteine. L-cysteine is catalyzed by cysteine dioxygenase (CDO) to L-cysteinesulfinate, which converts to β-sulfinylpyruvate through transamination by aspartate aminotransferase (AAT), and finally spontaneously decomposes to pyruvate and SO_2. The present study explored endogenous SO_2 production, and AAT and CDO distribution in different rat tissue. SO_2 content was highest in stomach, followed by tissues in the right ventricle, left ventricle, cerebral gray matter, pancreas, lung, cerebral white matter, renal medulla, spleen, renal cortex and liver. AAT activity and AAT1 mRNA expression were highest in the left ventricle, while AAT1 protein expression was highest in the right ventricle. AAT2 and CDO mRNA expressions were both highest in liver tissue. AAT2 protein expression was highest in the renal medulla, but CDO protein expression was highest in liver tissue. In all tissues, AAT1 and AAT2 were mainly distributed in the cytoplasm rather than the nucleus. These observed differences among tissues endogenously generating SO_2 and associated enzymes are important in implicating the discovery of SO_2 as a novel endogenous signaling molecule.

1. Introduction

Sulfur dioxide (SO_2) is well known for its toxicological effects, for which it has been extensively reported in the past [1]. However, it was recently discovered that SO_2 could be produced endogenously from sulfur-containing amino acid L-cysteine in mammals [2]. L-cysteine is catalyzed by cysteine dioxygenase (CDO) to L-cysteinesulfinate, which converts to β-sulfinylpyruvate through transamination by aspartate aminotransferase (AAT), then spontaneously decomposes to pyruvate and SO_2 [3]. Additionally, H_2S can be catalyzed to SO_2 by nicotinamide adenine dinucleotide phosphate oxidase [4] or by the reduction of thiosulphate [3]. SO_2 is metabolized to sulfite and oxidized to sulfate by sulfite oxidase, and finally excreted in urine [2].

Currently, the biological effects of endogenous SO_2 are not fully understood in mammals. In neutral pH [3], SO_2 dissociates to its sulfite derivatives ($NaHSO_3/Na_2SO_3$, 1:3 M/M), which is regulated to maintain homeostasis *in vivo*. Serum sulfite is significantly increased in patients with acute pneumonia and renal failure [5,6]. Balazy et al. [7] found that carbonyl sulfide and SO_2 could be produced in porcine coronary artery. Our research group discovered that endogenous SO_2 and AAT existed in vascular and cardiac tissues [8]. Previous studies have reported the vasorelaxing effect of SO_2 [8–14]. SO_2 can also regulate cardiac function in rats [15] and inhibit L-calcium channels in cardiomyocytes [16]. Additionally, SO_2 can improve pulmonary vascular structural remodeling [17,18], protect myocardium against isoproterenol-induced injury [19], and have an anti-atherogenic effect in rats [20]. Considering these discoveries, we proposed that SO_2 might be an important mediator in the cardiovascular system [21–24]. However, the generation of endogenous SO_2 in the lung, stomach, small intestine, liver, pancreas, spleen, renal cortex, renal medulla, cerebral gray matter and cerebral white matter has not been clear. Therefore, detecting the production of SO_2 and its related enzymes, such as AAT and CDO, in different rat tissues is becoming important for further studies exploring the biological effects of SO_2. Therefore, the present study was designed to determine the endogenous SO_2 pathway in different tissues of rats.

2. Materials and methods

2.1. Animal preparation

Animal care and experimental protocols complied with the Animal Management Rule of the Ministry of Health, China and

Abbreviations: SO_2, sulfur dioxide; AAT, aspartate aminotransferase; CDO, cysteine dioxygenase.

* Corresponding author at: Department of Pediatrics, Peking University First Hospital, Beijing 100034, China. Fax: +86 10 66530532.
E-mail address: junbaodu1@126.com (J. Du).

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the Animal Care Committee of Peking University First Hospital, Beijing, China. Eight male Wistar rats (body weight 250 ± 10 g) were obtained from the Experimental Animal Center, Peking University Health Science Center, Beijing, China. The rats were housed under special pathogen-free conditions, and kept at a temperature of 22 °C with 40% humidity and a 12 h light/12 h dark cycle.

2.2. Preparation of different tissue samples in rats

Eight male Wistar rats were anaesthetized by use of urethane (1 g/kg) by intraperitoneal injection, and then the tissues of the left and right ventricle, lung, stomach, small intestine, liver, pancreas, spleen, renal cortex, renal medulla, cerebral gray matter, and cerebral white matter were harvested rapidly for the following analyses. Samples were frozen and stored in liquid nitrogen. The tissues were homogenized in 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4, 10 mL/g tissue), and then centrifuged at 12,000*g* for 30 min at 4 °C. The supernatants obtained were prepared for SO_2 content, AAT activity and protein assay determination. Protein concentration in tissues was measured using the Coomassie brilliant blue method [25]. For *in situ* hybridization and immunohistochemical analyses, tissues were fixed in 4% polyoxymethylene.

