Erythrocyte Oxidative Stress in Patients With Calcium Oxalate Stones Correlates With Stone Size and Renal Tubular Damage

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OBJECTIVE To investigate how erythrocyte oxidative stress relates to renal tubular damage and calcium oxalate stone size in patients as oxidative stress has been demonstrated to be associated with stone formation in disease progression.

METHODS The study included 29 controls, 29 patients with kidney stones, and 28 patients with ureteral stones. Venous blood samples were collected to measure the expression and activity of antioxidant enzymes in the isolated erythrocytes. A 24-hour urine sample was collected to measure urinary chemistry. The cellular levels of oxalate and the oxidative stress marker malondialdehyde (MDA) were determined to examine their correlations with stone size and renal tubule damage.

RESULTS Calcium oxalate stone deposition and high free radical levels in venous blood associated with high levels of urinary oxalate, glutathione S-transferases tubular damage markers, and MDA and low urinary citrate levels. Compared with the erythrocytes of controls, the erythrocytes of stone groups had significantly lower levels and activities of antioxidant proteins, namely, reduced glutathione, catalase, and copper- or zinc-superoxide dismutase. The ureteral stone group also had significantly lower erythrocyte glutathione peroxidase levels and glutathione reductase activity than the controls. Erythrocyte oxalate levels correlated positively with erythrocyte MDA levels and negatively with erythrocyte antioxidant protein activities. Erythrocyte oxidative stress, as indicated by cellular MDA levels, also correlated well with urinary glutathione S-transferases and stone size.

CONCLUSION These results suggest that oxalate-mediated oxidative stress in erythrocytes might contribute to the tubular damage and stone accumulation in patients with hyperoxaluria. UROLOGY 83: 510.e9–510.e17, 2014. © 2014 Elsevier Inc.

Inadequate scavenging and increased levels of reactive oxygen species (ROS) typically result in redox imbalance and tissue damage in the kidney.[1] Multiple cellular regulatory proteins that are involved in removing ROS have been identified, including superoxide dismutases (SODs), glutathione peroxidase (GPx), and catalase.[2,3] We previously showed that the hyperoxaluria seen in patients with calcium oxalate (CaOx) stones can induce tubular cell injury and that this injury is because of the production of free radicals.[4] In addition, we found that intrarenal oxidative stress in experimental hyperoxaluric rats was enhanced during CaOx crystal deposition.[1] The oxidative stress seen in the kidney of hyperoxaluric rats is because of a decrease in the enzymatic activities of antioxidant proteins such as SOD and GPx.[4,5] Furthermore, direct infusion of oxalate into the kidney causes an intrarenal increase in superoxide formation.[6] These observations indicate that the increased oxidative stress of renal tissues in nephrolithiasis is caused by high oxalate levels and CaOx crystal accumulation.

The SOD, GPx, and catalase enzymes in erythrocytes play a central role in antioxidant defense.[2,7] The reduced form of glutathione (GSH) is also important in antioxidant defense because GPx activity is limited by the propensity of glutathione reductase (GR) to regenerate GSH so that a normal ratio of GSH to oxidized glutathione (GSSG) is maintained.[7,8] This reaction is an important antioxidant mechanism that serves to prevent the oxidative stress that arises from GSH depletion.[7,8] Because of these functions, erythrocytes are considered to be an important detoxifying system in the circulation. It is possible that erythrocytes suffer oxidative stress in patients with CaOx stones. Supporting this possibility is that

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postischemic renal injury and conditions such as diabetes or sickle cell anemia are known to be associated with oxidant-induced damage to erythrocytes.[8] When erythrocytes are overexposed to ROS, their cellular components are disrupted, and this leads to the functional impairment of erythrocytes. This phenomenon is termed erythrocyte oxidative stress (EOS).[7,9] EOS depletes the natural antioxidants of erythrocytes, while activating redox-sensitive transcription factors such as procoagulant tissue factors and proinflammatory mediators. Consequently, this promotes endothelial dysfunction and cardiovascular disease.[7,10-12] However, whether EOS also promotes stone formation in humans remains to be determined.

This issue was addressed by the present study of patients with renal and ureteral CaOx stones. The presence of ROS in the blood was assessed by a chemiluminescence (CL) assay, and EOS was measured by examining the erythrocyte levels of oxalate and the lipid peroxide malondialdehyde (MDA). How these EOS markers related to each other, erythrocyte antioxidant enzyme levels and activities, stone size, and renal tubular damage was then assessed.

