

Interdependency of Cystathione γ -Lyase and Cystathione β -Synthase in Hydrogen Sulfide–Induced Blood Pressure Regulation in Rats

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BACKGROUND

Hydrogen sulfide (H_2S), an endogenous vasoactive agent, is produced by cystathionine γ -lyase (CGL) and cystathionine β -synthase (CBS) enzymes. This study was conducted to evaluate the relative contribution of these enzymes in regulating systemic arterial pressure.

METHODS

Sprague–Dawley rats were chronically treated with CGL inhibitor, DL-propargylglycine (PAG, 37.5 mg/kg/day; intraperitoneally, i.p.) or CBS inhibitor, aminooxyacetic acid (AOA, 8.75 mg/kg/day; i.p.) or in combination for 4 weeks and the effects on arterial pressure (tail-cuff plethysmography) and renal excretory function (24 h urine collections using metabolic cages) were assessed once in a week. Changes in renal blood flow (RBF; Ultrasonic flowmetry) and glomerular filtration rate (GFR; Inulin clearance) were assessed in acute experiments in anesthetized rats at the end of treatment period.

RESULTS

Compared to vehicle treated control group, only the rats with combination therapy showed a decrease in urinary sulfate excretion

rate (248 ± 47 vs. 591 ± 70 nmol/24 h; marker for endogenous H_2S level) which was associated with an increase in mean arterial pressure (MAP; 130 ± 2 vs. 99 ± 2 mm Hg). Urine flow and sodium excretion were also increased in combination group as consequent to the increase in MAP. GFR did not alter due to these treatments but RBF was lowered (4 ± 0.3 vs. 7 ± 0.4 ml/min/g) only in the combination group compared to the control group.

CONCLUSION

These findings indicate that a deficiency in one enzyme's activity could be compensated by the activity of the other to maintain the endogenous H_2S level, the deficiency of which modulates systemic and renal vascular resistances leading to the development of hypertension.

Keywords: aminooxyacetic acid; blood pressure; DL-propargylglycine; hypertension; hydrogen sulfide; renal hemodynamics; sodium excretion

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Hydrogen sulfide (H_2S), a gaseous molecule is known to be synthesized endogenously in the mammalian tissues from the amino acid L-cysteine. Although considered as an environmental toxic gas, endogenously produced H_2S is now appreciated as gaseous signalling molecule alongside nitric oxide (NO) and carbon monoxide that modulates many physiological functions of the human body at molecular and systemic levels.¹ It is generated mainly by the action of two tissue specific enzymes named cystathionine γ -lyase (CGL) and cystathionine β -synthase (CBS) on the substrate L-cysteine. At present, the distribution of CGL and CBS throughout the body is not well characterized. However, it is reported that CGL is the major H_2S producing enzyme in the cardiovascular system and is present in the vascular smooth muscle as well as the

endothelium.^{2,3} On the other hand, CBS is mainly present in nervous tissues; although, its presence is also reported in many vascular tissues.⁴ Recently, it has also been suggested that another enzyme, 3-Mercaptopyruvate sulfurtransferase, which is specifically localized to neuronal tissue, is also involved in the generation of this gaseous molecule in the brain.⁵ However, only CGL and CBS enzymes are mainly present in liver and kidney tissues that are important for not only the metabolism of sulfur containing amino acids, but also the production of H_2S , which may exert various functions in different tissues.⁶

Recent studies have implicated an important role for endogenously formed H_2S as a critical regulator of cardiovascular homeostasis. H_2S has been characterized as autocrine/paracrine messenger implicated in the control of vascular tone. It has been demonstrated that H_2S -donor compound, sodium hydrogen sulfide (NaHS), causes dose-dependent relaxations of the isolated rat aorta^{2,7,8} and mesenteric arteries.⁹ Administration of NaHS, both acutely and chronically, caused reduction in blood pressure in hypertensive rats induced by NO synthase inhibition.¹⁰ However, conflicting findings

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were reported in mice with disrupted CGL enzyme as it was observed in one study³ that CGL knock-out mice developed hypertension after seventh week of age but other study¹¹ have shown that such knock-out mice were usually normotensive. Although it is generally accepted that the action of CGL in the cardiovascular system is responsible for the effect of H₂S on cardiovascular function, the activity of CBS may also play an important role in modulating vascular tone via its effects on autonomic nervous system.¹² It is reported that H₂S is critical in maintaining the excitatory/inhibitory balance in neurotransmission and thus serves as a novel neuromodulator.¹³ Although no specific mechanism has yet been identified, it may be possible that H₂S exerts an inhibitory effect on sympathetic nerve activity and thus modulates vascular tone to maintain blood pressure homeostasis.¹² However, the relative contribution of CGL- and CBS-mediated production of H₂S in the regulation of blood pressure and the deficiency of their activities in the pathophysiology of hypertension is not yet clearly known.