2.3. Determination of plasma and tissue concentration of SO_2

SO_2 concentrations were measured using high-performance liquid chromatography (HPLC, Agilent 1200 series, Agilent Technologies, Palo Alto, CA, USA) [5,26]. The tissue sample for SO_2 determination was prepared in the same manner as previously reported [8,19]. The supernatant was used for HPLC. The column (4.6 × 150 mm C18 reverse-phase column, Agilent Technologies, Palo Alto, USA) was first equilibrated with a buffer (methanol: acetic acid: water = 5:0.25:94.75 by volume, pH 3.4). The sample loaded onto the column was resolved by a gradient of methanol for 0–5 min, 5%; 5–10 min, 5–35%; 13–30 min, 35–45%; 17–22 min, 45–100%; 22–27 min, 5% at a flow rate of 0.8 mL/min. Sulfite–bimane was detected by excitation at 392 nm and emission at 479 nm. Quantification was carried out using the standardization of Na_2SO_3.

2.4. Measurement of AAT activity

AAT activity in plasma and tissue homogenates was determined by a Hitachi 7600 Automatic Biochemistry Analyzer (Tokyo, Japan). Tissue AAT activity was expressed as IU/g protein.

2.5. Determination of AAT1, AAT2 and CDO mRNA in tissues by quantitative real-time RT-PCR

Total RNA in rat tissues was extracted using the Trizol reagent and reverse-transcribed by oligo $(dT)_{15}$ primer and M-MLV reverse transcriptase. The reaction for the real-time PCR (final volume of 25 μL) was mixed with 2.5 μL of 10× PCR buffer, 1 μL of 7.5 μmol/L forward and reverse primer, 1 μL of 2.5 mmol/L dNTP mixture, 0.25 μL of *Taq* DNA polymerase, and 2 μL of rat tissue cDNA. The sequence of the primers and probes is shown in Table 1. The PCR condition was set to predenaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. An extraction of the passaged viruses of all genotypes available was at 10-fold (1×10^{-1}–1×10^{-6}) dilution and analyzed by both real-time and reverse-transcribed PCR methods. β-actin in each sample was used to calibrate the sample amount used for the determination [19].

2.6. Determination of AAT and CDO protein expression in tissues by Western blot analysis

The different rat tissues were homogenized and lysed. Equal amounts of proteins were boiled and separated by SDS–PAGE and transferred onto nitrocellulose membranes. The primary antibody dilutions were 1:500 for AAT1, 1:5000 for GAPDH and AAT2, and 1:500 and 1:4000 for two groups of CDO, respectively. Secondary antibodies were used as follows: HRP-labeled Santa Cruz goat anti-mouse and Santa Cruz goat anti-rabbit at dilutions of 1:6000 and 1:8000 for two groups of CDO, 1:4000 for AAT1, 1:20000 and 1:40000 for GAPDH and AAT2, respectively. Immunoreactions were visualized using electrochemiluminescence (ECL) and exposed to X-ray film (Kodak Scientific), and then quantified by use of AlphaImager (San Leandro, CA, USA) [27].

2.7. Expression of AAT1, AAT2 and CDO in different tissues using immunohistochemical analysis

We detected the expression of AAT1, AAT2 and CDO in different tissues by using immunohistochemical analysis. Sections of tissues were dewaxed by dimethylbenzene, and washed thrice with phosphate-buffered saline (PBS) each for 5 min, and then treated with 3% H_2O_2 for 10 min. The slides were also washed thrice with PBS (5 min each), then blocked with goat serum working fluid at 37 °C for 30 min, and incubated overnight at 4 °C with AAT1, AAT2 or CDO antibodies (dilutions of 1:30, 1:300, and 1:300, respectively). Slides were then thrice rinsed with PBS (5 min each). Biotinylated anti-rabbit or anti-mouse IgG was incubated for 15 min (AAT1) or 60 min (AAT2 and CDO) at 37 °C. After the slides were rinsed in PBS for three times, the sections were stained with 3,30-diaminobenzidine (DAB) to develop color. The sections were dehydrated through a graded ethanol series and made transparent in dimethylbenzene. Positive signals were defined as brown granules in tissues under light microscopy. For negative controls, primary incubation was performed with non-immune goat serum instead of primary antibodies [18].

