Implementation of an *in vitro* Model System for Investigation of Reperfusion Damage after Renal Ischemia

Christoph Sauvant^{1,*}, Reinhard Schneider^{2,*}, Hildegard Holzinger¹, Sylvia Renker², Christoph Wanner² and Michael Gekle³

¹Physiologisches Institut, Bay. Julius-Maximilians Universität Würzburg, ²Universitätsklinikum Würzburg, Medizinische Klinik und Poliklinik I. Abt. Nephrologie, Würzburg, ³Julius-Bernstein-Institut für Physiologie, Universität Halle-Wittenberg, *C. Sauvant and R. Schneider contributed equally to the manuscript and both have to be considered as first authors

Key Words

Renal ischemia • Reperfusion damage • Proximal tubule • Ischemic acute kidney injury • Apoptosis • Necrosis • Inflammation • Fibrosis • Dedifferentiation • Inducible NO-Synthase • *In vitro*

Abstract

Ischemic acute kidney injury (iAKI) is a common event in organ transplantation and may occur during severe surgery. To gain mechanistic insights into ischemia-induced alterations at the level of proximal tubule cells we set up an *in vitro* model of ischemia and reperfusion using the rat proximal tubule cell line NRK-52E. In this particular model we simultaneously applied acidosis, hypoxia and aglycemia together for 2h, using low volume buffer systems and a hypoxia chamber. Thereafter reperfusion was mimicked by subsequently culturing the cells for up to 48h under standard conditions. In order to validate the system we investigated whether effects that take place in existing *in vivo* models of ischemia and reperfusion can be observed. Namely, induction of necrosis, apoptosis and of ischemia reperfusion induced protein (IRIP), dedifferentiation (α SMA), inflammation

(MCP-1), inducible NO-synthase (iNOS), release of PGE₂ and basolateral uptake of organic anions. In fact, all parameters developed as described for the *in vivo* situation during reperfusion after ischemia. Taken altogether we have established an *in vitro* model of proximal tubule cell reperfusion damage after ischemia, showing typical changes described *in vivo*. Additionally, our model system is suitable for isolated application of the typical insults associated with ischemia (e.g. acidosis alone, hypoxia alone, aglycemia alone), in order to obtain more insight into the mechanistic events that lead to reperfusion damage in the kidney on the cellular level.

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Introduction

Ischemic acute kidney injury (iAKI) is a common event in organ transplantation and may occur during severe surgery [1]. Renal ischemia is responsible for approx. 50% of acute renal failure cases [2] and can

result from kidney transplant, trauma, obstructive uropathy, surgery and cardiopulmonary bypass [3]. The development of ischemic acute kidney injury (iAKI) is associated with poor prognosis particularly if dialysis is required and is leading to increased morbidity and mortality [1].

It is well known that the proximal tubule (namely the S3 segment) is sensitive to ischemic insult [4]. Therefore most approaches in setting up an *in vitro* model of renal ischemia were performed using proximal tubular cells [3]. As we are investigating the regulation of proximal tubular transport during reperfusion after renal ischemia in a rat model of iAKI [5], we are particularly interested in the effect of reperfusion after ischemia on the latter cell population. For this purpose, we set up an *in vitro* model ischemia using a rat proximal tubular cell line (NRK-52E cells).

In general, ischemia is thought to consist of a combination of the following major insults: hypoxia, hypercapnia induced acidosis, limited nutrient availability and waste removal impairment [3]. Upon reperfusion cellular injury continues to occur or is even elevated [6, 7].

With respect to our research aims the model ischemia should include as much of the major insults (mentioned above) as possible. Moreover, the possibility of an isolated and controlled application of the single insult should be given. This is not the case in the established and useful *in vitro* models of renal ischemia using mineral oil overlay technique [8, 9]. Therefore we decided to establish a model consisting of defined low volume buffer systems and a hypoxia chamber to simulate ischemia. After ischemia application of serum free growth medium under standard cell culture conditions is performed in order to mimic reperfusion. In this particular model we applied acidosis (pH 6.6), hypoxia (O_2 fraction below 1%) and aglycemia to the cells for 2h. After ischemia, we subsequently cultured the cells for up to 48h under standard conditions.