We hypothesized that as CGL enzyme is present mostly in the vascular tissue, H₂S production induced by the CGL activity would play a more predominant role in the regulation of systemic blood pressure compared to the activity of CBS which is primarily located in the nervous tissue. In this study, we examined the effects of chronic administration of the inhibitors of CGL (DL-propargylglycine; PAG)¹⁴ and CBS (aminooxyacetic acid; AOA)⁶ in rats for a 4-week period to determine the relative contribution of these enzymes in regulating blood pressure. As the kidney plays a key-regulatory role in maintaining systemic blood pressure, the effects of chronic inhibition of CGL and CBS on renal hemodynamic and excretory function are also assessed in this study.

METHODS

All of the experimental procedures described in this study were approved by and performed in accordance with the guidelines and practices established by the Tulane University Animal Care and Use Committee. These experiments were conducted on male Sprague–Dawley rats weighing 250–300 g body weight (8 week-old) that were purchased from Charles River Laboratories (Wilmington, MA), housed in a temperature- and light-controlled room in the Tulane University Vivarium, and allowed free access to a standard diet and tap water. The rats were divided into four groups ($n = 6$ in each group) according to chronic drug treatment as described below:

Group 1	Control	Vehicle (saline) treated
Group 2	PAG	Treated with PAG (37.5 mg/kg/day intraperitoneally, i.p.) alone
Group 3	AOA	Treated with AOA (8.75 mg/kg/day i.p.) alone
Group 4	PAG + AOA	Treated with both PAG and AOA

The doses of PAG (37.5 mg/kg/day i.p.) and AOA (8.75 mg/kg/day i.p.) for chronic daily injections were considered as

these doses were calculated based on the assumption that the injected would be completely absorbed into the blood circulation and reach a circulating concentration around 5 mmol/l and 1 mmol/l for PAG and AOA respectively. These concentrations were shown to cause maximal inhibitory effect on H₂S production *in vitro* in rat aortic¹⁴ and in liver tissue.⁶ These rats were exposed to the experimental procedures for a week prior to the beginning of the actual experiments in order to minimize the effects of environmental and procedural stresses on the experimental results. Both chronic studies at the conscious state and acute experiments under anesthesia at the end of chronic experimental periods were performed in these rats.

Chronic experiments. In the chronic studies, blood pressure was measured using tail-cuff plethysmography, and 24-h urine collections were made using metabolic cages in conscious rats. Baseline blood pressure and urine collections were obtained on the day prior to the start of daily drug treatment. During the 4-week treatment period, blood pressure and 24-h urine collections were made at the end of each week. On each occasion, blood pressure was measured first and then the rats were placed in metabolic cages for 24 h for urine collections. After noting the volume of urine-output, all urine samples were kept frozen (–20 °C) until analyzed for sulfate, sodium, and potassium concentrations in the urine.

Acute experiments. At the end of the 4-week chronic experimental period, the rats were anesthetized with thiobutobarbital sodium (Inactin; Sigma-Aldrich, St Louis, MO) at a dose of 100 mg/kg i.p. Supplemental doses of anesthesia were administered throughout the experimental period as required. The rats were placed on a servo-controlled surgical table that maintained body temperature at 37 °C. Tracheostomy was performed, and the animals were continuously provided with a mixture of 95% O₂ and 5% CO₂ through the endotracheal tube (PE-200).¹⁵ The right jugular vein was cannulated for the intravenous administration of an isotonic saline solution containing 1% albumin and 7.5% inulin (Inutest, Laevosan, Linz/Donau, Austria) as well as for supplemental doses of anesthesia. The right femoral artery was catheterized, and the cannula was connected to a pressure transducer to allow for continuous monitoring of arterial blood pressure using the AcqKnowledge data acquisition system (Biopac). The left kidney was exposed via a left-flank incision and placed in a lucite cup, and the left ureter was cannulated with a PE-10 catheter for urine collection. An ultrasonic flow probe (Transonic System) was placed on the left renal artery to measure total renal blood flow (RBF).¹⁵ Laser-Doppler flow probes (500 μm OD, Periflex 4001; Perimed) were used to measure the regional blood flow in the kidney.^{15,16} Cortical blood flow was measured by a Laser-Doppler flow probe placed on the surface of the kidney, and medullary blood flow was measured by a needle Laser-Doppler flow probe inserted ~3 mm deep into the left kidney. After a 60-min equilibration period following completion of surgical procedures, urine collections for two consecutive 30-min clearance periods were made with collections of corresponding blood samples and recordings of

hemodynamic parameters. All blood and urine samples were kept frozen (-20°C) until analyzed for concentrations of inulin.

