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Identification of agents that reduce renal hypoxia–reoxygenation injury using cell-based screening: Purine nucleosides are alternative energy sources in LLC-PK1 cells during hypoxia

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ABSTRACT

Acute tubular necrosis is a clinical problem that lacks specific therapy and is characterized by high mortality rate. The ischemic renal injury affects the proximal tubule cells causing dysfunction and cell death after severe hypoperfusion. We utilized a cell-based screening approach in a hypoxia–reoxygenation model of tubular injury to search for cytoprotective action using a library of pharmacologically active compounds.

Oxygen–glucose deprivation (OGD) induced ATP depletion, suppressed aerobic and anaerobic metabolism, increased the permeability of the monolayer, caused poly(ADP-ribose) polymerase cleavage and caspase-dependent cell death. The only compound that proved cytoprotective either applied prior to the hypoxia induction or during the reoxygenation was adenosine. The protective effect of adenosine required the coordinated actions of adenosine deaminase and adenosine kinase, but did not requisite the purine receptors. Adenosine and inosine better preserved the cellular ATP content during ischemia than equimolar amount of glucose, and accelerated the restoration of the cellular ATP pool following the OGD. Our results suggest that radical changes occur in the cellular metabolism to respond to the energy demand during and following hypoxia, which include the use of nucleosides as an essential energy source. Thus purine nucleoside supplementation holds promise in the treatment of acute renal failure.

Introduction

Acute tubular necrosis (ATN),[1] defined as acute renal failure (ARF) due to ischemic or toxic renal injury, is a clinical syndrome that commonly occurs in critically ill patients in the intensive care units and is often associated with various co-morbidities in the elderly.

During the last decades a considerable amount of research has been devoted to elucidating the pathophysiology of ATN with the ultimate goal to facilitate the development of therapeutic interventions that either prevent ARF, ameliorate the severity of tubular injury following an acute renal insult, or accelerate the recovery of established ATN. Despite the major advances in our understanding of pathogenic events in ischemia–reperfusion injuries and that of ATN, neither the clinical outcomes nor the therapeutic approaches changed considerably over the past decades. This is also illustrated by the fact that, while the therapeutic arsenal noticeably expanded in the treatment of ischemia–reperfusion based illnesses of other organs, saline-induced intravascular volume expansion remained the basis of ARF therapy supplemented with renal replacement therapy. A recent metaanalysis showed that even some of the traditionally employed treatment modalities like renal vasodilators and diuretics lacked any beneficial effect [1,2].

The "imperfect" experimental models that do not accurately simulate the clinical disease were generally accounted for the failure to translate basic, laboratory-derived, mechanistic insights into clinical practice and led to the development of more complex

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[1] *Abbreviations used:* Acetyl-CoA, acetyl coenzyme A; ADA, adenosine deaminase; ADP, adenosine diphosphate; AK, adenosine kinase; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CTL, control; ECAR, extracellular acidification rate; ER, endoplasmic reticulum; GTP, guanosine triphosphate; NAD^+, nicotinamide adenine dinucleotide; NO, nitric oxide; OCR, oxygen consumption rate; OGD, oxygen–glucose deprivation; PARP, poly(ADP-ribose) polymerase; PRE, pre-treatment; POST, post-treatment; SERCA, sarco-endoplasmic reticulum calcium ATPase.

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models with little success [3,4]. The strategy to transfer successful therapeutic approaches of similar, ischemia–reperfusion diseases of other organs equally failed in ARF, including those targeting the renal vasculature suggesting an inherent difference in the mechanism of ATN. From recent advances only the antioxidant *N*-acetylcystein holds promise for reducing the oxidative stress in the kidney, especially in radiocontrast injury [1,5].

The critical area of ischemic renal injury is the proximal tubule. According to widely accepted beliefs the high energy requiring transport function of this segment renders the epithelial cells within this part vulnerable to ischemic kidney injury, since the transport needs can rapidly deplete the cellular ATP content. Also, the renal microcirculation (namely the peritubular capillaries coming from the efferent arterioles) supplying these segments is implicated in the high susceptibility to injury, as the provider of insufficient blood flow during the reperfusion due to microcirculatory changes [6]. However, on a simply theoretical basis, this view is dubious since the transport demand rapidly decreases with the reduction of blood flow and filtration rate. Also, the high energy consumption is reflected in dense capillary structures along the tubules that may provide superior blood supply compared to what most cells receive in the body. Furthermore, the tubular epithelium benefits from glucose and other energy source supplies from the tubular side, and possesses high-affinity transport systems to take up these compounds. The lack of sufficient energy supply during the reperfusion is further opposed by the fact of glucosuria in ARF [7] confirming the presence of adequate glucose supply via the filtrate. These notions strongly support the idea that the proximal tubule epithelial cells, in fact, may enjoy better conditions than most other cells of the kidney during and after an ischemic period, suggesting that the acute tubular necrosis is rather based on unique and intrinsic functional characteristics of these cells than their cellular environment.