In order to validate the model ischemia and reperfusion applied to proximal tubular cells we tested whether it leads to effects described in existing models of ischemia and reperfusion. Even more importantly we tested whether it leads to effects typical for reperfusion after iAKI *in vivo*. In order to investigate this we measured induction of necrosis, apoptosis and of ischemia-reperfusion induced protein (IRIP), α -smooth muscle actin (α SMA), monocyte chemoattractant factor 1 (MCP-1), inducible NO-synthase (iNOS), release of PGE_2 and the effect on organic anion uptake.

Materials and Methods

Cell culture

NRK-52E cells were cultured on culture dishes or permeable supports (Falcon, Becton Dickinson Labware, Franklin Lakes, USA). The cells were maintained at pH 7.4 and 37°C, and gassed with 95% air/5% CO_2 . All studies were performed between passage 60 and 100. The seeding density was $0.4 \cdot 10^6 \text{ cm}^{-2}$. The medium was changed every third day and the cells were used for experiments at day 10 after seeding. All experiments were performed with cells that were serum starved for 24 hours before the experiments. Cells were seeded on 6well or 24well plates, culture dishes or permeable supports as appropriate.

In vitro ischemia reperfusion model

For experiments, cells were grown in cell culture dishes ($\varnothing$ 3.5cm). Control cells were exposed to bicarbonate-HEPES buffered Ringer solution adjusted to pH 7.4 ($NaHCO_3$ 24.0 mM, Na_2HPO_4 0.8 mM, NaH_2PO_4 0.2 mM, NaCl 86.5 mM, KCl 5.4 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 0.8 mM, HEPES 20 mM; pH adjustment with 1N NaOH) containing 5 mM glucose and kept at 37°C and ambient pO_2 partial pressure. Model ischemia was applied by exposing the cells to a bicarbonate-MES buffered Ringer at pH 6.6 ($NaHCO_3$ 4.5 mM, Na_2HPO_4 0.8 mM, NaH_2PO_4 0.2 mM, NaCl 106.0 mM, KCl 5.4 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 0.8 mM, MES (morpholinoethanesulfonic acid) 20 mM; pH adjustment to 6.6 with 1N NaOH) without glucose and at an O_2 fraction below 1%. Buffer volumes were 1ml per culture dish. Cells were exposed for 2h to either control or ischemic (pH 6.6, aglycemia, O_2 fraction below 1%) conditions and then cultivated under standard cell culture conditions (2ml per culture dish) representing the reperfusion period for 6, 24 or 48h. Hypoxia was applied to the cells using a hermetic chamber filled with gas consisting of 95% N_2 and 5% CO_2 (see acidosis). During filling, the fraction of O_2 was measured and filling was continued until the O_2 fraction in the chamber fell below 1%. An O_2 fraction below 1% represents a pO_2 of below 7.6 mmHg which is well in the range of the measured values in clamped kidneys [10] and well below the critical pO_2 for oxygen consumption in the kidney cortex [11]. The O_2 fraction was stable throughout the 2h of incubation as determined. Acidosis is well described to happen during renal ischemia *in vivo* and a pH of 6.6 is described in the renal tissue [12]. Stability of pH was assured by using a bicarbonate-MES buffered Ringer in a 5% CO_2 atmosphere. Correct pH was moreover assured by measurement before and after the incubation period. Aglycemia mimics the absence of glucose due to its consumption without redelivery in ischemia and was applied by using a glucose-free buffer (as mentioned above).

PGE_2 -ELISA

PGE_2 in the supernatant was determined by competitive ELISA technique using the Correlate-EIA™ PGE_2 Enzyme Immunoassay Kit from Assay Designs (Ann Arbor, Michigan, USA). Measurement and calculation were done in a 96well plate according to the manufacturers instructions. In brief, 100 μ l of either sample were given into a single well and 100 μ l of a

supplied solution containing conjugate (PGE₂ attached covalently to alkaline phosphatase) and PGE₂ antibody were added. The plate was incubated at room temperature for 2h on a shaker and the wells were washed three times afterwards. Then 200 µl substrate solution was added for 45 min and subsequently 50 µl stop solution was added before the optical density was determined at 405 nm in a multiwell-multilabel counter (Victor², Wallac, Turku, Finland). PGE₂ in the supernatant was then calculated and given as the amount of PGE₂ per cell protein.

