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Metabolomic characterization of renal ischemia and reperfusion in a swine model☆

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ABSTRACT

Acute kidney injury (AKI) is a serious complication in hospitalized and transplanted patients, and is mainly caused by ischemia/reperfusion (I/R). However, the current diagnosis of AKI based on acute alterations in serum creatinine or urine output is late and unspecific. To identify new systemic biomarkers for AKI, we performed serum and urine metabolomic profile analyses during percutaneous unilateral renal I/R in a well-controlled swine model. For this, serial serum and urine samples obtained during the pre-ischemia, ischemia and reperfusion periods were analyzed by ^{1}H nuclear magnetic resonance at 600 MHz. Through the metabolic profiles over I/R, we identified eight serum metabolites that increased with ischemia and recovered to basal values after reperfusion, delineating the ischemic period. In addition, we identified 13 urinary metabolites that changed during the early reperfusion reflecting the ischemic kidney, being able to differentiate between pre-ischemia and post I/R periods. All selected metabolites are described in terms of disease pathophysiology (change of energetic pathway and oxidative stress), which suggest that these serum and urinary metabolites are candidate AKI biomarkers. Interestingly, the selected metabolites allowed us to identify, well described NFκB, leptin, INF-γ and insulin pathways, and a new pathway (Huntingtin) that had not been previously implicated in renal I/R. Huntingtin showed different fragment patterns in ischemic versus non-ischemic kidneys. Therefore, the metabolomic profile found in renal I/R led to the identification of candidate disease biomarkers and a new pathway associated with renal injury.

1. Introduction

Acute kidney injury (AKI) is a serious complication observed in 11.9% of kidney transplants [41], approximately 30% of post cardiac surgery [3] patients and 39% of patients in intensive care [48], which significantly increases hospital mortality and prolongation of hospital stay [5]. AKI can be caused by contrast medium, medications, surgery and, mainly by ischemia generated by a decrease in renal perfusion (hypotension and hypovolemia) [47]. Despite technical progress in the management of AKI over the last 50 years, mortality rates have remained unchanged at around 50% [69].

AKI is defined as the abrupt decrease in kidney function determined by acute alterations in serum creatinine or urine output [42]. However, changes in serum creatinine are only detected when there is, at least, a 50% reduction in glomerular filtration rate (GFR) [54]. In addition, serum creatinine levels depend on muscular mass, age, gender, metabolism and hydration. Serum creatinine may also be overestimated by tubular secretion when the GFR is low [45]. New studies have been performed to discovery others biomarkers, but the results are generally inconclusive, because they are influenced by different causes of AKI such

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as sepsis, radiographic contrast, nephrotoxins [56] and other comorbidities associated with the disease [6]. In addition, ischemia/reperfusion (I/R) studies using animals have only been conducted in rodents [35,63], which have a higher metabolic rate than humans [29].

Aiming at the identification of the overall metabolic status of a particular tissue, at a particular moment, metabolomics may be well suited for the identification of new biomarkers of this condition [2,67]. This is done through the quantification of metabolite levels, reflecting the activation, or not, of several concurrent metabolic pathways [2,19].

Therefore, we aimed to identify changes in the serum and urinary metabolite profile during renal I/R. Taking advantage of the fact that pigs have similar metabolic rates and high homology with humans, we assessed metabolic changes caused only by AKI in a swine model of percutaneous, unilateral, renal I/R, [29,36]. Our findings provide new candidates to biomarkers and new insights about new proteins, metabolites and their interactions in renal I/R.

2. Materials and methods

2.1. Animals

Pigs (15–20 kg) were fasted overnight prior to surgery and were given water *ad libitum*. All experiments were carried out in accordance with the ethical principles in animal research of the Brazilian College of Animal Experimentation and were approved by the Institutional Ethics Committee (CAPPesq Protocol 179/11). Five juvenile female pigs were used as a model of unilateral kidney ischemia/reperfusion [39]. Briefly, selective endovascular catheterization of the right renal artery was performed using a 6F multipurpose guide-catheter and baseline angiography was acquired under fluoroscopic guidance. Total occlusion of the right renal artery was obtained by placing an angioplasty balloon-catheter into the right renal artery and inflating it for 120 min (ischemia), followed by deflation (reperfusion) for 24 h. The ischemic and contralateral kidneys were collected after this period. Serial serum was sampled from the inferior vena cava above the renal veins and urine was sampled directly from the bladder throughout the experiment. In a previous study we have characterized the used swine model of renal I/R. Briefly, the animals showed changes characteristic of AKI. There were increased serum creatinine, serum NGAL, fractional excretion of sodium, potassium and chloride and increased glucose and protein in urine. The most important result for the diagnosis to AKI was based on histological analysis, which clearly showed acute tubular necrosis. In this swine model, an increase of nitrated protein in serum and urine was also observed. Potentials limitations of our model was the lack of collect of serum and urine of each kidney independently and the lack of a sham group. The analysis of metabolites of two kidneys together was chose to discovery the early biomarkers to AKI. The initial time used as a control (pre ischemia) was performed just prior to the occlusion of the renal artery, after the beginning of surgery, but we have not controlled for systemic changes following only the surgical procedure. The use of a sham control group could provide time-series information on the surgical wound consequences [39].