This scenario prompted us to reconsider some of the widely accepted beliefs in ARF and focus our studies on the tubular epithelial cells. We chose a cellular model of tubular injury that employs true hypoxia and performed a systematic search for compounds that protect against the injury. The use of cell-based screening approach with the evaluation of the overall protection against fatal injury permitted a variety of actions exerted by the wide array of molecules by interacting with multiple targets simultaneously without the need to act on any of the currently known targets in renal injury [8,9]. Also, this methodology allowed a direct comparison of the efficacy of the tested compounds on the eventual outcome, thus provided us with an estimate of the contribution of various pathways in the development of tubular injury.

Materials and methods

Reagents

A library of 1280 pharmacologically active compounds (LOPAC) was obtained from Sigma–Aldrich (St. Louis, MO). The library includes drug-like molecules in the field of cell signaling and neuroscience. The compounds are dissolved at 10 mM in dimethylsulfoxide (DMSO) and dilutions were made either in DMSO or in phosphate-buffered saline (PBS, pH 7.4) to obtain 0.5% DMSO in the assay volume. Adenosine (ADE), inosine (INO), glucose, PJ34, 8-cyclopentyl-1,3-dipropylxanthine, 8-(3-chlorostyryl)caffeine, alloxazine, MRS 1523, erytro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), [4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido(2,3-d)pyrimidine (ABT 702), oligomycin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), antimycin A were purchased from Sigma–Aldrich (St. Louis, MO), necrostatin-1 (NEC) from Calbiochem (EMD BioSciences, San Diego, CA), carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone (Z-VAD-fmk) from Promega (Madison, WI). All compounds were dissolved in DMSO except for adenosine, inosine and glucose which were dissolved in culture medium.

Cell culture

LLC-PK1 porcine kidney proximal tubular cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT) containing 1 g/l glucose supplemented with 10% fetal bovine serum (PAA Laboratories, Dartmouth, MA), 4 mM glutamine and 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Cells (3000/well) were plated into 96-well tissue culture plates and 100,000 cells were plated into 60-mm culture dishes and cultured for 4 days at 37 °C in 5% CO_2 atmosphere.

In vitro model of acute tubular necrosis

Culture medium was replaced with DMEM containing no glucose prior to the induction of hypoxia. In the pre-treatment assay the drugs were added at 50 μM concentration in 5% of the culture volume (final concentration of DMSO was 0.5%). Culture plates were placed in gas-tight incubation chambers (Billups-Rothenberg Inc., Del Mar, CA) and the chamber atmosphere was replaced by flushing the chamber with 95% N_2/5% CO_2 mixture at 25 L/min flow rate for 5 min. The hypoxia was maintained by clamping and incubating the chambers for 20 h (or for the indicated period) at 37 °C. All assay plates subjected to hypoxia included vehicle-treated control wells with glucose-free medium (OGD) or medium containing 5 mM glucose (CTL). After hypoxia, glucose and serum concentration was restored by supplementing the culture medium with glucose and FBS and the cells were incubated for 24 h at 37 °C at 5% CO_2 atmosphere. In the post-treatment assay the drugs were added immediately after the hypoxia at 50 μM final concentration in 5% of the culture volume.

Viability assays

MTT viability assay

The MTT assay was performed as previously described with slight modification [10]. Briefly, cells were dissociated with 0.05 mM EDTA (at a final concentration of 2.5 μM) at 37 °C for 15 min at 5% CO_2 atmosphere, then 1/10 volume FBS containing 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Calbiochem, EMD BioSciences, San Diego, CA) was added in 1/10 volume to reach final concentration of 0.5 mg/ml, and the cells were incubated for 3 h at 37 °C at 5% CO_2 atmosphere. The converted formazan dye was detected at 570 nm with background measurement at 690 nm and viable cell count was calculated using a calibration curve created with serial dilutions of LLC-PK1 cells.

Alamar blue cell viability assay

Following the 24-h-long recovery period, the cells were pretreated with EDTA at a final concentration of 2.5 μM for 15 min at 37 °C to allow complete dye uptake. Then FBS was added to the cells to neutralize EDTA and Alamar blue (resazurin, 7-hydroxy-3*H*-phenoxazin-3-one-10-oxide) at a final concentration of 10 mg/ml. The cells were incubated for 3 h at 37 °C at 5% CO_2 atmosphere and fluorescence was measured on Synergy2 reader (Ex/Em: 530/590 nm) (Biotek, Winooski, VT, USA). The viability was calculated using dilution series of LLC-PK1 cells for calibration.