Caspase-3 activity assay

Caspase-3 activity was measured according to the manufacturer's instructions (Clontech Laboratories GmbH, Heidelberg, Germany) with slight modifications [13]: cells were washed once with PBS buffer (4°C) and incubated with 150 µl cell lysis buffer for 10 min on ice, harvested, and centrifuged at 16000g for 10 min at 4°C. 60 µl of the supernatant was incubated with 50 µmol/l DEVD-AFC (Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin; final concentration) for 60 min at 37°C, and fluorescence of the cleaved product, 7-amino-4-trifluoromethylcoumarin (AFC), was measured at 400 nm excitation and 505 nm emission wavelength using a multiwell-multilabel counter (Victor², Wallac, Turku, Finland). Cleaved AFC was quantified by a calibration curve using known AFC concentrations. As a control, cell extracts were incubated as described above but in the presence of caspase-3 inhibitor zDEVD-CHO. No activity could be found under these conditions. Protein content was determined with bicinchoninic acid assay (Pierce) using bovine serum albumin as a standard.

Realtime-R(everse)T(ranscriptase)-PCR

RNA from NRK-52E cells was extracted using Qiagen RNA Isolation Kit (Qiagen, Hilden, Germany). RNA concentration was determined and cDNA was synthesized using iScript cDNA synthesis kit (Biorad, CA, USA) according to the manufacturer's instructions. In brief, RT-PCR was performed according to iQ SYBR-Green Supermix RT-PCR system protocol (Biorad, CA, USA). Initial denaturation was performed at 95°C for 3 min. PCR amplification was performed as described in the respective references. iNOS was determined as described in [14]. For iNOS, the primers were 5'-GCA GGT TGA GGA TTA CTT CTT CCA-3' (sense) and 5'-GCC CTT TTT TGC TCC ATA GGA AA-3' (antisense), resulting in an 94 bp RT-PCR-product. MCP-1 was determined as described in [15]. For MCP-1, the primers were 5'-CCC AGAAAC CAG CCAAC-3' (sense) and 5'-TGC TGC TGG TGA TTC TCT-3' (antisense), resulting in an 223 bp RT-PCR-product. For α-SMA, the primers were 5' - gat cac cat cgg gaa tga acg - 3' (sense) and 5' - ctt aga agc att tgc ggt gga - 3' (antisense), resulting in an 389 bp RT - PCR - product, according to [16]. For IRIP, the primers were 5' - gct caa caa aga cct gaa ccc c - 3' (sense) and 5' - gca agt ggt ccc cca aac at - 3' (antisense), resulting in an 102 bp RT - PCR - product, according to the predicted size for the rat analogue of IRIP from mouse presented in [17]. COX primers were chosen according to Ivanov et al. [18]. For COX-1, the primers were 5' - taa gta cca ggt gct gga tgg - 3' (sense) and 5' - ggt ttc ccc tat

aag gat gag - 3' (antisense), resulting in an 244 bp RT - PCR - product. For COX-2, the primers were 5' - tac aag cag tgg caa agg cc - 3' (sense) and 5' - cag tat tga gga gaa cag atg gg - 3' (antisense), resulting in an 283 bp RT - PCR - product. For β-actin, the primers were 5' - tct aca atg agc tgc gtg tg - 3' (sense) and 5' - tac atg gct ggg gtg ttg aa - 3' (antisense), resulting in an 129 bp RT - PCR - product. The RT-PCR products were tested for correct size by agarose gel electrophoresis and melting point analysis. Quantification was performed using the ΔΔC_T method using β-actin as reference gen and expression in control cells was normalized to 1.

Fluorescein uptake

For transport experiments, NRK-52E cells were seeded on 24well filters (Falcon, Becton Dickinson Labware, Franklin Lakes, USA) and grown until confluency. The volumes of the apical and basolateral compartments were 0.2 ml and 0.5 ml, respectively, in order to avoid hydrostatic pressure differences. Ischemia was induced as described above, using the latter volumes. Fluorescein uptake was determined as described [19]. In brief, the cells were washed three times with phosphate buffered Ringer (PBS; 138 mmol/l NaCl, 1 mmol/l NaH₂PO₄, 4 mmol/l Na₂HPO₄, 4 mmol/l KCl, 1 mmol/l MgCl₂, 1 mmol/l CaCl₂, 5 mmol/l glucose, pH 7.4). Transport measurements were performed in phosphate buffered ringer at pH 7.4 and 37°C. Fluorescein uptake transport was determined by measuring the uptake of 10 • 10⁻⁶ M fluorescein after 2 min. After that, cells were washed 4 times with ice-cold PBS until no fluorescein was detectable in the washing solution. The cells were then lysed in 1 ml 0.1 % Triton - X100 in 20 mM MOPS (3-(N-morpholino)propanesulfonic acid) buffered at pH 7.4 and fluorescence was counted in a multiwell - plate-reader (Victor², Wallac Instruments, Finland). Counts were corrected for extracellular binding and unspecific adhesion to the cells by subtraction of fluorescein counts on cells at 4°C. Fluorescein counts were normalized to protein content in the lysate measured by BCA protein assay (Pierce, Rockford, USA).

