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Identification and dissection of the Nrf2 mediated oxidative stress pathway in human renal proximal tubule toxicity

Anja Wilmes [a,1], Daniel Crean [b,1], Sonia Aydin [a], Walter Pfaller [a], Paul Jennings [a,*], Martin O. Leonard [b]

[a] Division of Physiology, Department of Physiology and Medical Physics, Innsbruck Medical University, Innsbruck, Austria
[b] School of Medicine and Medical Science, UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland

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ABSTRACT

The identification and dissection of cellular stress mechanisms is fundamental to understanding the susceptibility of the kidney to chemicals and pharmaceuticals and for the development of renal biomarkers indicative of sub lethal injury. Here, we utilised whole genome DNA microarrays in an attempt to uncover molecular mechanisms of response to nephrotoxin exposure.

Human renal proximal tubular cells (HK-2) were treated for 12 h and 48 h with 5 μM Cadmium (Cd), 30 μM Diquat Dibromide (Diq), and 5 μM Cyclosporine A (CsA). Nephrotoxin treatment resulted in an alteration of a total of 4608 transcripts. Ingenuity Pathways Analysis™ revealed the anti-oxidant and detoxification Nrf2 pathway as the most significantly enriched signaling pathway in the selected dataset. Activation of this transcription factor was confirmed as nuclear translocation and paralleled the temporal alterations of compound induced H_2O_2 production. Transcriptomics, western blot and immunofluorescence showed an induction of both HO-1 and NQO1 with Cd and Diq exposure, but not with CsA treatment. Knockdown of Nrf2 by siRNA, reduced compound induced NQO1 mRNA to basal levels and attenuated toxin induced HO-1 mRNA expression. siRNA knock down of HO-1, but not NQO1, enhanced Cd induced H_2O_2 production and Cd induced toxicity.

Using an un-biased transcriptomic approach we have identified the Nrf2 pathway as the most significant signaling response in renal epithelial cells challenged with nephrotoxin. This study highlights the importance of this pathway and particularly HO-1 in renal epithelial adaptation to oxidative stress.

1. Introduction

The kidney represents a major target for toxic xenobiotics due to its role in the control of body fluid and electrolyte homeostasis. The high blood perfusion rate (20% of cardiac output) and the capability to metabolise, concentrate, extract and secrete toxic compounds, make the kidneys extremely vulnerable to a wide variety of toxins. Xenobiotic induced nephrotoxicity is a major cause of both acute and chronic renal failure and thus represents a significant challenge to the health care and pharmaceutical industries.

Due to the functional and biochemical heterogeneity of the nephron, the susceptibility to a particular nephrotoxic insult will vary among nephron segments. The epithelial cells of the proximal nephron are target sites for a wide variety of nephrotoxic chemicals due to a large number of transport systems (Fanos and Cataldi, 2001) and the presence of xenobiotic metabolising enzymes such as cytochrome p-450 monooxygenases, NADPH-cytochrome c reductase, glucuronyl transferase, sulfotransferases, glutathione S-transferases, cysteine conjugate β-lyase, and prostaglandin H synthase (Lohr et al., 1998). Another variable for susceptibility is the intracellular concentration of glutathione (GSH) and GSH dependent enzyme activity, which is highest in proximal tubule and decreases progressively down the nephron (Guder and Ross, 1984; Mohandas et al., 1984). Additionally, the proximal tubule has a high requirement for oxygen which makes this area of the nephron especially sensitive to oxygen deprivation (Leonard et al., 2006). For these reasons the proximal tubule is one of the main renal cell types studied in the context of nephrotoxicity.

In vitro cell culture technology, although not perfect, allows the study of specific pathways at a cellular level without the influence of higher order systems. A major advantage of cell culture technology is that it is ideally suited to whole genome transcriptomic analysis (Jennings et al., 2009) and RNA silencing. Here, we employed these techniques in an attempt to identify and dissect pathways associated with proximal tubular injury. Human proximal tubular epithelial cells (HK-2 cell line) were treated with three unrelated chronic nephrotoxins; the heavy metal and environmental pollutant cadmium, the contact herbicide diquat dibromide and the

* Corresponding author. Address: Division of Physiology, Department of Physiology and Medical Physics, Innsbruck Medical University, Fritz-Pregl Strasse 3, 6020 Innsbruck, Austria. Tel.: +43 512 9003 70826, fax: +43 512 9003 73800.
E-mail address: paul.jennings@i-med.ac.at (P. Jennings).
[1] These authors contributed equally to this work.

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immunosuppressive calcineurin inhibitor cyclosporine A. Using bioinformatic pathway analysis we demonstrated the NF-E2 related factor 2 (Nrf2) pathway as the most significantly altered pathway with nephrotoxin exposure. Furthermore we demonstrated a functional role for Nrf2 activation in the regulation of HO-1 and NQO-1 gene expression and also revealed a protective role for HO-1 in nephrotoxin induced ROS generation and proximal tubular injury.

2. Methods

2.1. Cell culture

The human proximal tubular epithelial HK-2 cell line (Ryan et al., 1994) was obtained from the American Type Culture Collection (ATCC No. CRL-2190, Manassas, VA) and maintained in a 1:1 mixture of Dulbecco modified Eagle's medium and Ham's F-12 basal medium (DMEM/F-12, 11966-025 and 21765-029, respectively, from Gibco, Invitrogen) containing 5 mM glucose supplemented with 10 ng/ml human recombinant EGF, 36 ng/ml hydrocortisone, 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 5 ng/ml sodium selenite, 2 mM Glutamax (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in a 37 °C at atmospheric O_2 with 5% CO_2 in a humidified environment, fed three times weekly and sub-cultivated by trypsinization when near confluence. Cells between passage 6 and 21 were used for this study.