Measurement of proteins level and expressions of CGL and CBS in the renal tissue. At the end of the experiments, 29 or 30 days following the start of the treatment with inhibitors, rats were killed and the right kidneys were sagittally sectioned to use one half fixed in formalin for immunohistochemistry for localization CGL and CBS proteins in the renal tissues, and the other half was snap-frozen for eventual western blot analysis. CGL and CBS immunohistochemistry were performed in formalin fixed renal tissue sections ($3\mu\text{m}$) by peroxidase technique using specific antibodies for CGL (cat no H00001434-M01; Abnova) and CBS (cat no H00000875-M01; Abnova) respectively at 1:3,000 and 1:500 dilutions to determine the distribution of the specific proteins and their changes during chronic treatments with PAG and AOA. Semi-quantitation of immunoreactivity was performed using NIS Elements AR version 3.0 software; (Nikon). In addition, whole kidney samples were used to measure CGL and CBS protein levels by western blot. The proteins were electrophoretically transferred to a nitrocellulose membrane (Invitrogen, Carlsbad, CA) using iBlot (Invitrogen), blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) followed by the incubation overnight at 4°C with the primary antibodies mouse anti-CGL (1:500 dilution) and mouse anti-CBS (1:1,000 dilution). Detection was accomplished using an Odyssey fluorescent scanner.

Analytical procedures for blood and urine samples and statistical analysis. Urinary excretion rate of sulfate ($U_{\text{S}}V$) is determined by analyzing urine samples acquired from the chronic experiments using Zinc-trapping and colorimetry method.^{17,18} The changes in $U_{\text{S}}V$ are considered as an indicator for assessing the status of endogenous H_2S production in the animals used in this study. The concentrations of sodium and potassium in urine samples were determined by flame-photometry. Spectrophotometry was used to determine inulin levels in the urine and blood samples obtained during the acute experiment. The value of inulin clearance was considered as glomerular filtration rate (GFR). Data are expressed as means \pm SE. Statistical comparisons of differences in means were done by one-way ANOVA followed by Holm-Sidak *post hoc t*-tests. Differences in the mean values were deemed significant at $P < 0.05$.

RESULTS

Effect of chronic PAG and AOA treatment on $U_{\text{S}}V$

These effects are summarized in **Figure 1**. Inhibition of CGL or CBS by chronic treatments of PAG or AOA alone had no significant effect on $U_{\text{S}}V$. However, the combination therapy of these inhibitors resulted in significant decreases in $U_{\text{S}}V$ after the second week of treatment period (240 ± 18 vs 591 ± 70 nmol/24h; $P < 0.05$) compared to values at day 0 (D0) which remains unaltered on the fourth week of treatment (248 ± 50 nmol/24h; $P < 0.05$). This finding indicated that the dose of combination therapy was effective in causing substantial

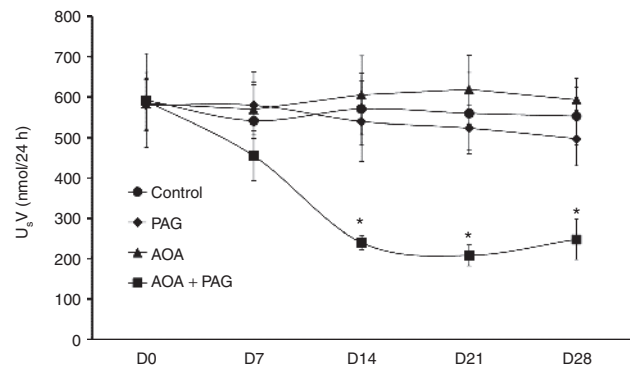


Figure 1 | Urinary excretion rate of sulfate ($U_{\text{S}}V$) responses to chronic inhibition of H_2S production (data from the chronic experiments). PAG, DL-propargylglycine (CGL inhibitor); AOA, aminooxyacetic acid (CBS inhibitor). * $P < 0.05$ vs. values on day 0 (D0). AOA, aminooxyacetic acid; CBS, cystathionine β -synthase; CGL, cystathionine γ -lyase; PAG, DL-propargylglycine.

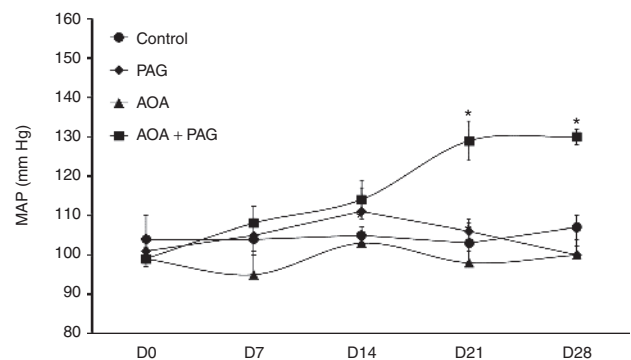


Figure 2 | Mean blood pressure (MBP) responses to chronic inhibition of H_2S production (data from the chronic experiments). * $P < 0.05$ vs. values on day 0 (D0). AOA, aminooxyacetic acid (CBS inhibitor); CBS, cystathionine β -synthase; CGL, cystathionine γ -lyase; PAG, DL-propargylglycine (CGL inhibitor).

inhibition ($>50\%$) of endogenous H_2S production. Although changes in $U_{\text{S}}V$ is considered as an indicator for assessing the changes in endogenous H_2S production, it should be noted here that $U_{\text{S}}V$ may also represent the dietary and other metabolic sources of sulfur.¹⁹ However, as it was ensured that the animals of similar age and sex in different groups were given identical diet and housed in identical condition, the contribution of other sources of sulfur was deemed minimal in causing the observed changes in $U_{\text{S}}V$.