2.8. Statistical analysis

Results are expressed as mean ± SEM. The analyses were done using SPSS 13.0 (Chicago, IL, USA). Differences of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of the endogenous SO_2 content in different tissues in rat

The distribution of SO_2 content in respective rat tissue is shown in Fig. 1A. Among the different tissues, SO_2 concentration was highest in tissue of the stomach, followed by tissue of the right ventricle, left ventricle, cerebral gray matter, pancreas, lung, cerebral white matter, renal medulla, spleen, renal cortex and liver.

3.2. Characterization of AAT activity in different tissues in rat

The tissue distribution of AAT, a key SO_2-generating enzyme, is shown in Fig. 1B. Unlike SO_2 content distribution, AAT activity was highest in the left ventricle, then right ventricle, cerebral gray matter, cerebral white matter, renal medulla, renal cortex, liver, intestine, pancreas, lung, spleen and stomach.

Table 1
Primers and TaqMan probes used in quantitative real-time PCR for the measurement of AAT1, AAT2, CDO and β-Actin cDNAs in rats.

cDNA	Oligonucleotide	Sequence	Product size (bp)
AAT1	Forward primer	5′-CCAGGGAGCTCGGATCGT-3′	121
	Reverse primer	5′-GCCATTGTCTTCACGTTTCCTT-3′	
	TaqMan probe	5′-CCACCACCCTCTCCAACCCTGA-3′	
AAT2	Forward primer	5′-GAGGGTCGGAGCCAGCTT-3′	121
	Reverse primer	5′-GTTTCCCCAGGATGGTTTGG-3′	
	TaqMan probe	5′-TTTAAGTTCAGCCGAGATGTCTTTC-3′	
CDO	Forward primer	5′-GGGAAAATCAGTGTGCCTACATT-3′	121
	Reverse primer	5′-GCATGGCATGTATCGAAAGGT-3′	
	TaqMan probe	5′-TTACATCGAGTAGAGAACGTCAGCCACACAGAG-3′	
β-Actin	Forward primer	5′-ACCCGCGAGTACAACCTTCTT-3′	80
	Reverse primer	5′-TATCGTCATCCATGGCGAACT-3′	
	TaqMan probe	5′-CCTCCGTCGCCGGTCCACAC-3′	

TaqMan probe labeled with FAM at the 5′end and TAMRA at the 3′ end. AAT: aspartate aminotransferase; CDO: cysteine dioxygenase.

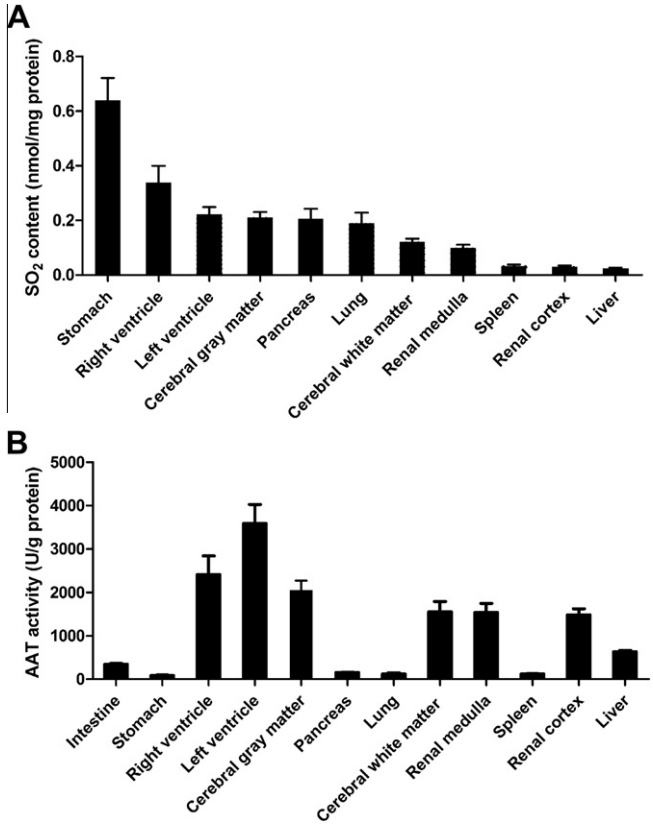

Fig. 1. Comparison of SO_2 content and AAT activity in different rat tissues (mean ± S.E.M). (A) SO_2 content was highest in the stomach, followed by the right ventricle, left ventricle, cerebral gray matter, pancreas, lung, cerebral white matter, renal medulla, spleen, renal cortex and liver. (B) AAT activity was highest in the left ventricle, then right ventricle, cerebral gray matter, cerebral white matter, renal medulla, renal cortex, liver, intestine, pancreas, lung, spleen and stomach.

