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Pre-stimulation of the kallikrein system in cisplatin-induced acute renal injury: An approach to renoprotection

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ABSTRACT

Antineoplastic treatment with cisplatin is frequently complicated by nephrotoxicity. Although oxidative stress may be involved, the pathogenic mechanisms responsible for renal damage have not been completely clarified. In order to investigate the role of the renal kinin system in this condition, a group of rats was submitted to high potassium diet to stimulate the synthesis and excretion of tissue kallikrein 1 (rKLK1) previous to an intraperitoneal injection of 7 mg/kg cisplatin. A significant reduction in lipoperoxidation, evidenced by urinary excretion of malondialdehyde and renal immunostaining of hidroxy-nonenal, was accompanied by a decline in apoptosis. Coincident with these findings we observed a reduction in the expression of renal KIM-1 suggesting that renoprotection may be occurring. Stimulation or indemnity of the renal kinin system deserves to be evaluated as a complementary pharmacological measure to diminish cisplatin nephrotoxicity.

Introduction

Cisplatin is an important antineoplastic agent used in the treatment of solid tumors. Its use is frequently limited by its nephrotoxicity, observed in approximately 1/3 of patients after a single dose, making necessary a reduction of dosage or interruption of therapy. Clinical and histopathological findings of renal injury induced by cisplatin are those observed during acute renal failure secondary to tubular necrosis (Pabla and Dong, 2008), but the cellular and molecular mechanisms involved have been disclosed only recently. Cisplatin gets into the cell by passive or facilitated transporter; there, it induces DNA changes that activate apoptotic signaling and forms adducts with reduced glutathione weakening its intracellular concentration with the corresponding increase in reactive oxygen species (ROS). ROS amplify DNA damage by inducing translocation of Bax from the cytosol to the mitochondria, depleting the ATP synthesis and liberating cytochrome C to activate the intrinsic apoptotic pathway. Cisplatin also favors the expression of pro-inflammatory cytokines such as TNF-α, TGF-β, RANTES, MIP-2 and IL-1β, contributing to produce an enhanced inflammatory response.

A protective role of the renal kallikrein–kinin system (KKS) has been described in several models of renal damage (Chao et al., 1998; Hirawa et al., 1999; Schanstra et al., 2002; Tu et al., 2008) including reduction of ROS (Zhang et al., 2004) as it has been shown in a model of acute renal damage induced by gentamicin (Bledsoe et al., 2006). High potassium intake has a stimulatory effect on the synthesis/secretion of kallikrein and expression of the kinin B2 receptor (Obika, 1987; Vio and Figueroa, 1987; Ardiles et al., 2006; Ardiles et al., 2013), being the enzymatic activity of urinary kallikrein and its renal immunohistochemical good expressions of the activity of renal kallikrein–kinin system. The following experiments append evidence supporting this role of the KKS, up-regulated by rich potassium diet, by reducing oxidative stress (OS) and apoptosis in a rat model of cisplatin nephrotoxicity.

Methods

Animals and experimental model. Male adult Sprague–Dawley rats (200–250 g) obtained from the University Breeding Centre (Department of Anatomy, Histology and Pathology, Universidad Austral de Chile) were maintained at constant room temperature with a 12 h light/dark cycle, unrestricted access to normal food and hydration *ad libitum*. The Universidad Austral Animals Ethics Committee approved all experimental procedures.

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Groups of study

- CT (Control group, $n = 20$): They represented the experimental model of cisplatin nephrotoxicity receiving a single intraperitoneal dose of 7 mg/kg of cisplatin (Pfizer) and sacrificed in groups of five immediately before cisplatin and 1, 3, and 7 days after the injection.
- KCl (Potassium group, $n = 20$): They received 2% potassium chloride (KCl) in drinking water to stimulate renal kallikrein (Ardiles et al., 2006; Vio and Figueroa, 1987) for 4 weeks prior to cisplatin and interrupted after injection to avoid hyperkalemia during induced acute renal failure. The cisplatin dose and days of sacrifice were as the control group.

Urine collection and tissue preparation. Twenty four hour urine collections were performed in individual metabolic cages, while food was withheld to avoid fecal contamination, and water was offered *ad libitum*. Animals were euthanized by aortic exsanguination under anesthesia, serum was obtained and kidneys were removed, decapsulated and washed with saline to obtain horizontal slices that were fixed in 4% formalin-PBS and embedded in paraffin wax.

Serum and urine chemistry. Creatinine was measured by a modified Jaffé reaction and electrolytes with an ion-selective autoanalyzer. Proteinuria was quantified with a colorimetric method (Pyrogallol red) and values were expressed as mg/mg of urinary creatinine to avoid differences in urinary volumes.

Urinary kallikrein assay. Urinary kallikrein activity was determined by the amidase method (Amundsen et al., 1979) using the synthetic substrate D-Val-Leu-Arg-*p*-nitroanilide (Sigma). Details may be found published (Ardiles et al., 2006). Values were expressed as mU/mg of urinary creatinine.

Urinary malondialdehyde assay. Urinary malondialdehyde (MDA) was quantified by the thiobarbituric acid reaction (Ohkawa et al., 1979) and values were expressed as nmol of MDA/mg of urinary creatinine.