Effect of chronic treatment of PAG and AOA on mean arterial blood pressure

The changes in mean arterial pressure (MAP) in each group of rats are illustrated in **Figure 2**. It was observed that the treatment with PAG or AOA alone for 4 weeks did not alter MAP in rats. However, in the group of rats that were treated with combination therapy of both PAG and AOA, MAP increased gradually from 99 ± 2 mm Hg at D0 to 130 ± 1 mm Hg ($P < 0.05$) at the end of 4-week treatment period. However, significant difference was noted at the end of third week (130 ± 2 ; $P < 0.05$) but not at the end of second week (114 ± 5 mm Hg; $P = \text{n.s.}$) treatment period when compared to the values at D0.

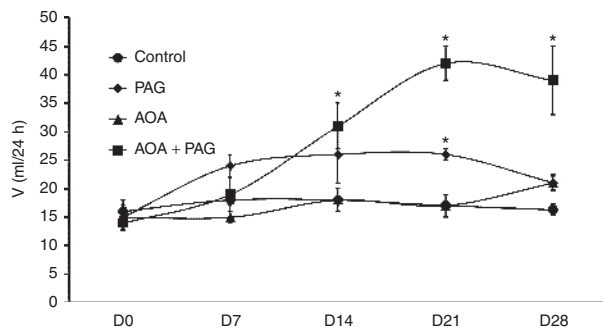


Figure 3 | Urine flow (V) responses to chronic inhibition of H₂S production (data from the chronic experiments). **P* < 0.05 vs. values on day 0 (D0). AOA, aminooxyacetic acid (CBS inhibitor); CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; PAG, DL-propargylglycine (CGL inhibitor).

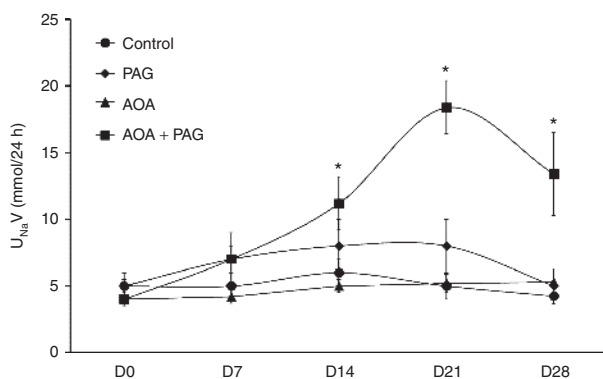


Figure 4 | Urinary excretion rate of sodium (U_{Na}V) responses to chronic inhibition of H₂S production (data from the chronic experiments); **P* < 0.05 vs. values on day 0 (D0). AOA, aminooxyacetic acid (CBS inhibitor); CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; PAG, DL-propargylglycine (CGL inhibitor).

It was noted that the changes in U_SV (significant changes at the end of second week) were preceded the changes in MAP (significant changes at the end of third week).

Effects of chronic treatment of PAG and AOA on urine flow, urinary excretion of sodium and potassium

The results of these measurements are summarized in **Figures 3** and **4**. Urine flow (V; **Figure 3**), urinary excretion rate of sodium (U_{Na}V; **Figure 4**) and urinary excretion of potassium did not alter during treatment of PAG or AOA alone. However, in the combined therapy group, V was increased gradually from 12.7 ± 0.8 ml/day on D0 to 38.8 ± 5.4 ml/day (*P* < 0.05) at the fourth week treatment period. Similar to the response on V, urinary excretion rate of sodium (U_{Na}V; **Figure 4**) were also increased in combination therapy group. U_{Na}V was increased gradually from 3.6 ± 0.5 mmol/day on D0 to 13.4 ± 3.1 mmol/day (*P* < 0.05) at the fourth week treatment period. However, the changes in urinary excretion of potassium during combination therapy were not statistically different during the treatment period (2.4 ± 0.2 mmol/day on D0 to 3.2 ± 0.3, 5.1 ± 0.7, 4.5 ± 0.4, and 4.3 ± 1.2 mmol/

Table 1 | Renal hemodynamic parameters after 4 weeks of H₂S inhibition (data from the acute experiments).