Other methods

Cell number and cell volume were determined using a Coulter Counter. Protein content was measured by BCA protein assay (Pierce, Rockford, USA) (see the results section or the figure legends for additional details on experimental protocols). LDH release was measured according to [20] at room temperature in a photometer.

Data analysis

Data are presented as means ± SEM respectively. The *n* value is given in the text or in the figures and represents the number of supports used for the respective experiments (wells, filters, culture dishes). Statistical significance was determined by unpaired Student's *t*-test or ANOVA as appropriate. Differences were considered statistically significant when *P* < 0.05.

Materials

If not stated otherwise, chemicals used were from Sigma.

Fig. 1. A: Effect of reperfusion after model ischemia on NRK52-E cell protein. Protein during reperfusion in ischemic cells is given as % of the respective time controls. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for up to 48h. n is given in the figure. Statistical significance is indicated by * (indicating difference to respective time control) or # (indicating difference to ischemia effect after different reperfusion time intervals) respectively. B: Effect of reperfusion after model ischemia on necrosis in NRK52-E cells. Necrosis was determined by release of cytosolic lactate dehydrogenase (LDH) into cell culture medium. LDH release during reperfusion in ischemic cells is given as difference to the respective time controls. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for up to 48h. n is written above or below the respective bars. Statistical significance is indicated by * (indicating difference to respective time control) or # (indicating difference to ischemia effect after different reperfusion time intervals) respectively. C: Effect of reperfusion after model ischemia on apoptosis in NRK52-E cells. Apoptosis was determined by activity of caspase-3 in cell lysates. Caspase-3 activity during reperfusion in ischemic cells is given as difference to the respective time controls. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for up to 48h. n is written above or below the respective bars. Statistical significance is indicated by * (indicating difference to respective time control) or # (indicating difference to ischemia effect after different reperfusion time intervals) respectively.

Results

Model ischemia leads to moderate protein loss

To simulate key features of renal ischemia, NRK-52E cells were subjected to pH 6.6, aglycemia and hypoxia for 2h in a buffer volume of about 100 μ l per cm^2 growth area (as described in detail in the methods section and as discussed later on). Control cells were treated for 2h with buffer at pH 7.4, normoglycemia and normoxia at the same volume in parallel. Following aspiration of the respective experimental buffers the reperfusion phase was simulated by addition of culture medium without serum and subsequent standard culture for up to 48h (200 μ l per cm^2). Protein content per culture dish was determined as a measure for total cell mass (mean protein content per culture dish before ischemia: $973.8 \pm 61.0 \mu\text{g}$; $n=27$; protein content was not different 6h after the 2h treatment

period). Cell protein in ischemia treated dishes was normalized to protein content of the respective time controls for every single experiment by a priori matching. As presented in Fig. 1A, ischemia induced a slight but significant decrease in protein content in NRK-52E cells 48h after model ischemia.

Cell protein loss by apoptosis and necrosis

Necrosis as well as apoptosis have been described to occur during reperfusion in renal epithelial cells due to ischemia [21]. To gain evidence on the mechanism of protein loss we thus investigated the release of cytosolic lactate dehydrogenase (LDH) as a measure of unspecific cell death (necrosis) and the activity of caspase-3 as a

Fig. 2. A: Effect of reperfusion after model ischemia on the amount of α SMA mRNA in NRK52-E cells. α SMA was determined by realtime PCR as described in the methods section and is used as a marker for dedifferentiation. α SMA mRNA is normalized to β -actin mRNA. α SMA signal in control cells is set as 1 and the respective signal in ischemic cells is given as the relative amount compared to these particular time controls. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for up to 48h. n is written above or below the respective bars. Statistical significance is indicated by * (indicating difference to respective time control) or # (indicating difference to ischemia effect after different reperfusion time intervals) respectively. B: Effect of reperfusion after model ischemia on the amount of IRIP mRNA in NRK52-E cells. IRIP was determined by realtime PCR as described in the methods section. IRIP mRNA is normalized to β -actin mRNA. IRIP signal in control cells is set as 1 and the respective signal in ischemic cells is given as the relative amount compared to these particular time controls. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for up to 48h. n is written above or below the respective bars. Statistical significance is indicated by * (indicating difference to respective time control) or # (indicating difference to ischemia effect after different reperfusion time intervals) respectively.

