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Reduced albumin in the renal cortex of ethylene glycol-treated rats

Jui-Lung Shen[a,b,c], Kee-Ming Man[a,d,e], Yung-Hsiang Chen[f,g], Chiao-Hui Chang[f,g], Yuan-Ju Lee[h], Huey-Yi Chen[f,g], Kao-Sung Tsai[f], Fuu-Jen Tsai[f,g], Wen-Chi Chen[f,g,*], Hsuan-Fu Kuo[i]

[a] Department of Anesthesiology, Tung's Taichung Harbour Hospital, Taichung, Taiwan
[b] Centre for General Education, Feng Chia University, Taichung, Taiwan
[c] Department of Dermatology, Taichung Veteran General Hospital, Taichung, Taiwan
[d] Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan
[e] Graduate Institute of Geriatric Medicine, Anhui Medical University, Hefei, China
[f] Graduate Institute of Integrative Medicine, School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung, Taiwan
[g] Departments of Medical Research, Obstetrics and Gynecology, Pediatrics, and Urology, China Medical University Hospital, Taichung, Taiwan
[h] Department of Urology, National Taiwan University Hospital, Taipei, Taiwan
[i] Department of Internal Medicine, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan

*Corresponding author, e-mail: wgchen@mail.cmu.edu.tw, medsnail@hotmail.com

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ABSTRACT: Ethylene glycol (EG), a frequently used lithogenic agent, causes calcium oxalate (CaOx) crystal deposition in the renal cortex. In this study, we compared the protein expression pattern in kidneys of rat with EG-induced renal lithiasis with that of normal rats. Ten male Sprague-Dawley rats were divided into 2 groups. In the control group ($n = 5$), rats were fed with normal drinking water; in the EG group ($n = 5$), rats were fed with 0.75% EG in drinking water. After 4 weeks, rats fed with EG showed CaOx deposition in the renal cortex. The renal cortex proteins were extracted and separated by two-dimensional electrophoresis (2-DE). Comparative analyses of the respective spot patterns from the 2-DE output were performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Among the proteins identified, reduced albumin in rat renal lithiasis was confirmed by immunohistochemical analysis. Our results demonstrated that the amount of albumin was reduced in EG-treated rat kidneys. Subsequent crystal formation may be due to the loss of inhibitors or decreasing the remaining amount of albumin in the tissues, suggesting that albumin may play an important role in modulating EG-induced stone formation.

KEYWORDS: calcium oxalate, protein expression, proteomics, urolithiasis

INTRODUCTION

Urolithiasis is a complex process. Many theories have been proposed to define the cause of stone formation, including the effects of nucleation, promoters, and lack of inhibitors[1]. As inhibitors, proteins may play a role in defence against nephrocalcinosis. Albumin, for example, may play a role as an inhibitor to decrease stone formation[2]. However, some urinary proteins may also promote stone formation[3]. Recently, we isolated albumin from calcium oxalate (CaOx) stones and found that albumin can induce CaOx nucleation. The role of albumin in stone formation is still controversial and needs to be further investigated.

Rodgers et al investigated the difference in albumin levels between white and black patients with kidney stones because of higher incidence rates of kidney stones found among white patients. They discovered molecular differences in albumin, for example, albumin derived from black subjects had stronger inhibitory effect on CaOx formation than that from white subjects[4]. Hence molecular strength may influence the stone formation rate. This means that albumin inhibits crystal binding and may induce crystal formation under some conditions. We speculated whether the rat model of urolithiasis also demonstrates the same phenomenon to further verify the role that albumin plays in the formation of calcium stones.