Treatment groups	RBF (ml/min/g)	GFR (ml/min/g)	CBF (PU)	MBF (PU)
Control (n = 6)	7.0 ± 0.4	1.0 ± 0.05	554 ± 15	232 ± 36
PAG (n = 6)	7.0 ± 1.2	0.94 ± 0.06	476 ± 21	175 ± 38
AOA (n = 5)	7.2 ± 1.0	0.91 ± 0.03	485 ± 48	218 ± 39
AOA + PAG (n = 5)	3.5 ± 1.2*	0.92 ± 0.04	375 ± 26*	134 ± 19

AOA, aminooxyacetic acid; CBF, cortical blood flow; GFR, glomerular filtration rate; MBF, medullary blood flow; PAG, DL-propargylglycine; PU, perfusion unit; RBF, renal blood flow. **P* < 0.05 vs. values in control group.

day at the end of first, second, third, and fourth week period of treatment respectively.)

Effects of chronic treatment of PAG and AOA on renal blood flow and glomerular filtration rate

The results obtained from acute experiments at the end of 4-week treatment period, are provided in **Table 1**. The total RBF was significantly lower in rats with combination therapy compared to that in the vehicle treated group as well as in groups treated with PAG or AOA alone. Similarly, regional blood flow (cortical blood flow and medullary blood flow) was also lower in the combination therapy group compared to the values in other groups. However, the values of GFR were not significantly different in any of these treatment groups compared to the vehicle treated group.

Immunohistochemistry analysis of CGL and CBS in renal tissues

CGL and CBS immunoreactivity and densitometric analysis in kidney slices are illustrated in **Figure 5** and **Figure 6**, respectively. As illustrated in **Figure 5a**, CGL was mainly located in renal vasculature as well as in distal nephron segments in tissues taken vehicle treated control rats. Mean data from the densitometric analysis of the intensity of CGL immunoreactivity in these kidney sections are illustrated in **Figure 5e**. It was observed that CGL immunoreactivity increased nearly threefold in PAG treated rats and nearly twofold in rats subjected to combination therapy compared to vehicle treated group. This enhancement in CGL immune-expression in combined treatment group was significantly lower than that in PAG treated group. There was no change in CGL intensity in AOA treated group. On the other hand, CBS immunostaining was observed exclusively in intracellular compartment of proximal tubular cells in control rats (**Figure 6a**). Similar distribution, though with markedly increased immunointensity, was observed in kidney sections from rats treated with combination therapy. It should be noted that CBS immunoreactivity was mainly observed at the brush border but not intracellularly in the proximal tubular cells in rats treated with either PAG or AOA alone. This CBS immunoreactivity was absent in the glomeruli and other tubular segments as well as in renal vasculature. Mean data from the densitometric analysis of the CBS intensity in these kidney sections are illustrated in **Figure 6e**. CBS immunoreactivity was slightly but significantly increased in