This model was developed with the main objective to discovery new biomarkers for renal I/R. Our model is one of unilateral kidney ischemia and assumes that for a new biomarker to be sensitive to subtle change; it must be identified through sampling from the whole blood or urine, as opposed to selectively from one of the renal veins, or collector systems. With this, we aim at identifying molecules with abrupt concentration changes that could be found systemically (blood and urine) and generate more sensitive biomarkers.

For metabolomic analysis we used serum and urine from 5 different periods: pre-ischemia (immediately before occlusion), ischemia (60 min), 0.5 h post-reperfusion, 4 or 6 h post-reperfusion and 11 h post-reperfusion. These collected times were chosen based on representative points of the increase in serum creatinine.

2.2. ^{1}H nuclear magnetic resonance (NMR) spectroscopic analysis

A nuclear magnetic resonance (NMR) spectroscopy platform was used for metabolite measurement.

2.2.1. Sample preparation

Serum and urine were filtrated using 3 kDa cutoff filters (Amicon Ultra, Millipore, Carrigtwohill, Co. Cork, Ireland) to remove high molecular weight such as proteins and lipids. Before use, the filters were washed three times with deionized water (1 h per cycle, 13680xG at 4 °C), in order to remove substances such as glycerol.

For the serum sample, 350 μL were added to filters and centrifuged for 2 h, 13680xG at 4 °C. The serum filtrate (200 μL) was diluted in 280 μL of deionized water, mixed with 60 μL of phosphate buffer (1 M pH 7.4), 5 mM of TSP (3-(Trimethylsilyl) propionic acid-d_4 sodium salt – Ref: 269,913, Sigma-Aldrich, Saint Louis, MO, USA) and 60 μL of deuterium oxide (D_2O 99% Sigma; Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA). For the urine sample, 4 mL were centrifuged for 1 h and 3005xG at 4 °C. The filtrate (480 μL) was mixed with 60 μL of same buffer with TSP than serum and 60 μL of D_2O. Samples (600 μL) were added to 5 mm NMR tubes.

2.2.2. NMR data acquisition and metabolite identification

One-dimensional (1D) ^{1}H NMR spectra were acquired at 599.844 MHz with an Inova AS 600 spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA) with a cryogenic probe, using a standard presaturation pulse sequence for water suppression with solvent irradiation on the relaxation delay (1.5 s) and mixing time of 100 ms. NMR spectra were acquired using 256 scans with 64 k points and a spectral width of 16 ppm, an acquisition time of 4 s, and a total pulse recycle delay of 5.42 s. The FIDs were multiplied by an exponential function corresponding to a 0.3 Hz line broadening prior to Fourier transformation. All spectra phase and baseline were corrected manually and metabolites were identified and quantified using Chenomx NMR Suite 7.6 (Chenomx Inc., Edmoton, Alberta, Canada), a commercial spectral fitting software containing an NMR spectral reference library of 304 compounds.

2.3. SDS-PAGE and immunoblotting

Nuclear protein extraction from the ischemic and contralateral kidneys were performed in accordance with Kyroycheva and collaborators [28] and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Seventy-five milligrams of renal cortex (ischemic or not) were washed twice with 500 μL of phosphate buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) to remove the blood excess. Then, cortices were homogenized with Dounce homogenizer on ice for 10 min in 700 μL of cytoplasmic buffer (10 mM Tris-HCl (pH 7.6), 1.5 mM MgCl2, 10 mM KCl, 2 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride and 1:300 (v/v) phosphatase inhibitor cocktails (P8340, P5726, P0044, Sigma-Aldrich, St. Louis, MO) and centrifuged for 10 min at 10621xG at 4 °C. The supernatant was discarded and the pellet was resuspended in 300 μL of nuclear buffer (0.42 M KCl, 20 mM Tris-HCl (pH 7.6), 20% glycerol, 1.5 mM MgCl2, 2 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, and 1:1000 (v/v) phosphatase inhibitor cocktails). After 10 min on ice, the suspension was centrifuged for 30 min at 10621xG at 4 °C and the supernatant with nuclear proteins was collected. Fifty micrograms of nuclear protein determined by the Bradford method (BioRad #500-0001) were used in 10% of SDS-PAGE. Proteins were then transferred to a PVDF microporous membrane and used for immunoblotting. The PVDF membrane was incubated with rabbit monoclonal *anti*-Huntingtin (Abcam, Cambridge, MA, USA, #ab109115). Antibody was visualized using an enhanced chemiluminescence system. Signal magnitude was calculated using the Image J software.