3.3. Characterization of AAT1, AAT2 and CDO mRNA in different tissues by quantitative real-time PCR

The relative mRNA expression of AAT 1 and AAT 2 paralleled that of the total AAT. The tissue distribution of AAT1 mRNA in different tissues is shown in Fig. 2A. AAT1 mRNA was highest in the left ventricle, followed by the right ventricle, cerebral gray matter, cerebral white matter, intestine, liver, renal medulla, renal cortex, pancreas and stomach. There was very little AAT1 mRNA in spleen and lung tissue (Fig. 2A).

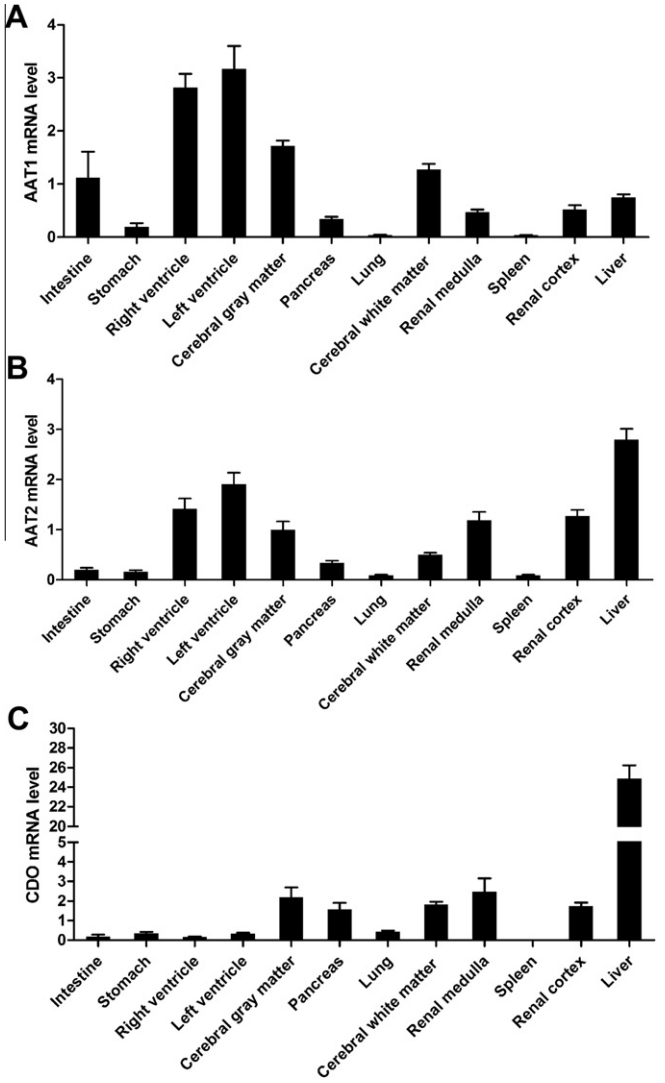

Fig. 2. Comparison of AAT1, AAT2 and CDO mRNA levels in different rat tissues. (mean ± S.E.M). (A) AAT1 mRNA was highest in the pancreas, then right ventricle, cerebral gray matter, cerebral white matter, liver, spleen, renal medulla, intestine, lung, stomach, left ventricle and renal cortex. (B) AAT2 mRNA was highest in the liver, then left ventricle, right ventricle, renal cortex, renal medulla, cerebral gray matter, cerebral white matter, pancreas, intestine, stomach, lung and spleen. (C) The expression levels of CDO mRNA were highest in the liver, followed by the renal medulla, cerebral gray matter, cerebral white matter, renal cortex, pancreas, lung, stomach, left ventricle, intestine, right ventricle, and spleen.