Ethylene glycol (EG) is the most frequently used lithogenic agent. Using EG in a rat model is a stable model to induce CaOx crystal deposition in kidneys and can be used to mimic CaOx formation in human kidneys. We have also used this model to study stone disease[5–7]. Mass spectrometry is a powerful analytical tool that measures the mass and sequence of peptides[8], and is widely used in proteomic studies to analyse the peptide sequences of a group of proteins extracted from tissues[9]. In our previous study, we extracted low-molecular-weight proteins from CaOx stones and measured their characteristic patterns using mass spectroscopy[10]. In this study, to further investigate the molecular changes underlying rat renal lithogenesis induced by chronic EG treatment, we compared the protein expression patterns between rat renal tissues with lithiasis induced by EG and normal rat renal tissues. To do this, we used two-dimensional electrophoresis (2-DE) combined with surface-enhanced matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

MATERIALS AND METHODS

Animal study

Ten male Sprague-Dawley rats (6 weeks of age) were purchased from BioLASCO Co. (Taipei, Taiwan), each weighing approximately 250–300 g. They were housed in the animal centre at the China Medical University (Taichung, Taiwan) acclimated to a room temperature of 25 °C with a 12 h light, 12 h dark cycle and fed standard commercial rat chow. All experimental protocols were approved by the Institutional Animal Care and Use Committee of China Medical University (No. 101-108-N, 20 Dec 2011). Rats were randomly divided into two groups. Group 1 ($n = 5$) served as the normal control and received deionized distilled water and fed normal chow. Group 2 ($n = 5$) had free access to drinking water containing 0.75% EG as a stone inducer throughout the entire 4-week experimental period. We recorded body weights of rats before and after the experiment. All rats were killed at the end of the experiment under ether anaesthesia. Both kidneys from each rat were harvested and weighed. Right-sided kidneys were fixed in formalin, embedded in paraffin and stained with haematoxylin and eosin Y solution. We then examined the histological sections of kidneys for crystal deposits with a polarized light microscope. Further immunohistochemical staining was also performed after identification of special protein from the results of the proteomic study.

Collection of serum and urine samples

We aspirated about 2 ml blood from rat tail vessels and put it into a heparin-containing test tube. For further collection of 24-h urine sample, rats were housed in metabolic cages for one day after blood sampling. Samples were stored in 4 °C container before transportation to laboratory. After 4 weeks, the experimental animals were killed and serum and urine samples were collected again. Body weight was recorded before killing the rats. Weight of harvested rat kidney was also recorded. Hitachi-7150 and Roche-Omnic analysers were used for biochemical studies of urine and blood samples which include calcium, free calcium, phosphorus, and pH value.

Harvested right kidneys were put in 10% formalin and embedded by paraffin. Then the tissue sections were stained by haematoxylin-eosin solution. Histological sections of kidneys were examined with a polarized light microscope for the presence of CaOx crystal deposits at a magnification factor of 100×. The extent of crystal deposition was according to the previous reported semi-quantitatively subjective scoring system and our ImageScoring software which graded from 0–3^+ (where 0 = none, 1^+ = few, 2^+ = several, and 3^+ = many crystal deposits).

Extraction of rat kidney proteins

Rat left kidneys were immersed into liquid nitrogen immediately after harvest. A slice of 1/4 kidney including cortex and medulla was cut for the extraction. Sample was washed with phosphate buffered saline (PBS) three times. Then, samples were grounded in a mortar under liquid nitrogen and with 20 µl protease inhibitor (cocktail; Sigma-Aldrich Inc., St. Louis, MO). The powder was put into 500 ml buffer A solution (8 M Urea 24 g + 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate $C_{32}H_{58}N_2O_7S$) 2 g + 0.02% bromophenol blue 100 µl, and add double destilled water to 50 ml in total). Lysate samples were centrifuged for 15 min (4 °C, 10 000g). The supernatants were placed in 1.5 ml Eppendorf tube with acetone (sample:acetone = 1:3) for protein precipitation. After centrifugation, 200 µl Buffer A was added to the precipitate and the protein concentration was determined by Bradford dye-binding method (absorbance wavelength 595 nm).