measure for regulated cell death (apoptosis). As presented in Fig 1B and Fig 1C, ischemia induced necrosis and apoptosis was detected solely after 48h of model ischemia in NRK-52E cells, which is in accordance with protein loss shown above.

Fig. 3. Effect of reperfusion after model ischemia on the amount of iNOS mRNA in NRK52-E cells. iNOS was determined by realtime PCR as described in the methods section. iNOS mRNA is normalized to β -actin mRNA. iNOS signal in control cells is set as 1 and the respective signal in ischemic cells is given as the relative amount compared to these particular time controls. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for up to 48h. n is written above or below the respective bars. Statistical significance is indicated by * (indicating difference to respective time control) or # (indicating difference to ischemia effect after different reperfusion time intervals) respectively.

Fig. 4. Effect of reperfusion after model ischemia on the amount of MCP-1 mRNA in NRK52-E cells. MCP-1 was determined by realtime PCR as described in the methods section. MCP-1 mRNA is normalized to β -actin mRNA. MCP-1 signal in control cells is set as 1 and the respective signal in ischemic cells is given as the relative amount compared to these particular time controls. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for up to 48h. n is written above or below the respective bars. Statistical significance is indicated by * (indicating difference to respective time control) or # (indicating difference to ischemia effect after different reperfusion time intervals) respectively.

Fig. 5. A: Effect of reperfusion after model ischemia on the release of PGE₂ from NRK52-E cells. PGE₂ was determined by ELISA as described in the methods section. PGE₂ signal during reperfusion in ischemic cells is given as relative signal compared to the respective time controls. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for up to 48h. n is given in the graph. Statistical significance is indicated by * (indicating difference to respective time control) or # (indicating difference to ischemia effect after different reperfusion time intervals) respectively. B: Effect of reperfusion after model ischemia on the amount of COX-1 mRNA in NRK52-E cells. COX-1 was determined by realtime PCR as described in the methods section. COX-1 mRNA is normalized to β -actin mRNA. COX-1 signal in control cells is set as 1 and the respective signal in ischemic cells is given as the relative amount compared to these particular time controls. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for up to 48h. n is written above or below the respective bars. Statistical significance is indicated by * (indicating difference to respective time control) or # (indicating difference to ischemia effect after different reperfusion time intervals) respectively. C: Effect of reperfusion after model ischemia on the amount of COX-2 mRNA in NRK52-E cells. COX-2 was determined by realtime PCR as described in the methods section. COX-2 mRNA is normalized to β -actin mRNA. COX-2 signal in control cells is set as 1 and the respective signal in ischemic cells is given as the relative amount compared to these particular time controls. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for up to 48h. n is written above or below the respective bars. Statistical significance is indicated by * (indicating difference to respective time control) or # (indicating difference to ischemia effect after different reperfusion time intervals) respectively.

Model ischemia leads to appearance of ischemia reperfusion markers

It is well known that dedifferentiation occurs during reperfusion after renal insult due to ischemia [22]. As a result, appearance of α - smooth muscle actin (α SMA) is widely described during the reperfusion phase in vivo [23]. Actually, α SMA-mRNA was found to be upregulated after model ischemia in NRK-52E cells after 6h and 24h of reperfusion. This ischemia induced α SMA-mRNA was no more present after 48h (Fig. 2A).

Ischemia Reperfusion Induced Protein (IRIP) is a recently described protein that is induced by renal ischemia in vivo [17]. In NRK-52E cells, IRIP-mRNA was upregulated 6h after application of a 2h model ischemia. 24h after ischemia IRIP-mRNA is back to the respective control levels. No more effect on IRIP-mRNA level was detected 48h after model ischemia.