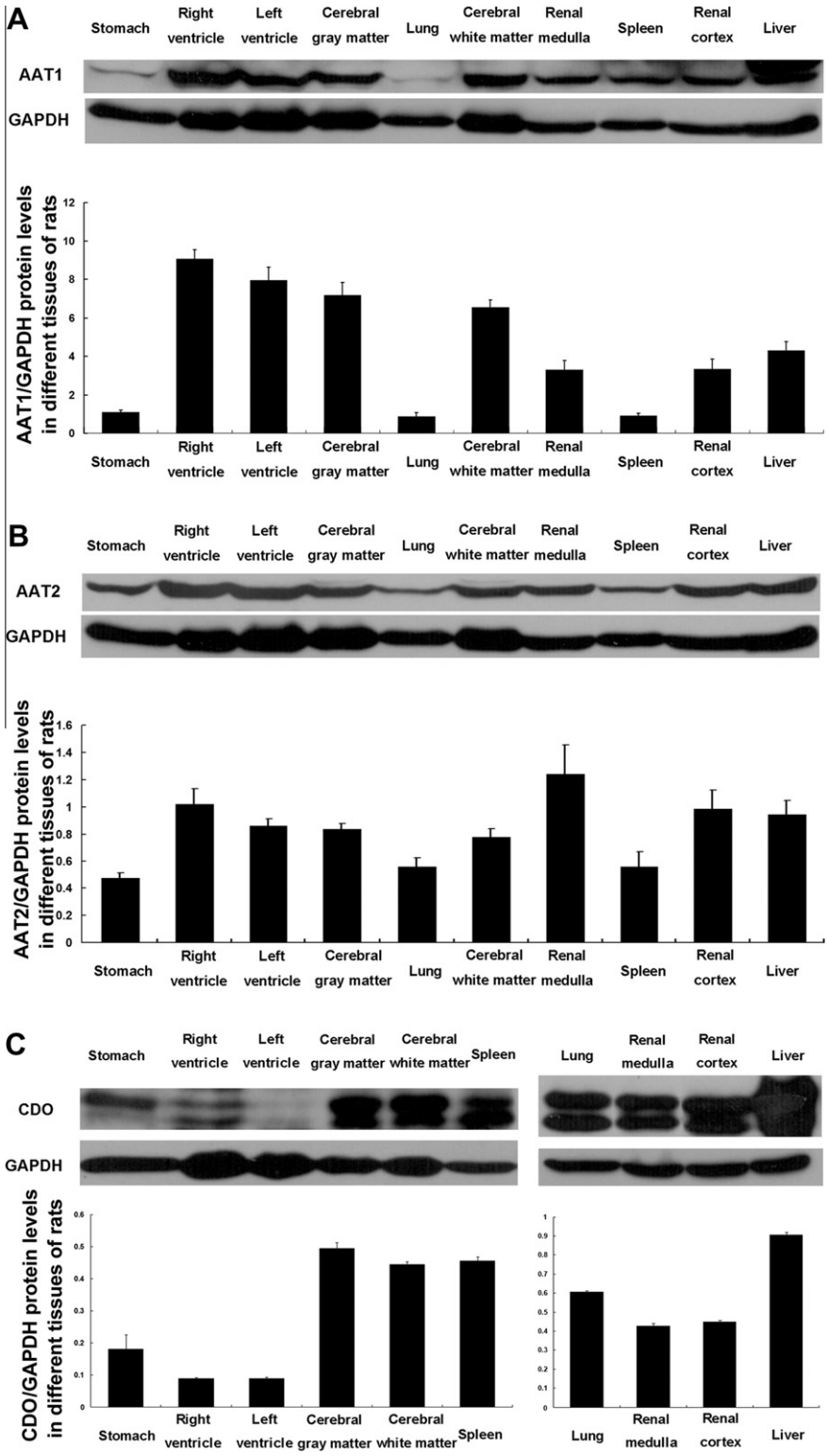

Fig. 3. Comparison of AAT1, AAT2 and CDO expressions in different rat tissues using Western blot analysis. (mean ± S.E.M) (A) AAT1 protein expression was highest in the right ventricle, followed by the left ventricle, cerebral gray matter, cerebral white matter, liver, renal cortex, renal medulla, stomach, spleen and lung. (B) AAT2 protein expression was highest in the renal medulla, then the right ventricle, renal cortex, liver, left ventricle, cerebral gray matter, cerebral white matter, lung, spleen and stomach. (C) CDO expression is much higher in the liver, lung, renal cortex and renal medulla than in the stomach, cerebral gray matter, spleen, cerebral white matter, left ventricle and right ventricle.

AAT2 mRNA was highest in the liver, followed by the left ventricle, right ventricle, renal cortex, renal medulla, cerebral gray matter, cerebral white matter, pancreas, intestine, stomach, lung and spleen (Fig. 2B).

The expression levels of CDO mRNA were highest in the liver, followed by the renal medulla, cerebral gray matter, cerebral white matter, renal cortex, pancreas, lung, stomach, left ventricle, intestine, right ventricle and spleen (Fig. 2C).

Table 2
The expression of AAT1, AAT2 and CDO in different tissues in rat by using immunohistochemical analysis.