Two-dimensional gel electrophoresis

About 200 µg of proteins in 350 µl rehydration buffer were applied to pH 3–10, 17 cm IPG strip (Bio-Rad ReadyStrip), followed with IEF as rehydration for

12 h, 500 V for 0.5 h, 1000 V for 1 h, 3000 V for 1.5 h, 8000 V for 3 h, and 8000 V for 3 h until total voltage hours of 36 500 was achieved. Before the SDS-PAGE, strips were equilibrated in 6 M urea, 50 mM Tris-HCl (pH 8.8), 30% (v/v) glycerol, 2% SDS, 0.002% trace bromophenol blue and 1% dithiothreitol for 15 min and then the same buffer containing 2% iodoacetamide instead for the second 15 min. After equilibrium, strips were loaded to 10% SDS-PAGE for electrophoresis.

Silver staining was performed by PlusOne Silver Staining kit (General Electric Company GE, Fairfield CT) after SDS-PAGE. We scanned and analysed the gels by Bio-Rad GS-800 Image Densitometer (Hercules CA) ready for the comparison the difference.

In-gel digestion of proteins by trypsin

After scanning the gels, each spot of interest was cut into 1 mm cubes and washed with double distilled water twice on a vortex for 10 min. Then, the gel was aspirated into microtube with a 1.5 ml pipette prewashed with 100% acetonitrile (ACN, C_2H_3N). The gel was washed with 100 µl wash buffer (25 mM NH_4HCO_3, 50% ACN) for 15 min. After aspirating the solution, 200 µl silver destain solution (silver destain solution 0.1 g potassium ferricyanide, 0.15 g, sodium thiosulphate in 10 ml Milli Q water) was added into the tube. The solution was removed and the gel was rinsed with 200 µl 25 mM ammonium bicarbonate buffer. The procedure of destaining was repeated twice until the gel turned transparent. The gel was then immersed in 100 µl 100% ACN. The gel was air dried 1 h after removal of ACN solution. The gel was then treated with 3 µl trypsin solution (20 ng/µl trypsin, 25 mM NH_4HCO_3, pH 8) at 37 °C for overnight. Then, 2 µl 100% ACN and 1% trifluoroacetic acid (TFA) was added and sonicated for 10 min. The supernatant was ready for mass spectroscopic study.

Protein identification was performed by MALDI-TOF (Ultraflex III TOF/TOF, Brucker, Germany) in proteomics Core of Laboratory (China Medical University). Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometer (MALDI-TOF MS, Ultraflex III, Brucker, Germany) was used to analyse the sample with α-cyano-4-hydroxycinnamic acid (CHCA matrix). The peak list of MS spectra was processed by FLEXANALYSIS v.3.0 (Bruker Daltonics) software. The extracted peak list was searched against a Swissport (release 51.0) using MASCOT v.2.2.04 (Matrix Science, London). Parameters were selected as Taxonomy-Rattus; enzyme-trypsin; fixed modifications-carbamidomethyl (C); variable modifications-oxidation (M); Precursor peptide tolerance ± 100 ppm.

Table 1 Average body weight gain and kidney weight between control and EG group.[†]

	Control group	EG group
Body weight gain (g)	180 ± 13	$131 \pm 23^{*}$
Left kidney (g)	1.8 ± 0.3	1.8 ± 0.3
Right kidney (g)	1.9 ± 0.5	1.7 ± 0.2