Histological evaluation. Four micrometer thick sections stained with Schiff's periodic acid were examined under optical microscopy to evaluate glomerular sclerosis, casts, evidences of acute tubular damage, tubular atrophy and inflammatory infiltrate in a semiquantitative scale (0: absent, 1: mild (<25% of the sample), 2: moderate (25%–50% of the sample) and 3: severe (>50% of the sample).

Immunohistochemistry. Dewaxed tissue sections (4 μm thick) were treated with 3% hydrogen peroxide to inhibit endogenous pseudoperoxidase activity. Sections were treated with Protein Block Serum Free (DakoCytomation) prior to incubation with each of the following primary antibodies: rabbit polyclonal anti-urinary kallikrein (Calbiochem), rabbit anti-MDA (Alpha Diagnostic), mouse anti-4-hidroxy-2-nonenal (Oxis Research), rabbit anti-nitrotyrosine (Upstate) 1/100, rabbit anti-Cu/Zn superoxide dismutase (Stressgen), or M30 Cytodeath (Roche) that detects caspase-cleaved cytokeratin 18. Antibodies were diluted with 1% BSA 1% and incubation was performed overnight at 4 °C. Bound antibodies were detected with the aid of the LSAB + System (Dakocytomation) and peroxidase activity was developed using dyaminobenzidine (Vector Laboratories). Finally, sections were counterstained with hematoxylin. Negative controls included omission of primary antibody or its replacement by non-immune immunoglobulins at the same dilution. The KS 300 Imaging System 3.0 (Zeiss, München-Hallbergmoos, Germany) was used to quantify immunocytochemical staining as previously described (Ardiles et al., 2006; Ardiles et al., 2013).

Western blotting. Four animals were randomly selected from both groups at days 3 and 7. Kidneys from adult healthy rats, neither submitted to potassium nor cisplatin, were used as source of normal renal tissue. Kidneys were homogenized with 10 ml of lysis buffer per gram of tissue, containing a cocktail of protease inhibitors. Protein content was quantified by the Bradford method and 50 μg of each sample was separated on 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. Proteins were transferred onto PVDF membranes that were incubated with 1:4000 dilution of a monoclonal antibody directed to caspase 9 (Cell Signaling). After washing and incubation with a peroxidase-labeled anti-mouse IgG, the membrane was exposed to luminol-hydrogen peroxide in the darkness for 5 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Millipore) was used to control protein load in each well. The software ImageJ was used to convert films images to a gray scale of 8-bits. Intensity values for immunoreactive bands were calculated as integrated density using the proportion of active/inactive caspase 9, divided by GAPDH value and expressed as relative density.

KIM-1 RT-PCR. Four animals were randomly selected from both groups at days 3 and 7. Kidneys of adult rats (non-submitted to neither potassium nor cisplatin) were used as normal controls. Kidney tissue was immersed in trizol and homogenized on ice; 200 μl of chloroform was added to 1 ml of extraction solution, mixed and centrifuged at 12,000 × *g* for 15 min. The superior phase was mixed with isopropanol (1:1) and centrifuged at 12,000 × *g* for 10 min. The pellet was washed with 75% ethanol air dried and suspended in 40 μl of nuclease-free water. DNAase I treatment was performed to avoid traces of genomic DNA and divalent cations. Extracted RNA was mixed with oligodT and random primers, incubated for 5 min at 70 °C and then on ice. After 30 s centrifugation, reverse transcriptase buffer 5×, 25 mM $MgCl_2$, dNTPs, RNAsin, reverse transcriptase enzyme and nuclease-free water were added and incubated for 1 h at 37 °C. Enzyme inactivation was carried out by incubation at 75 °C × 15 min. For PCR, cDNA was mixed with buffer Green Gotaq 5×, mix of 5 mM dNTPs, Taq DNA polymerase, cDNA and nuclease-free water (final volume 20 μl) and 10 mM of KIM-1 primer forward: 5′ GGG GTG GGT CAC CCT GT 3′, and primer reverse: 5′ GTG GGC CTT GTG GTT GTG GGT 3′). Primers were designed to amplify a 371 bp segment corresponding to positions 680–1051 (AF035963.1) obtained at NCBI (National Center for Biotechnology Information), Initial denaturation step was at 94 °C for 5 min, followed by 26 cycles of amplification (94 °C × 30 s, 57 °C × 35 s and 72 °C for 45 s) and a final extension at 72 °C for 5 min. GAPDH was used as housekeeping gene. PCR products were visualized by agarose gel electrophoresis using ethidium bromide. Finally, the gel image was converted to gray-scale of 8-bits using the ImageJ program, expressing values as integrated density normalized by GAPDH values.

Statistical analysis. The non-parametric Mann–Whitney test for unpaired samples or Wilcoxon's test for paired comparisons were used to examine differences between groups and non-parametric Spearman correlation to evaluate association between variables using GraphPad InStat 3.01 (Windows 95/NT, San Diego CA, USA). Values are expressed as mean ± SE and p values <0.05 were considered significant.

Results

Urinary electrolytes before cisplatin injection evidenced the ingestion of a high potassium diet by the KCl group with changes after cisplatin injection. Serum potassium was also higher in the same animals during evolution of the acute renal damage. Reduction in renal function after cisplatin was evidenced as an increase in creatinine without significant differences between both groups (Table 1).

Histological evaluation of potassium pretreated rats, before cisplatin injection, did not show differences when compared with control animals. Presence of distal tubular casts (mainly in the cortex),