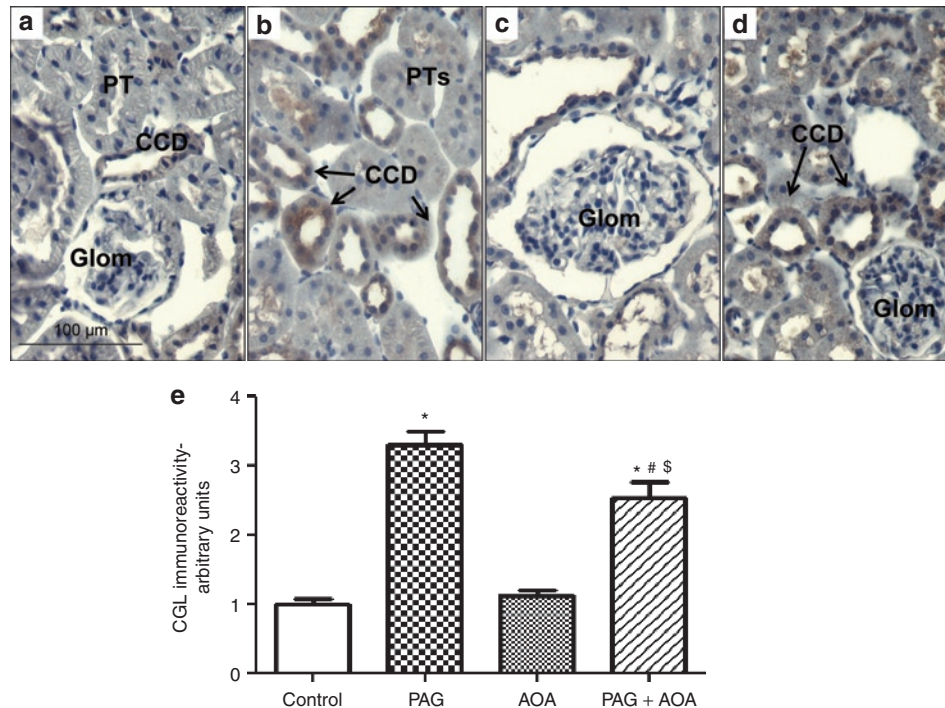


Figure 5 | CGL Immunoreactivity and Densitometric analysis in kidney slices: CGL immunoreactivity (Brown staining with 3,3'-diaminobenzidine) in paraffin embedded kidney sections (5 μ m) from controls (**a**) and treated groups (**b-d**) was observed in the apical aspect of the renal vasculature and in collecting ducts cells. Mean data from the densitometric analysis of the CGL intensity in these kidney sections from control rats (panel **a**) and the rats treated with either PAG (panel **b**), AOA (panel **c**) or combination of both (PAG + AOA; panel **d**) are illustrated in **e**. Values are mean \pm s.e. * P < 0.0001 vs. control; # P < 0.0001 vs. PAG; \$ P < 0.0001 vs. AOA. Magnification: 400X. AOA, aminooxyacetic acid; CD, collecting duct; CGL, cystathionine γ -lyase; Glom, glomerulus; PAG, DL-propargylglycine; PT, proximal tubule.

combination therapy group compared to PAG alone treated group.

Western blot analysis of CGL and CBS in renal tissue

Further quantification of the levels of these enzyme proteins in the renal tissues by western blot analysis revealed that PAG and PAG plus AOA treatments significantly increased CGL protein levels (25 kDa)²⁰ in whole kidney samples compared to controls (PAG: 3.0 ± 0.3 ; PAG + AOA: 1.7 ± 0.4 vs. control: 1.0 ± 0.1 -fold change; P < 0.01; n = 4 in each cases) but not AOA alone (1.0 ± 0.1) (Figure 7a). In contrast, these treatments did not alter the protein levels of CBS (63 kDa)²¹ in the kidney (PAG: 0.7 ± 0.04 ; AOA: 1.1 ± 0.3 ; PAG + AOA: 0.8 ± 0.1 ; vs. control: 1.0 ± 0.1 -fold change; P = 0.02; n = 4 in each cases) (Figure 7b). These results of the protein levels quantified by western blot were comparable with those observed with immunohistochemistry analysis as above.

DISCUSSION

In the present investigation, we have demonstrated that chronic administration of the inhibitors of both CGL and CBS enzymes in combination for 4 weeks in rats resulted in a decrease in $U_{S\dot{V}}$ which is considered as an indicator for endogenous H_2S production. Although the doses of these inhibitors independently were shown to be effective in preventing H_2S generation in rat aortic¹⁴ and liver⁶ tissues, the level of $U_{S\dot{V}}$ was not altered by these doses when given individually in this

study. The changes in $U_{S\dot{V}}$ were also associated with increases in MAP in the combination therapy group. It was noted that the changes in $U_{S\dot{V}}$ (at the second week) preceded the changes in MAP (at the third week) indicating that a deficiency in H_2S production leads to an increase in arterial pressure. These findings demonstrate that the reduction in H_2S production in the vascular system occurred only when activities of both H_2S producing enzymes (CGL and CBS) were simultaneously blocked. These results suggest that the deficiency in the activity of one enzyme could be compensated by the activity of the other in maintaining the endogenous production level of H_2S .

Although $U_{S\dot{V}}$ or blood pressure did not alter during 4 weeks of treatment with PAG alone in this study, the same dose administration of this CGL inhibitor in a previous study in rats¹⁴ reported a reduction in plasma level of H_2S and associated increase in blood pressure at the end of more than 5 weeks treatment period. This apparent discrepancy seems related to the difference in the period of treatment with the inhibitor. CGL expressions in the renal tissue were also not reduced (rather increased) during 4-week period of PAG treatment in the present study. However, the results from the previous study¹⁴ indicate that CGL expression as well as the reductions in tissue and plasma H_2S levels in the aortic tissue was decreased only after 5-week period of treatment with the enzyme inhibitor. These results may indicate a time dependency of this inhibitor's ability on reducing endogenous H_2S production that contributes to increase in arterial pressure.