[†] data are expressed as mean ± S.D.
[*] $p < 0.05$

Immunohistochemical staining of the rat kidney

Sections of formalin-fixed, paraffin-embedded tissues mounted on poly-d-lysine-coated slides were deparaffinized in xylene and rehydrated through serial baths of alcohol and water. The slides were placed in 6 M urea and heated in a microwave oven at 700 W for 5 min. The hydrated sections were then treated in methanol containing 0.3% hydrogen peroxide for 30 min to eliminate endogenous peroxidase activity and were then washed in PBS. The primary antibody used in this study was a monoclonal antibody to albumin (ALB, P-20, sc-46293, Santa Cruz, USA) (1:250 dilution). The polyclonal antibody-treated slides were rinsed in PBS solution and incubated with a biotinylated secondary antibody (Signet, USA). The slides were washed in PBS, and then incubated with an avidin-biotinperoxidase complex (Signet, USA) for 30 min. After washing with PBS, a chromogenic reaction was developed by incubating the slides with a freshly prepared solution of 3,(3)-diaminobenzidine tetrahydrochloride (0.04%) and hydrogen peroxide (0.03%). A brownish yellow substrate in the cytoplasm indicated a positive reaction to albumin.

Statistical analysis

The significance of differences in mean values between the groups was determined with the Student's t-test. The data are expressed as means ± S.D. A comparison giving a p value < 0.05 was considered statistically significant. The software used for analysis was the Statistical Package for the Social Science (SPSS for Windows, release 15.0, SPSS Inc., Chicago, Illinois, USA).

RESULTS

After 4 weeks, the average body weight gain in EG-treated rats (131 ± 23 versus 180 ± 13 g, respectively) were significantly lower than the controls ($p = 0.007$). Kidney weight was the same in both groups (Table 1).

Table 2 Comparison of biochemical parameters between experimental and control groups.[†]

		Control group	EG group
Before the experiment			
Urine	Phosphorus (mg/dl)	22.2 ± 0.8	22.6 ± 0.4
	Free calcium (mmol/l)	0.8 ± 0.4	1.1 ± 0.4
	pH	6.5 ± 0.5	6.5 ± 0.2
Serum	Calcium (mg/dl)	6.9 ± 1.4	6.9 ± 1.1
	Phosphorus (mg/dl)	22.0 ± 2.1	21.4 ± 2.0
After the experiment			
Urine	Phosphorus (mg/dl)	21.0 ± 1.1	19.5 ± 0.7
	Free calcium (mmol/l)	0.4 ± 0.2	0.2 ± 0.1
	pH	6.9 ± 0.5	6.7 ± 0.3
Serum	Calcium (mg/dl)	11.2 ± 0.3	10.7 ± 0.6
	Phosphorus (mg/dl)	12.5 ± 1.3	9.5 ± 2.3[a]
	Free calcium (mmol/l)	1.1 ± 0.1	1.1 ± 0.1
	pH	7.1 ± 0.2	7.1 ± 0.1
	Albumin (g/dl)	4.0 ± 0.1	4.0 ± 0.9

[†] data are expressed as mean $\pm$ S.D.
[a] $p < 0.05$

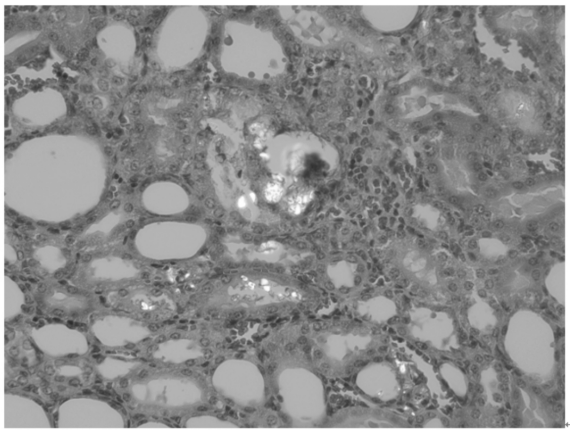

Fig. 1 Pathology of rat kidney treated with EG. Birefringent CaOx crystals were shown within tubules; lymphocyte and red blood cell infiltrations were also presented.

There were no differences among controls and EG rats in serum albumin and calcium or in urinary phosphorus, free calcium, and urine pH (Table 2). Although urinary free calcium was lower in the EG-treated group than in the control group, it was only a marginally significant difference ($p = 0.059$). There was a significant difference in serum phosphorus between the EG-treated group (9.5 ± 2.3 mg/ml) and the control group (12.5 ± 1.3 mg/ml, $p = 0.015$) (Table 2).