Induction of iNOS during reperfusion after ischemia

Upregulation of inducible NO-synthase (iNOS) is a hallmark in renal ischemia reperfusion injury [24] and excess NO generated by iNOS is thought to play a pivotal role in this process. As iNOS is expressed in the proximal tubule [25], we investigated the effect of a model ischemia on the amount of iNOS-mRNA in NRK-52E cells. Actually, iNOS mRNA was elevated after a 24h reperfusion period, whereas no change was detected before and after this particular reperfusion phase (Fig. 3).

Induction of monocyte chemoattractant factor 1 (MCP-1) during reperfusion after ischemia

Induction of monocyte chemoattractant factor 1 (MCP-1) is described to occur during reperfusion after

Fig. 6. Basolateral uptake of organic anions into NRK52-E cells after model ischemia and 48h reperfusion. Fluorescein uptake was determined as described in the methods section. Fluorescein is given as pmol per mg cell protein and uptake time. Cells were seeded on permeable supports and treated as described in the methods section. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for 48h. n is written above or below the respective bars. Statistical significance is indicated by * (indicating difference to respective time control).

renal ischemia [26]. Furthermore MCP-1 expression is described in proximal tubular cells [27, 28]. Therefore, we measured whether this is also the case in our *in vitro* model system. Ischemia led to an about 2.5fold increase in MCP-1 mRNA after a 24h reperfusion period (Fig. 4). Expression of MCP-1 then declined to around 1.4fold expression as compared to the control cells after 48h.

Ischemia induced generation of PGE₂ and downregulation of organic anion transport

It is well known that PGE₂ levels increase in the renal cortex during reperfusion after ischemia [29, 30]. Therefore, we investigated whether an increased amount of PGE₂ is detectable in our system, too. Model ischemia led to an increase of PGE₂ present in the supernatant after 48h of reperfusion as compared to non ischemic cells (Fig. 5A). Therefore, similar to what happens in kidney cortex, PGE₂ induction also occurs in proximal tubular cells after model ischemia.

In general it is thought that renal PGE₂ generation after renal ischemia is accomplished by upregulated COX-

2, while regulation of COX-1 is negligible, because the common pathophysiology of inflammation is assumed to be involved [31]. However, proximal tubular cells are thought not to express COX-2 in substantial quantities, although a basic expression of COX-2 [32] is described in these cells [33]. In human allograft rejection showing characteristics of AKI, COX-1 was described to be upregulated in tubular epithelial cells [34]. Therefore, we investigated the expression of both COX-2 and COX-1 during reperfusion. As indicated in Fig. 5B, COX-1 is upregulated with time during reperfusion, while expression of COX-2 declines in parallel (Fig. 5C). This indicates that the increased amount of PGE₂ may be due to an increase in COX-1. However, we are aware that due to the specific half-life of COX-2 protein, COX-2 may still play a substantial part in PGE₂ generation after 48h.

PGE₂ leads to a downregulation of organic anion uptake transport in NRK52-E cells [19] and downregulation of proximal tubular organic uptake transport is also described *in vivo* after iAKI [5]. Therefore, we investigated whether downregulation of organic anion uptake also happens 48h after a model ischemia. As presented in Fig. 6 basolateral organic anion uptake (as determined by fluorescein uptake) is reduced due to ischemia in post ischemic cells.

Discussion

Undoubtedly, it is not possible to use *in vitro* techniques to create an exact replica of the *in vivo* environment, and this is therefore not the primary goal of such efforts. However, *in vitro* methodologies offer several advantages over *in vivo* experimentation and therefore are suitable to complement *in vivo* studies. Arguably, the most striking advantage of an *in vitro* model is that it provides an environment where specific stimuli can be isolated, controlled, and tested to access their contribution to physiological or pathophysiological events [3]. Therefore, we set up an *in vitro* model ischemia followed by simulated reperfusion using a rat proximal tubular cell line (NRK-52E cells).

As already mentioned in the introduction, ischemia is thought to consist of a combination of the following major insults: hypoxia (I), hypercapnia induced acidosis (II), limited nutrient availability (III) and waste removal impairment (IV) [3]. Upon reperfusion cellular injury continues to occur or is even elevated [6, 7].

Hypoxia (I) was applied as mentioned in the methods section. An O₂ fraction below 1% represents a pO₂

of below 7.6 mmHg which is well in the range of the measured values in clamped kidneys [10] and well below the critical pO_2 for oxygen consumption in the kidney cortex [11].