Tissues	AAT1	AAT2	CDO
Intestine	Bellows surfaces and smooth muscle layer	Bellows surfaces and smooth muscle layer	Bellows surfaces and smooth muscle layer, stronger in bellows surfaces
Stomach	Bellows surfaces and smooth muscle layer	Bellows surfaces and smooth muscle layer	Bellows surfaces and smooth muscle layer
Myocardium	Myocardial cells	Myocardial cells	Myocardial cells
Pancreas	Acinar cells and islet cells	Acinar cells and islet cells	Acinar cells and islet cells
Lung	Alveolar epithelium, alveolar septum cells, vascular smooth muscle layer and bronchial smooth muscle layer	Alveolar epithelium, alveolar septum cells, vascular smooth muscle layer and bronchial smooth muscle layer	Alveolar epithelium, alveolar septum cells, vascular smooth muscle layer and bronchial smooth muscle layer
Kidney	Small artery and distal luminal surface	Glomerulus, renal capsule and renal tubule, the highest in renal glomerulus and distal convoluted tubule	Renal glomerulus, renal tubule (distal convoluted tubule was significantly higher than the proximal tubule)
Spleen	Blood vessels and individual cells in red pulp	Red and white pulp, more obviously in the junction area	Splenic trabeculae, blood vessels and red pulp
Liver	Hepatic cells	Hepatic cells	Hepatic cells, more obviously in central lobular vein around the vein

AAT: aspartate aminotransferase; CDO: cysteine dioxygenase.

3.4. Characterization of AAT1, AAT2 and CDO protein expression in different tissues by Western blot analysis

Next, we detected AAT1, AAT2 and CDO protein expressions in different tissues by Western blot analysis. The distribution of AAT1 protein expression in different tissues is shown in Fig. 3A. AAT1 protein expression was highest in the right ventricle, followed by the left ventricle, cerebral gray matter, cerebral white matter, liver, renal cortex, renal medulla, stomach, spleen and lung (Fig. 3A).

Unlike the distribution of AAT1 protein expression, AAT2 protein expression was highest in the renal medulla, then the right ventricle, renal cortex, liver, left ventricle, cerebral gray matter, cerebral white matter, lung, spleen and stomach (Fig. 3B).

We also detected CDO protein expression in different tissues by Western blot analysis, the distribution of which is shown in Fig. 3C. CDO protein expressions in the liver, lung, renal cortex and renal medulla were much more abundant than expression in the cerebral gray matter, spleen, cerebral white matter, stomach, left ventricle and right ventricle (Fig. 3C).

3.5. Expression of AAT1, AAT2 and CDO in different tissues by using immunohistochemical analysis

Protein locations of AAT1, AAT2 and CDO in different tissues stained by immunohistochemical analysis are shown in Table 2 and Fig. 4.

4. Discussion

CDO and AAT are two important enzymes in the generation of SO_2 [8,28]. CDO catalyzes the oxidation of L-cysteine to L-cysteine-sulfinate, which then converts to β-sulfinylpyruvate under AAT catalysis, and finally decomposes to SO_2 [3]. CDO is one of the highly regulated metabolic enzymes that respond to diet and play a critical role in the regulation of cellular cysteine concentration [28,29]. According to its localization, AAT consists of two types of isozymes, AAT1 and AAT2. AAT1 is localized in the cell cytoplasm and AAT2 is localized in the cell mitochondria [30]. Both are present in all but erythrocyte in animal tissue [31]. There are some differences between AAT1 and AAT2 in functional effects. For example, both AAT1 and AAT2 are involved in the malate–aspartate shuttle. However, ketoglutarate and aspartate react together to form glutamate and oxaloacetate in a reaction catalyzed by AAT1, while glutamate and oxaloacetate react together to form ketoglutarate and aspartate as catalyzed by AAT2 [31]. Up to now, the distribution of SO_2 and its generating enzymes have not been understood clearly in different organs. Thus, the present study explored the endogenous production of SO_2 and its important generating enzymes AAT and CDO in different tissues of rat.