Histological studies revealed that there was no calcification in the kidneys of normal control rats. As expected, crystal deposits were found in the kidneys of EG-treated rats. Under polarized light microscopy, the crystals exhibited a birefringent appearance (example shown in Fig. 1), in which multiple crystals are seen in both renal cortex and medulla. Pathological changes were also noticed, including the presence of intratubular crystals, collecting-duct crystal plugs, tubular epithelial damage, and interstitial inflammation. The crystal formation rates for control and EG groups were 0.0% and 100%, respectively (Table 3).

Table 3 CaOx crystal formation rate between experimental and control group.

	Control group	EG group
Rat number	5	5
Number of rats with crystal	0	5
Incidence (%)	0	100

Table 4 Results of the comparison of protein spots.

Spot No.	Protein ID	p value
1	GNAQ_RAT	> 0.05
2	DNM1L_RAT	> 0.05
3	IQUB_RAT	> 0.05
4	DESM_RAT	> 0.05
5	GNAQ_RAT	> 0.05
6	MYH4_RAT	> 0.05
7	DC1L2_RAT	> 0.05
8	RAB19_RAT	> 0.05
9	T11L2_RAT	> 0.05
10	VINC_RAT	> 0.05
11	COQ5_RAT	> 0.05
12	RAB19_RAT	> 0.05
13	VINC_RAT	> 0.05
14	T11L2_RAT	> 0.05
15	H1T_RAT	> 0.05
16	T11L2_RAT	> 0.05
41	ID[a]	> 0.05
42	ID[b]	> 0.05
43	ID[c]	> 0.05
44	albumin	< 0.05

[a] Transmembrane protein 44 isoform a (predicted)
[b] Spindlin-like protein 2 (SPIN-2)
[c] Chondroitin sulphate proteoglycan 6, isoform CRA_c

Reproducible high-resolution 2-DE protein maps were obtained from the kidney tissues of all rats. Following trypsin digestion, protein expression spots in the EG-treated kidney tissues were identified by MALDI-TOF-MS, using 2D IMAGE-MASTER software. 2-D gel electrophoresis revealed albumin as one of the differing protein expression spots; the other protein expression spots remain unidentified. After a Mascot web search with probability-based Mowse scoring, we set the protein scoring level of significance to less than 5% to make a finding worthy of further study (Tables 4 and 5). Point no. 44 indicates higher protein expression in the gel profile

Table 5 List of searching results for albumin[†]

	Mass	Score	Expect	Queries matched	OS	GN	PE	SV
ALBU_RAT	70 682	79	9.7×10^{-5}	11	*Rattus norvegicus*[a]	Alb	1	2
PRPS1_RAT	35 325	16	1.9×10^{2}	3	*R. norvegicus*[b]	Prps1	1	2
GEPH_RAT	83 954	27	14	7	*R. norvegicus*[c]	Gphn	1	3
ARL1_RAT	20 513	22	43	3	*R. norvegicus*[d]	Arl1	1	1
TDIF1_RAT	36 897	22	51	4	*R. norvegicus*[e]	Dnttip1	2	1

[†] Search Parameters of Mascot: Type of search MS/MS - Ion Search; Enzyme - Trypsin; Fixed modifications - Carbamidomethyl (C); Variable modifications - Oxidation (M); Mass values - Monoisotopic; Protein Mass - Unrestricted; Peptide Mass - Tolerance ± 150 ppm; Fragment Mass - Tolerance ± 0.8 Da; Max Missed - Cleavages 1; Instrument type - MALDI-TOF-TOF.