2.2. Cytotoxicity determination

Supernatant lactate dehydrogenase (LDH) release was used to determine cytotoxicity. LDH activity was measured using a commercial colorimetric assay (Roche, Mannheim, Germany). ATP levels in methanol cellular extracts were measured using the ATPlite kit from PerkinElmer (6016731) according to the manufacturer's instructions.

2.3. Toxin exposure

Dose response curves were generated by exposing confluent HK-2 cells in 96 well plates to compounds for 48 h. Stock concentrations of compounds were, Cyclosporine A (CsA, Novartis) 10 mM in 100% ethanol, $CdCl_2$ (Cd, Sigma) 10 mM in PBS and 100 mM diquat dibromide (Diq, Sigma) in HBSS. Serial dilutions of 2-fold dilutions of compounds were used, starting at 50 μM (solubility limit) for CsA, 0.1 mM for Cd and 5 mM for Diq. For CsA, all treatment regimes had the same concentration of ethanol (0.5%). The next lowest dose before significant toxicity was selected for further investigations, which was 5 μM CsA, 5 μM Cd, and 30 μM Diq.

For all subsequent treatments cells were cultured in 35 mm diameter petri dishes unless otherwise indicated. Confluent monolayers were treated at 12, 24, and 48 h with 5 μM Cd, 30 μM Diq, and 5 μM CsA. Final concentration of ethanol for CsA treatment and control was 0.1%.

2.4. RNA isolation

RNA was isolated using the silica gel column RNeasy extraction method (Qiagen, Crawley, UK) according to the manufacturer's instructions. The quantity and quality of RNA retrieved was assessed by microcapillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Dublin, Ireland) in conjunction with the RNA 6000 Nano LabChip kit. Samples with an RNA Integrity Number (RIN) > 9.5 and an A260/A280 ratio between 1.8 and 2.2 defined as high quality RNA were chosen for further analysis.

2.5. Microarray analysis

Hybridization target preparations were performed according to protocols recommended by Affymetrix. Briefly, 5 μg total RNA was reverse transcribed into cDNA using an oligo(dT)-T7 promoter primer and transformed into double stranded cDNA by *E. coli* DNA polymerase using the Affymetrix one cycle cDNA synthesis kit. After purification of double stranded cDNA with the Affymetrix GeneChip Sample Cleanup Module, biotin-labeled cRNA was produced by T7 polymerase (Affymetrix IVT Labeling kit). After Agilent-based quantification and integrity control, 20 μg cRNA was fragmented by alkaline treatment (Affymetrix GeneChip Sample Cleanup Module) and 15 μg fragmented cRNA was added to the hybridization cocktail (300 μl final volume). Fragmented cRNA was hybridized to Human Genome U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA) for 16 h, washed and stained for 2 h with fluorescent detection reagent streptavidin–phycoerythrin. The arrays were washed and stained according to the recommended Fluidics Station protocol (EukGE-WS2 version 5_450). Fluorescence signal intensities from each feature on the microarrays were determined using the Affymetrix GeneChip Scanner 3000 and the GCOS software (version 1.2) according to the manufacturer's recommendations. DNA array data fulfils MIAME standards and was deposited to ArrayExpress (http://www.ebi.ac.uk/arrayexpress/; accession No. E-MEXP-2599).

2.6. Bioinformatic analysis

Affymetrix CEL files were uploaded onto the CARMA web tool (http://carmaweb.genome.tugraz.at/carma/) and condensed using the MAS5 algorithm (scaled to a median target intensity of 100). CEL files were loaded into the Microsoft Excel add-on BRB Array tools version 3.80 (Wright and Simon, 2003). Genes with a spot intensity of less than 20 were truncated to 20. A class comparison (two-sample *t*-test with nominal significance level of the univariate test set to 0.001) was used to select significantly altered probe sets compared to their time matched control. The union of all differentially expressed probe sets (DEPs) was used for pathway analysis using Ingenuity Pathways Analysis (IPA) 8 (Ingenuity® Systems, www.ingenuity.com). The significance of the association between the data set and the canonical pathway was measured in two ways: (1) The ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that are present in this canonical pathway. (2) The right-tailed Fisher's Exact Test was used to calculate *p*-values based on the number of Pathway Eligible Molecules in the pathway and its size, and also on the total number of Pathway Eligible Molecules analysed and the total number of molecules in the database that could have contributed to a pathway. Results were ranked on the associated *p*-value.

2.7. Hydrogen peroxide production

HK-2 cells were cultured in 24 well plates and treated as described. Cell culture supernatant was collected and assayed immediately. Equal volumes of supernatant and 0.16 mM Amplex Red Ultra (Molecular Probes) with 1 U/ml horseradish peroxidase (HRP) in PBS were mixed and incubated for 1 h at 37 °C. Fluorescence was measured at 540 nm excitation and 595 nm emissions. Standard fluorescence intensity was linearly proportional to H_2O_2 added to cell culture medium. Relative fluorescent units (RFU) were converted to percentage control values.

2.8. Western blot analysis

2.8.1. Nuclear cell lysates

Cells were washed twice in PBS and scraped into 400 μl of ice cold hypotonic buffer (10 mM HEPES–NaOH buffer, pH 7.9, containing