Acidosis (II) is well described to happen during renal ischemia *in vivo* and a pH of 6.6 is described in the renal cortical tissue during ischemia [12]. In fact, in our system acidosis occurs without hypercapnia as renal cortical pCO_2 during ischemia is around 100 mmHg in rats [10], whereas pCO_2 is around 40 mmHg in our model system. However, as acidosis is thought to be the most important (and best described) insult resulting from hypercapnia [3], we decided to focus on this parameter alone.

Limited nutrient availability (III) during model ischemia was guaranteed by using an ischemia buffer free of glucose (see methods). Consequently, after consuming the few glucose resources present in the cell before incubation a depletion of glucose and ATP occurs, which is well described to occur during renal ischemia [3].

Waist removal impairment (IV) of non volatile compounds, which occurs during renal ischemia, is achieved by restriction of buffer volume to 100 μ l per cm^2 during ischemia (1ml per 3.5 cm $\varnothing$ culture dish). This was confirmed in principle by determination of lactate accumulation (data not shown). This moreover represents the optimal ratio of media volume per surface area as proposed by Felder et al. [35], which leads to a minimum diffusion distance allowing equilibration of gas concentrations between cellular compartment and the gas atmosphere present in the hypoxia chamber. For reperfusion after ischemia an increased volume was chosen (200 μ l per cm^2 during ischemia; 2ml per 3.5 cm $\varnothing$ culture dish), in order to maintain glucose supply over the reperfusion period. The latter volume-surface-ratio was also shown to be suitable for gas exchange between the cells and the gaseous phase [36].

A number of studies investigating renal ischemia and reperfusion *in vitro* using proximal tubular cells are published (for review see [3]), most of them using proximal tubular cells from pig (LLC-PK1) or, to a lesser extent, cells of human origin. Most of these studies investigated apoptosis and necrosis, energy depletion or induction of HIF-1 as a measure of ischemia and/or ischemia induced reperfusion injury. Only few of them also determined release of $TNF\alpha$ or activation of $NF\kappa B$. In general, most of the studies cited in [3] only investigate very few parameters of renal injury in order to characterize the respective model systems. One model using NRK-52E

cells with mineral oil overlay technique is published, where the authors show cellular hypoxia, energy depletion and apoptosis [9]. Ensuing the authors strongly focussed on the effects of ischemia and reperfusion on cell-cell contacts [9, 37].

In our model system, we show cell death by necrosis and apoptosis during reperfusion after ischemia. Apoptosis is also described in most *in vitro* systems [3]. More importantly, the latter three phenomena also happen during reperfusion in renal epithelial cells after ischemia *in vivo* [21]. In order to further characterize our system, we decided to measure additional parameters which are well known to be changed during reperfusion after ischemia in the kidney cortex *in vivo*, but were not determined in the recent studies describing *in vitro* systems up to now [3].

As already mentioned in the results section dedifferentiation (as determined by α SMA induction) occurs during reperfusion after ischemia [22] and its appearance is widely described during the reperfusion phase *in vivo* [23]. Therefore, induction of α SMA in our model system indicates similarity to the *in vivo* situation. The same is true for IRIP, which was recently described to be induced after renal ischemia *in vivo* [17]. Upregulation of inducible NO-Synthase (iNOS) is a hallmark in renal ischemia reperfusion injury [24] and excess NO generated by iNOS is thought to play a major part in reperfusion injury [24, 38]. As nitric oxide is described to induce apoptosis in renal tubular epithelial cells [39], apoptosis after 48h reperfusion might be the consequence of iNOS induction 24h after ischemia. Inflammation is thought to play a major role in reperfusion damage after renal injury [31] and the proinflammatory MCP-1 is induced during reperfusion after renal ischemia [26] and is expressed in proximal tubular cells [27, 28]. Induction of both iNOS and MCP-1 is therefore additional evidence that our *in vitro* model system shows important characteristics of renal reperfusion damage *in vivo*.