[a] Serum albumin OS

[b] Ribose-phosphate pyrophosphokinase 1 OS

[c] Gephyrin OS

[d] ADP-ribosylation factor-like protein 1 OS

[e] Deoxynucleotidyltransferase terminal-interacting protein 1 OS

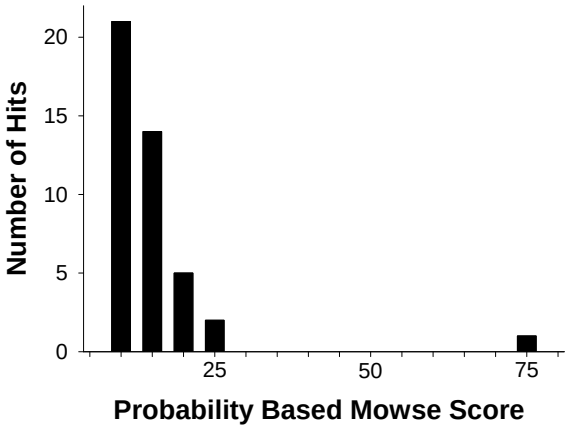

Fig. 2 Results from data bank of no. 44 which was indicated as albumin. Protein score is $-10 \log_{10} P$, where P is the probability that the observed match is a random event. Protein scores greater than 51 are significant ($p < 0.05$). Database: SwissProt 20 081 110 (400 771 sequences; 144 646 353 residues). Taxonomy: Rattus (7217 sequences). Timestamp: 24 Mar 2009 at 02:30:03 GMT. Top score: 79 for ALBU_RAT, serum albumin OS = *R. norvegicus*, GN = Alb, PE = 1, SV = 2.

of EG-treated rat kidney tissues. This spot was cut from one of the silver-stained gels and identified as albumin (Mass: 70 682, Score: 79) by MALDI-TOF-MS (Fig. 2). No other proteins were scored at less than 0.05.

Immunohistochemical staining was used to further validate the differential amount of albumin between the control and EG-treated rat kidneys. The expression of albumin in the EG group was markedly reduced compared with that in control group (Fig. 3).

DISCUSSION

We successfully induced CaOx crystallization in the kidneys of EG-treated rats. Several kidney proteins of interest differed in amounts between the control and EG-treated groups, as seen on 2-D SDS-PAGE. However, there were significantly fewer proteins of interest after comparing with the Mascot web data bank, except one. We found lower amount of albumin in the renal cortex in the experimental group than in the control group. These findings were further verified by mass spectroscopy. The results demonstrated that EG could decrease the amount of albumin in kidney.

Proteins play an important role in the pathophysiology of kidney stone formation[11]; moreover, some urinary proteins may promote stone formation. Recently, we demonstrated that albumin, the most abundant protein found in the stone matrix, can induce CaOx nucleation. The evidence supports a specific role of albumin in the constitution of the stone protein matrix[12]. Albumin is the most abundant protein in urine and is frequently seen within CaOx stones. Albumin was found to be the major protein from CaOx crystals induced in vitro in healthy controls and from stone-forming patients' urine[13–15]. As a major part of stone matrix protein, albumin has been hypothesized to be involved in urolithiasis. The role of albumin in CaOx stone formation may be related to its functions or may just be an incidental occurrence. Our previous proteomic studies on human kidney stones revealed albumin was abundant within CaOx stones[10]. However, the mechanism of how albumin is incorporated in CaOx stones is still unclear.

Albumin is a very important and unique protein in many species and plays an important role in main-