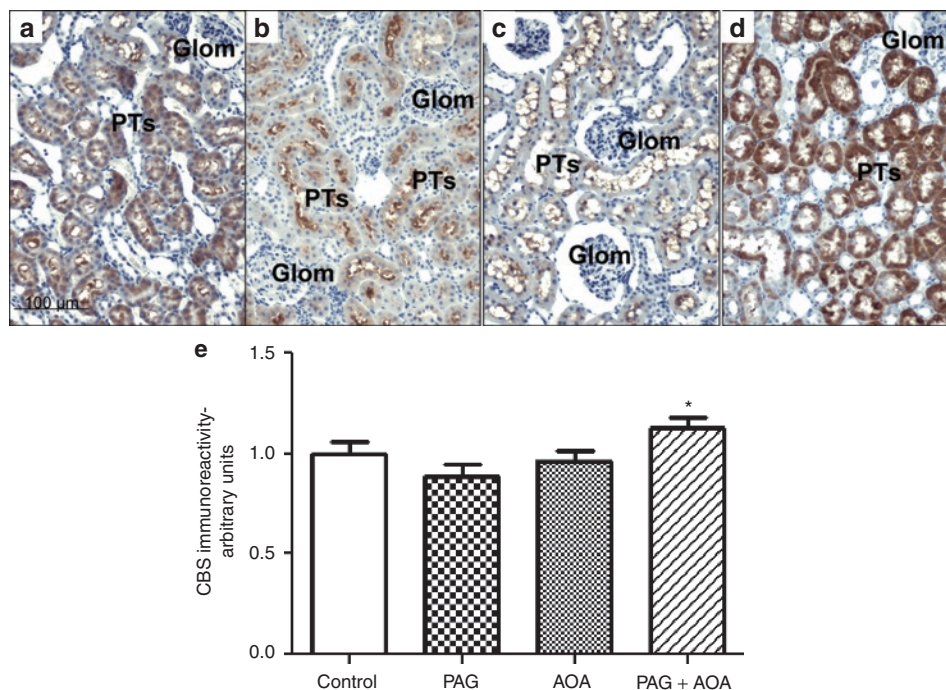


Figure 6 | CBS immunoreactivity and densitometric analysis in kidney slices: CBS immunoreactivity (Brown staining with 3,3-diaminobenzidine) was observed exclusively in intracellular compartment of proximal tubular (PT) cells of paraffin embedded kidney (5 μ m) sections of control rats. Similar distribution, though with marked increased immunointensity, was observed in kidney sections from rats treated with combination (PAG + AOA) therapy (panel **d**). It should be noted that CBS immunoreactivity was mainly observed at the brush border but not intracellularly in the PT cells in rats treated either PAG (panel **b**) or AOA (panel **c**) alone. Glomeruli, other tubular segments and renal vasculature were negative. Mean data from the densitometric analysis of the CBS intensity in these kidney sections are illustrated in **e**. Values are mean \pm s.e. * $P < 0.05$ vs. PAG treated group only. Magnification: 200X. AOA, aminooxyacetic acid; CBS, cystathionine β -synthase; CD, collecting duct; Glom, glomerulus; PAG, DL-propargylglycine.

It is also noted that a considerable time-delay exists in mediating the response to a deficiency in CGL activity and the consequent increases in blood pressure. The knock-out mice lacking the gene for CGL usually not hypertensive at early age^{3,11} but developed hypertension after at least after 7 weeks of age.³

A link between H₂S deficiency and an increase in arterial pressure has been suggested in many previous studies.^{10,22,23} A deficient level of plasma H₂S was reported in hypertensive rat models such as SHR when compared to the level in normotensive WKY rat models.¹⁴ Hypertension induced by treatment with NO synthase inhibitors in rats was also associated with a decrease in plasma H₂S level.¹⁰ On the other hand, systemic administration of the H₂S-donor compound NaHS resulted in hypotension in many previous studies.^{7,10,23} In one study, it was reported that the genetic deletion of CGL enzymes in mice resulted in nearly 50% reduction in the plasma level of H₂S, which was associated with ~20–25 mmHg increases in MAP.³ In the present study in rats, we also observed comparable changes in MAP (~25 mmHg) in response to combination therapy with PAG and AOA that resulted in nearly a 50% reduction in U_SV, indicating a similar reduction in the plasma level of H₂S in rats as reported in earlier studies on mice.³

The cardiovascular effects of H₂S have been demonstrated in experimental studies *in vivo*¹⁰ and *in vitro*.^{2,7–9} Most of these studies provided evidence that H₂S is a novel gase-

ous vasorelaxant agent, acting by directly increasing K_{ATP} channel currents and hyperpolarizing the cellular membrane.⁷ However, as opposed to other gaso-transmitters such as NO and carbon monoxide, H₂S-induced vasorelaxation is not mediated by the cyclic guanosine 3',5'-monophosphate signaling pathway, but rather by the cyclic adenosine 3',5'-monophosphate signaling pathway.^{24–26} It is suggested that H₂S is acting as a putative endothelium dependent hyperpolarizing factor, thus reducing the extracellular Ca²⁺ entry and relaxing vascular smooth muscle cells.²⁷ Although acting as a vasorelaxant, H₂S also caused inhibition of vascular smooth muscle cells' proliferation and induced apoptosis in some *in vitro* studies.^{28,29} Thus, a generalized vasoconstriction and structural remodeling by vascular smooth muscle cells' proliferation may be involved in the hypertensive response to the chronic H₂S deficiency in the present study.