Previously, it has been reported that the concentration of SO_2 in plasma is (15.54 ± 1.68) μmol/L in male Wistar rats [8]. Meng et al. reported a similar concentration of (16.77 ± 8.24) μmol/L SO_2 in plasma [9]. The concentration of sulfite in serum detected by HPLC is (4.87 ± 2.49) μmol/L in healthy donors. The reference range for total normal serum sulfite is 0–10 μmol/L [26]. The level of plasma AAT activity is (87 ± 18) U/L in rat [8]. The present study showed that SO_2 content was relatively high in the stomach and right ventricles compared with that in the renal cortex and liver. However, the activity of AAT, a key enzyme for SO_2 generation, was distributed differently compared with SO_2. AAT activity was highest in the left and right ventricles, and lowest in the stomach and spleen. Furthermore, the gene and protein expressions of AAT1 and AAT2 in different tissues were also detected by quantitative real-time RT-PCR and Western blot analysis, respectively. The distribution of mRNA and protein expressions differed between AAT1 and AAT2. AAT1 mRNA was highest in the left ventricle and lowest in the lung and spleen. However, AAT2 mRNA was highest in the liver while lowest in the lung and spleen. AAT1 protein expression was highest in the right ventricle and lowest in the lung and spleen. However, AAT2 protein expression was highest in the renal medulla and lowest in the lung and spleen. The data indicate that the SO_2/AAT pathway is abundant in the left and right ventricles, but not so much in the lung and spleen.

We further detected the expression of CDO, another key enzyme in SO_2 production, by Western blot analysis. CDO protein expression was higher in the renal medulla and lung than in the stomach, right ventricle, left ventricle, cerebral gray matter, cerebral white matter, and spleen. The expression levels of CDO mRNA were highest in the liver, and lowest in the intestine and spleen. The data are consistent with those of previous studies where high levels of CDO were expressed in the liver, and substantial amounts were also present in the kidney, lung, pancreas and adipose tissue of mice and rats [28].

In this study, we also explored protein localization of AAT1 and AAT2 in different tissues by immunohistochemical methods. AAT is reported to be abundant in endothelial cells and located in vascular smooth muscle cells adjacent to the endothelial layer [8]. We found that AAT1 was mainly distributed in the cytoplasm, not in the nucleus. In some tissues, the distribution of AAT2 was similar to that of AAT1. AAT1 and AAT2 were expressed on the smooth muscle layer of intestines, alveolar epithelium, alveolar septum cells and bronchial smooth muscle layer of lung, and evenly