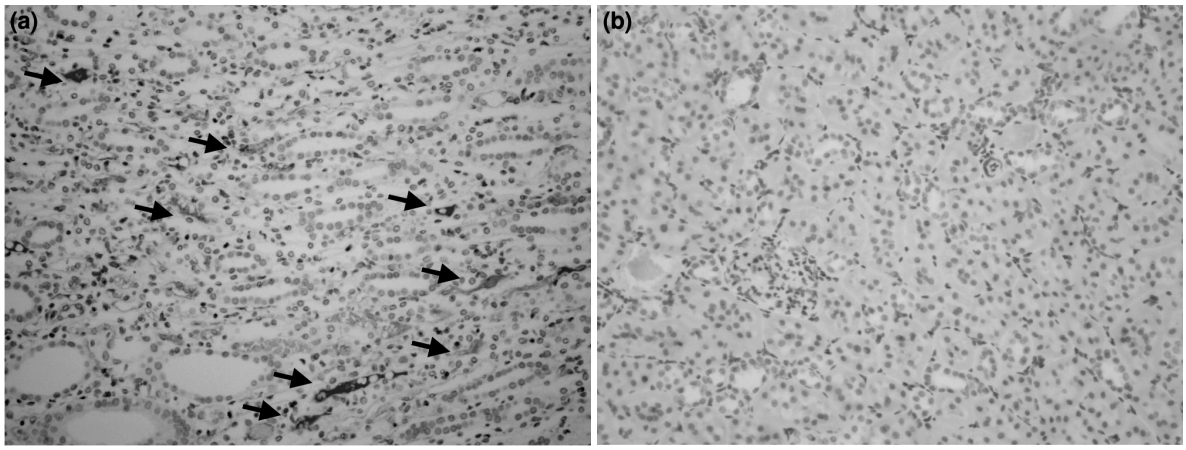

Fig. 3 Amount of albumin in rat kidney tissue: (a) expression of albumin in rat kidney of control group; (b) absence of expression of albumin in rat kidney of EG group.

taining a normal healthy body. Albumin is a major contributor to maintenance of colloid oncotic pressure and acts as a carrier protein. Lower amounts of albumin influence fluid and electrolyte imbalance. In the human body, albumin acts as free-radical scavenger, binds and transports important molecules or drugs, and comprises half of all serum proteins. Abnormal amounts of albumin may indicate poor health. Albumin is synthesized in the liver, however, EG does not adversely affect the liver with the chronic low doses. To rule out an effect on the liver, we measured the levels of albumin in the plasma samples and found that there were no significant changes (Table 2; normal range of albumin in rat: 3.8–4.8 g/dl), indicating that the effect of EG on renal albumin is most likely a kidney effect.

In contrast, there are several potential effects of albumin on the inhibition of CaOx crystal formation in kidneys. Albumin may act as a modulator in the formation of CaOx stones. Rodgers et al found that albumin had stronger inhibitory effect on CaOx formation in black subjects than in white subjects[4]. Structural differences may influence the function of albumin, which may explain the racial differences. However, the exact inhibitory function of albumin remains unknown. Liu et al studied the kinetics of bovine albumin to explain how albumin promotes the formation of CaOx crystals and facilitates ordered CaOx crystal assembly by suppressing supersaturation-driven interfacial structure mismatch[16].

The observational data are difficult to rationalize both the inhibitory and stimulatory effects of albumin. In relation to our results, the decrease in albumin in kidney tissue might be less inhibitory towards crystal formation as suggested. On the other hand, the decrease in albumin could simply be the consequence of more albumin being incorporated into the forming crystals, thus decreasing the remaining amount in the kidney tissues. In this case, albumin would facilitate stone formation rather than inhibit it. Thus albumin might be not really alleviating stone formation, but again is commingling within the stone matrix.

Our study has some advantages and limitations. We used an animal model of CaOx lithogenesis that can mimic stone formation in human kidneys, but cannot account for differences between humans and rats. Lithogenesis via oral intake of EG rarely occurs in humans. However, albumin is widely present in many species and may have the same functions across species. We found homology in rat albumin, which has a relative high conserved structure as compared to that in human (74% identity, BLASTp). Hence albumin might exert similar effects in human diseases.

In conclusion, our results clearly demonstrated that the expression of albumin was reduced in the kidneys of the EG-treated rat. Crystal formation may be due to the loss of inhibitors or decreasing the remaining amount of albumin in the kidney tissues. The mechanism of EG-mediated lithogenesis is partly via modulation of the amount of albumin in the kidney.

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