MATERIALS AND METHODS

Patients

Patients were eligible for enrollment if they had been diagnosed with renal or ureteral stones by imaging analysis and admitted to the urologic ward at National Taiwan University Hospital for surgical intervention between October 2003 and December 2004. The exclusion criteria were as follows: the presence of urinary tract infection, hyperparathyroidism, other inflammatory or malignant disease, glucose-6-phosphate dehydrogenase deficiency, diabetes mellitus, history of heart disease (hypertension atherosclerosis, myocardial infarction, and so forth), or chronic kidney disease with estimated glomerular filtration rate <30 mL/min/1.73 m^2, as these might potentially have an effect on whole body oxidative stress. None of the patients had undergone bowel resection or had recurrent stone development because of primary hyperoxaluria. All patients were admitted to the ward 1 day before the surgical intervention and informed thoroughly about the procedures. The ethics committee of the institution approved this study (9261701142), and informed written consent was obtained from all participants.

The control group consisted of 29 normal volunteers (54.2 ± 2.4 years). None of the controls had urolithiasis, as indicated by imaging studies, and none had a previous stone history. The patients were divided into 2 groups. The renal stone (RS) group consisted of 29 patients with renal stones (54.6 ± 2.6 years) in 1 kidney but without hydronephrosis or hydrocalycosis. The ureteral stone (US) group consisted of 28 patients (53.8 ± 2.3 years) who had ipsilateral hydronephrosis, as indicated by intravenous urography (IVU) 2-3 weeks after their first outpatient department interviews. One week after the IVU, patients returned to our outpatient department for confirmation of the presence of US. At the same time, patients with a confirmed IVU were included in the US group based on the exclusion criteria previously mentioned. All patients and controls were men. The stones in the patients were composed of both CaOx and calcium phosphate in varying proportions. The patients were diagnosed on the basis of ultrasonography of the abdomen and IVU. The radiograph of the abdomen was used to estimate the stone size (the longest diameter of the stone). There was at least a 7-10 day interval between IVU treatment and subsequent collection of blood and urine. Venous blood samples were collected after overnight fasting, and a 24-hour urine sample was collected from all patients while they followed a normal diet.

Biochemical Analysis and Renal Function

Blood glucose, cholesterol, and triglyceride levels were determined at the central laboratory of National Taiwan University Hospital. Creatinine (Cr), protein, and citrate levels in urine or plasma were measured by commercial kits as previously mentioned.[1] Commercial kits were used to determine the levels of stone-induced kidney damage markers, namely, the urinary α-isoenzyme of glutathione S-transferase (α-GST), which is a marker for proximal tubular damage, and the π-GST, which is a marker for distal tubular damage.[4] Renal function was evaluated by measuring Cr clearance. Urinary supersaturation with respect to CaOx was assessed by calculating the activity product of CaOx index, as proposed previously.[13] All assays were performed in duplicate and expressed per gram of urinary Cr.

Measurement of Free Radical Generation in Blood

The lucigenin-enhanced CL method is a reliable assay of ROS generation, as shown previously.[14] Briefly, a heparinized 0.5-mL sample of venous blood was collected and diluted with an equal volume of saline. The CL was measured in the absolutely dark chamber of the CLD-10 system (Tohoku Electronic Industrial Co, Sendai, Japan). After 100 seconds of stabilization, lucigenin or saline was added and mixed well with the blood. The CL of the blood sample was then counted continuously for a total of 600 seconds. The total CL counts were calculated by integrating the area under the curve, after subtracting the area under the curve of vehicle (saline) treatment.

Isolation of Erythrocytes and Antioxidant Assays

Another 5 mL of blood was collected in an ethylenediaminetetraacetic acid-prepared tube and centrifuged at 620*g* at 4°C for 20 minutes to remove the plasma. The erythrocytes were then washed 3 times with an equal volume of phosphate-buffered saline (pH 7.4) and centrifuged. The packed cells were diluted by adding saline to achieve a 30% hematocrit suspension, after which 0.05% Triton X-100 (Sigma, St Louis) was added in a 3:1 ratio. After 20 minutes of incubation, the mixture was centrifuged at 10,000*g* at 4°C for 10 minutes, and the supernatant was used to determine the antioxidant enzyme activities of GPx, Cu/ZnSOD, MnSOD, and GR, as described previously.[1] The levels of GSH and GSSG were measured by commercial kits as described previously.[1] All assays were performed in duplicate and expressed per gram of hemoglobin (Hb).

Protein Expression of Antioxidant Enzymes

The proteins in the erythrocyte lysates were obtained by using a commercial extraction kit (BioVision, Milpitas, CA) and subjected to electrophoresis, as described previously.[5,15] After blocking, the membranes were incubated with appropriately diluted antisera specific for Cu/ZnSOD and MnSOD (Calbiochem, Darmstadt, Germany), or catalase and GPx (The Binding Site, Birmingham, England, UK). After being washed, the membranes were incubated with horseradish peroxidase-conjugated rabbit antisheep or antigoat immunoglobulin G (IgG) antibodies (Vector, Burlingame, CA). The bound antibody