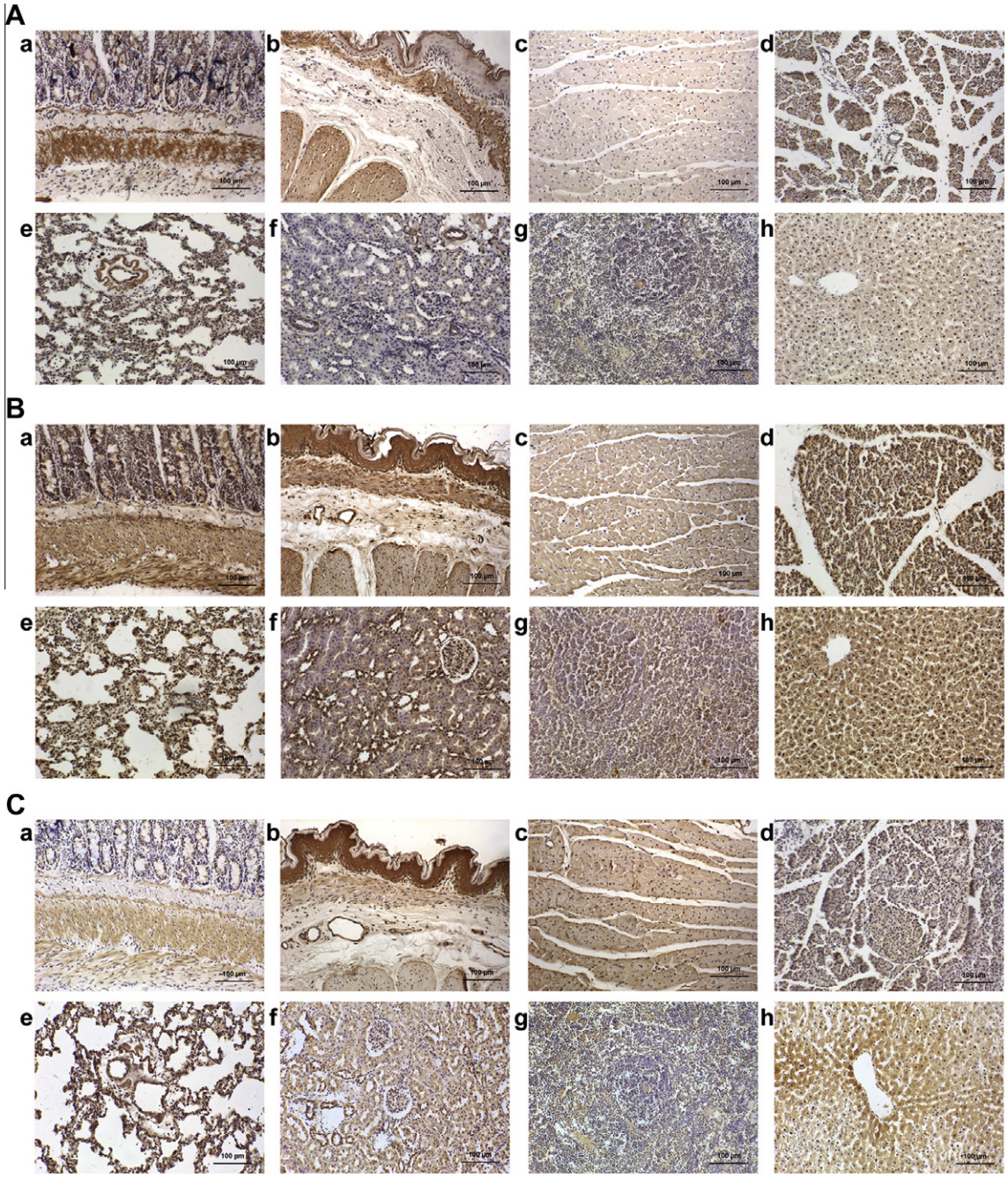

Fig. 4. Expression of AAT1, AAT2 and CDO in different rat tissues using immunohistochemistry. (A) AAT1: (a) Intestine, expressed on smooth muscle layer; (b) stomach, distributed on smooth muscle layer of stomach; (c) heart, distributed in a wide range of myocardial cells; (d) pancreas, distributed in the acinar and islet cells; (e) lung, distributed in the alveolar epithelium, alveolar septum cells, vascular smooth muscle layer and bronchial smooth muscle layer; (f) kidney, distributed in small arteries and the distal luminal surface; (g) spleen, expressed in blood vessels and individual cells in red pulp; (h) liver, expressed in a wide range of liver cells. (B) AAT2: (a) intestine, distributed on the smooth muscle layer of intestines; (b) stomach, expressed on the smooth muscle layer; (c) heart, distributed in a wide range of myocardial cells; (d) pancreas, evenly distributed in acinar and islet cells; (e) lung, expressed in the alveolar epithelium, alveolar septum cells, vascular smooth muscle layer and bronchial smooth muscle layer of lung; (f) kidney, distributed in renal glomerulus, renal capsule, renal tubule, glomerulus and distal convoluted tubule; (g) spleen, expressed in red pulp, white pulp, and more obviously in the junction area; (h) liver, expressed in a wide range of liver cells. (C) CDO: (a) intestine, distributed on the smooth muscle layer of intestines; (b) stomach, expressed on the smooth muscle layer; (c) heart, uniformly expressed in a wide range of myocardial cells; (d) pancreas, uniformly expressed in acinar and islet cells; (e) lung, in the alveolar epithelium, alveolar septum cells, vascular smooth muscle layer and bronchial smooth muscle layer of lung; (f) kidney, distributed in the renal glomerulus and renal tubule (expression was significantly stronger in the distal convoluted tubule than in the proximal tubule); (g) spleen, expressed in splenic trabeculae, blood vessels and red pulp; (h) liver, well-distributed in liver cells, mainly in the central venous and lobular vein.

distributed in acinar, myocardial and liver cells. However, in the spleen, AAT1 was distributed in blood vessels and individual cells in red pulp. AAT2 was uniformly expressed in red pulp and white pulp. More obvious expression was noted on the junction edge. In the kidney, AAT1 was expressed in small arteries and the distal luminal surface, while AAT2 was expressed in the renal glomerulus, renal capsule and glomeruli of the renal distal convoluted tubule ball.

CDO is one of the most highly regulated metabolic enzymes [28]. In our study, it was mainly distributed in the cytoplasm

rather than the nucleus, as determined by immunohistochemical analysis. CDO was mainly expressed in the smooth muscle layer of the intestines and in the alveolar epithelium, septum cells and bronchial smooth muscle layer of the lung. In the liver, CDO was mainly expressed in the central lobular vein around the vein of liver. In addition, CDO was distributed in the renal glomerulus, renal tubule, acinar cells and myocardial cells, and expressed in splenic trabeculae and red pulp. These data indicate that AAT and CDO, key enzymes for SO_2 generation, are widespread among different tissues.

The present study reported the distribution of SO_2 and its generating enzymes AAT and CDO in different tissues of the rat. The results indicated that endogenous SO_2, AAT and CDO were abundant in the ventricles but low in the lung and spleen. The observation that tissues can endogenously generate the gas SO_2 strongly supports its role as an endogenous gaseous signal molecule.

Acknowledgments

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