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In vitro evidence of the promoting effect of testosterone in kidney stone disease: A proteomics approach and functional validation

Channarong Changtong [a,b,c,1], Paleerath Peerapen [a,c,1], Supaporn Khamchun [a,b,c], Kedsarin Fong-ngern [a,c], Somchai Chutipongtanate [a,c], Visith Thongboonkerd [a,c,*]

[a] Medical Proteomics Unit, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
[b] Graduate Program in Immunology, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
[c] Center for Research in Complex Systems Science, Mahidol University, Bangkok 10700, Thailand

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ABSTRACT

Incidence of kidney stone disease in males is 2- to 4-fold greater than in females. This study aimed to determine effects of testosterone on kidney stone disease using a proteomics approach. MDCK renal tubular cells were treated with or without 20 nM testosterone for 7 days. Cellular proteins were extracted, resolved by 2-DE, and stained with Deep Purple fluorescence dye ($n = 5$ gels derived from 5 independent samples/group). Spot matching, quantitative intensity analysis, and statistics revealed significant changes in levels of nine protein spots after testosterone treatment. These proteins were then identified by nanoLC-ESI-Qq-TOF MS/MS. Global protein network analysis using STRING software revealed α-enolase as the central node of protein–protein interactions. The increased level of α-enolase was then confirmed by Western blotting analysis, whereas immunofluorescence study revealed the increased α-enolase on cell surface and intracellularly. Functional analysis confirmed the potential role of the increased α-enolase in enhanced calcium oxalate monohydrate (COM) crystal-cell adhesion induced by testosterone. Finally, neutralization of surface α-enolase using *anti*-α-enolase antibody successfully reduced the enhanced COM crystal–cell adhesion to the basal level. Our data provided in vitro evidence of promoting effect of testosterone on kidney stone disease via enhanced COM crystal–cell adhesion by the increased surface α-enolase.

Biological significance: The incidence of kidney stone disease in male is 2- to 4-fold greater than in female. One of the possible factors of the male preference is the higher testosterone hormone level. However, precise molecular mechanisms that testosterone plays in kidney stone disease remained unclear. Our present study is the first exploratory investigation on such aspect using a proteomics approach. Our data also provide a novel mechanistic aspect of how testosterone can impact the risk of kidney stone formation (i.e. the discovery that testosterone increases alpha-enolase expression on the surface of renal tubular cells that is responsible, at least in part, for crystal–cell adhesion).

1. Introduction

Kidney stone is a solid concretion of mineral crystals forming in the kidney. Nephrolithiasis or kidney stone disease is one of the most common urologic diseases affecting approximately 1–5% of the general population worldwide and the incidence is up to 20% of regional population, particularly in the Middle East [1]. Among several stone types, calcium oxalate monohydrate (COM) is the most common crystalline composition found in clinical calculi [2,3]. Although the stones can be removed by surgical and radiologic procedures, approximately 50% of the cases have recurrent stones within 10 years after the removal [4]. This reflects the inefficacy of kidney stone preventive strategy due to incomplete understanding of the complexity of pathogenic factors of kidney stone development.

Numerous factors, including age, gender, diets, hydration status, metabolic disorders, genetic abnormalities, social class, and economic level, are associated with the risk of kidney stone development [5]. Among these, gender has been proven to be a crucial factor—proportion of kidney stone incidence in males compared to females is about 2–4:1 [5,6]. Testosterone has been proposed as the key factor responsible for the male preference of this disease [7]. Beside epidemiologic kidney stone studies, several animal studies have provided strongly supportive evidence for this hypothesis. Lee YH et al. [8] have shown that testosterone could promote COM kidney stone formation in a rat model of nephrolithiasis. This promoting effect of testosterone has been

* Corresponding author at: Medical Proteomics Unit, Office for Research and Development, Siriraj Hospital, Mahidol University, 6th Floor – SiMR Building, 2 Wanglang Road, Bangkoknoi, Bangkok 10700, Thailand.
E-mail addresses: thongboonkerd@dr.com, vthongbo@yahoo.com (V. Thongboonkerd).
[1] Equal contributions by these authors.

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confirmed by Fan J et al. [9] in another rat model demonstrating that 43–88% of testosterone-treated rats developed COM crystal deposition in their kidneys.

Nevertheless, mechanisms of promoting effect of testosterone on COM kidney stone formation remained unclear. A number of previous studies have shown that testosterone could modulate general and specific renal cellular functions via oxidative stress, caspase-mediated apoptosis, oxalate synthesis, oxalate and uric acid excretion, decreased calcium reabsorption, and reduced production of osteopontin—a major COM crystal growth inhibitor [10–14]. These lines of evidence have suggested that kidney stone promoting the effects of testosterone may be mediated by response or changes in renal tubular cells. However, precise lithogenic response of renal cells upon testosterone exposure remained largely unknown. Understanding these mechanisms may be beneficial for prevention of COM kidney stone formation, particularly in males.