Associated with increases in MAP in the present study, there were also reductions in total RBF and regional blood flow (cortical blood flow and medullary blood flow) in the kidney due to this combination therapy, demonstrating that the reduction in H₂S formation resulted in increases in systemic as well as in renal vascular resistances. In the present study, there was no appreciable change in GFR though RBF decreased in rats chronically treated with combined administration of PAG and AOA. This indicates that there were proportionate reductions in vascular resistances in both afferent

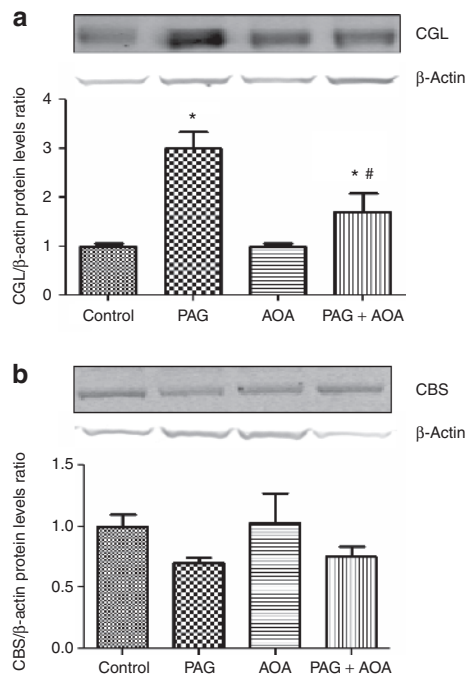


Figure 7 | Representative western blots showing CGL (Upper panel; **a**) and CBS (Lower panel; **b**) protein levels in whole kidney samples ($n = 4$ in each cases) from the control rats and the rats treated with either PAG (DL-propargylglycine; CGL inhibitor), AOA (aminoxyacetic acid; CBS inhibitor) or combination of both (PAG + AOA). Densitometric analyses were done against β actin, used as loading control. Control levels were defined as the unit. * $P < 0.01$ vs. control, # $P < 0.01$ vs. PAG. CBS, cystathionine β -synthase; CGL, cystathionine γ -lyase.

and efferent arterioles during chronic combination therapy that maintained the net glomerular pressure at a steady state³⁰ and thus, GFR remains unchanged. The increases in urine flow and sodium excretion in the rats treated with both PAG and AOA in combination is likely to be related to pressure-diuresis and natriuresis phenomenon as MAP increased in rats with this combination therapy.¹⁶ These data suggest that the hypertensive response to chronic H₂S deficiency may be primarily due to its' effect on systemic vascular resistance. However, as the renal excretory function could be influenced by the systemic effects of these inhibitors, it may not be possible to determine the specific functional effects of CGL and CBS inhibition in the kidney from the present results. It was also reported²³ that acute administration of PAG and AOA in combination directly in the kidney caused reductions in GFR, urine flow, and sodium excretion without appreciable change in RBF or systemic arterial pressure. Although these renal excretory changes to acute administration of CGL and CBS inhibitors²³ seem apparently contradictory with the findings in the present chronic study, it should be noted here that these responses to H₂S deficiency in both these studies were only observed when these inhibitors were administered in combination but not in alone. Thus the findings in these studies (23 and the current study) are supportive of the conclusion that endogenous H₂S level is interdependently maintained by the activity of these enzymes.

In the present study, it was observed that chronic PAG treatment enhanced CGL immune-expression as well as protein levels in the renal tissue that was slightly reversed but remained high at significant level in rats treated with combination therapy as determined by both immune-staining (Figure 5) and western blot analysis (Figure 7a). The reason for such enhancement of CGL protein expression during chronic PAG administration with or without AOA treatment is not yet clearly understood. However, it seems that inhibition of CGL enzymatic activity by PAG caused a compensatory stimulation of the synthesis of its own enzyme proteins. Such up-regulation of specific enzyme protein expressions in response to chronic treatment with its inhibitor agent may not be entirely unexpected. For example, angiotensinogen mRNA was also seen up-regulated during chronic treatment with angiotensin converting enzyme inhibitors.³¹ However, chronic AOA treatment alone in the present study did not show any marked changes in the protein levels of CBS or CGL in the renal tissue (Figures 6 and 7b). It is interesting to note that the immunohistochemistry analysis of renal tissues in the present study demonstrated that CGL was mainly located in renal vasculature as well distal nephron segments while CBS was located only in the proximal tubules. These data demonstrate distinctive tubular locations of CBS (proximal tubule) and CGL (distal tubules and collecting ducts) that warrants further experiments to determine the differential regulation of tubular function by these H₂S producing enzymes.

In conclusion, the findings in the present investigation indicate that the functional deficiency in the activity of one H₂S producing enzyme could be compensated by the activity of the other to maintain the endogenous H₂S production level. The present data demonstrate that chronic H₂S deficiency caused increases in vascular resistance leading to renal vasoconstriction and systemic hypertension. These data suggest that the hypertensive response to chronic H₂S deficiency may be primarily due to its effect of increasing systemic vascular resistance.

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