In this study, we hypothesized that testosterone might induce renal tubular cell response and activate crystal–cell interaction via changes in cellular protein expression responsible for functional deteriorations to promote kidney stone formation. Madin–Darby Canine Kidney (MDCK) cell line that was originated from kidney cortex and showed many properties of renal tubular epithelium [15,16] was used as an in vitro model in this study. Cellular proteome of testosterone-treated cells was compared with that of untreated (controlled) cells using two-dimensional gel electrophoresis (2-DE) followed by mass spectrometric protein identification and functional investigations of the identified altered protein that could be linked to the mechanisms of kidney stone formation.

2. Materials and methods

2.1. Testosterone preparation

Testosterone enanthate (Bayer Testoviron Depot 250 mg/ml, Bayer Healthcare Pharmaceuticals, Berlin, Germany) was dissolved in 100% fetal bovine serum (FBS) (GIBCO, Invitrogen Corporation, Grand Island, NY) to make a stock solution of 1 mM. The final concentration of 20 nM, which is within normal physiologic serum level and has been used widely in previous studies on various cell types [17–20], was used for all subsequent experiments.

2.2. Cell culture and testosterone treatment

Approximate 3×10^6 MDCK cells were inoculated in each 75-cm^2 tissue culture flask and maintained in a complete medium (Eagle's minimum essential medium (MEM) (GIBCO) supplemented with 10% FBS, 1.2% penicillin G/streptomycin, and 2 mM glutamine) in a humidified incubator at 37 °C with 5% CO_2 until confluent. Thereafter, the complete medium was refreshed and testosterone was added to make a final concentration of 20 nM. In parallel, the cells maintained in the complete medium (MEM supplemented with 10% FBS, 1.2% penicillin G/streptomycin and 2 mM glutamine) but without addition of testosterone served as the control. For both groups, the culture medium was refreshed every day to maintain a steady concentration of testosterone for 7 days ($n = 5$ independent cultures in each group).

2.3. Cell proliferation and trypan blue assay

Approximately 2.5×10^5 MDCK cells were cultivated with or without 20 nM testosterone for 1–7 days in 6-well plate polystyrene, cell culture cluster (Corning Inc.; Corning, NY), while culture medium was refreshed every day. At the given time points, all the cells harvested by trypsinization from each whole well were mixed with trypan blue stain and counted in a hemacytometer ($n = 3$ biological replications in each group). Total cell number representing cell proliferation and the number of trypan blue stained cells representing cell death are reported.

2.4. Cellular protein extraction

Cells from both controlled and testosterone-treated groups were harvested by scraping. The cells were washed with PBS three times and cell pellets were collected by centrifugation at 500 rpm, 25 °C for 5 min. Proteins were extracted by a lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS), 120 mM dithiothreitol (DTT), 40 mM Tris-HCl, and 2% ampholyte (pH 3–10) at 4 °C for 30 min with vortex every 5 min. Whole cell lysates were then centrifuged at 14,000 rpm, 4 °C for 5 min. The supernatants were collected and protein concentrations were measured by Bradford's method using Bio-Rad Protein Assay (Bio-Rad Laboratories; Hercules, CA).

2.5. 2-DE and staining

Proteins derived from each culture flask were resolved in each 2-D gel (100 μg total protein/each gel; $n = 5$ gels/group; a total of 10 gels were analyzed). Each protein sample was premixed with a rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 120 mM DTT, 40 mM Tris-base, 2% ampholytes (pH 3–10), and a trace of bromophenol blue to make a final volume of 150 μl. The mixture was rehydrated onto

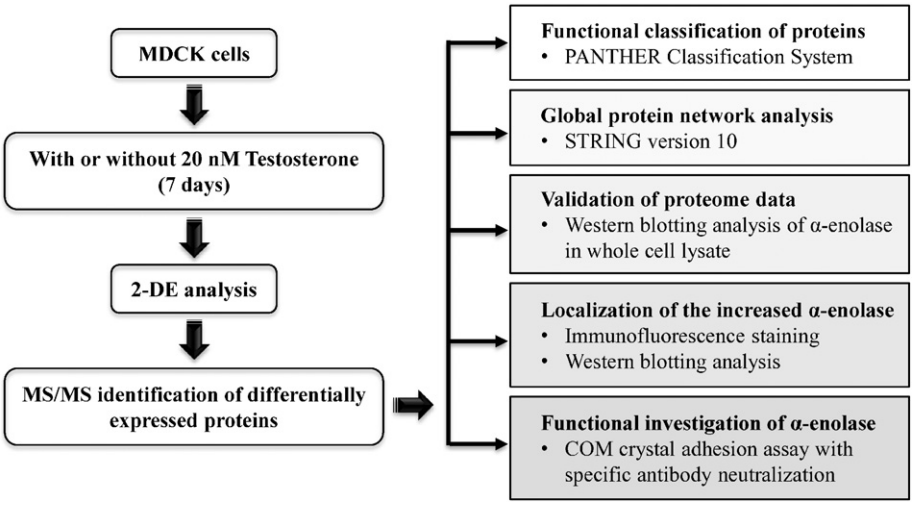

Fig. 1. Schematic diagram summarizing the workflow and experimental procedures in this study.