Finally, PGE_2 was elevated during reperfusion after ischemia in our system, which is well known to take place *in vivo* [29, 30]. Due to our data, the increase in PGE_2 observed in our system is most likely due to increase in COX-1. This is in accordance to an increase in COX-1 in tubular epithelial cells during human allograft rejection showing characteristics of AKI [34]. Interestingly, it is described that a substantial increase of NO (as is generated by iNOS) leads to reduced expression of COX-2 and an activation of COX-1 [40]. As iNOS is found to be upregulated in our system, we consider this as additional evidence for the role of

COX-1 in generation of PGE₂ therein. Moreover, preliminary data from our laboratory on organic anion uptake after model ischemia using isoform specific inhibitors of COXs, indicate that inhibition of COX-1, but not of COX-2 abolishes downregulation of organic anion uptake. Hence, additional different cell populations are present in the kidney cortex *in vivo* and moreover do invade after ischemia [31]. Therefore, our data are certainly no estimate of the relative impact of COX isoforms on the amount of PGE₂ in kidney cortex during reperfusion after ischemia *in vivo*. Nonetheless, the data are in agreement with the thought that basolateral organic anion uptake is downregulated during reperfusion by PGE₂ [5, 19]. The latter additionally indicates the occurrence of basic features of reperfusion after renal ischemia in our model system.

Taken altogether we have established an *in vitro* model of reperfusion damages after ischemia applying the most prominent insults associated with ischemia on a proximal tubular cell line from rat (NRK-52E). Therein, we could show typical damages described *in vivo* and partly in already established *in vitro* systems. Moreover, we could detect for the first time that typical events observed during reperfusion *in vivo* occur in an (our particular) model system *in vitro*, e.g. induction of IRIP,

iNOS, MCP-1 or PGE₂. Additionally, our model system now allows application of the typical insults associated with ischemia in an isolated manner (e.g. acidosis alone, hypoxia alone, aglycemia alone), in order to obtain more insight into the mechanistic events that lead to reperfusion damage in the kidney on the cellular level.

Abbreviations

NRK-52E cells (normal rat kidney epithelial cells); PGE₂ (prostaglandin E₂); iNOS (inducible nitric oxide synthase); MCP-1 (monocyte chemoattractant protein 1); IRIP (ischemia reperfusion induced protein); α SMA (α smooth muscle actin); LDH (lactate dehydrogenase); iAKI (ischemic acute kidney injury).

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References

- Mahon P, Shorten G: Perioperative acute renal failure. *Curr Opin Anaesthesiol* 2006;19(3):332-338.
- Star RA: Treatment of acute renal failure. *Kidney Int* 1998;54(6):1817-1831.
- Russ AL, Haberstroh KM, Rundell AE: Experimental strategies to improve *in vitro* models of renal ischemia. *Exp Mol Pathol* 2007;83(2):143-159.
- Witzgall R: The proximal tubule phenotype and its disruption in acute renal failure and polycystic kidney disease. *Exp Nephrol* 1999;7(1):15-19.
- Schneider R, Sauvant C, Betz B, Otremba M, Fischer D, Holzinger H, Wanner C, Galle J, Gekle M: Downregulation of organic anion transporters OAT1 and OAT3 correlates with impaired secretion of para-aminohippurate after ischemic acute renal failure in rats. *Am J Physiol Renal Physiol* 2007;292(5):F1599-1605.
- Tracey WR, Treadway JL, Magee WP, Sutt JC, McPherson RK, Levy CB, Wilder DE, Yu LJ, Chen Y, Shanker RM, Mutchler AK, Smith AH, Flynn DM, Knight DR: Cardioprotective effects of ingliforib, a novel glycogen phosphorylase inhibitor. *Am J Physiol Heart Circ Physiol* 2004;286(3):H1177-1184.
- Ueda N, Shah SV: Tubular cell damage in acute renal failure-apoptosis, necrosis, or both. *Nephrol Dial Transplant* 2000;15(3):318-323.
- Meldrum KK, Meldrum DR, Hile KL, Burnett AL, Harken AH: A novel model of ischemia in renal tubular cells which closely parallels *in vivo* injury. *J Surg Res* 2001;99(2):288-293.
- Covington MD, Bayless KJ, Burghardt RC, Davis GE, Parrish AR: Ischemia-induced cleavage of cadherins in NRK cells: evidence for a role of metalloproteinases. *Am J Physiol Renal Physiol* 2005;289(2):F280-288.

- 10 Nelimarkka O, Niinikoski J: Oxygen and carbon dioxide tensions in the canine kidney during arterial occlusion and hemorrhagic hypotension. *Surg Gynecol Obstet* 1984;158(1):27-32.
- 11 Leichtweiss HP, Lübbers DW, Weiss C, Baumgärtl H, Reschke W: The oxygen supply of the rat kidney: measurements of intrarenal pO₂. *Pflügers Arch* 1969;309(4):328-349.
- 12 Holloway JC, Phifer T, Henderson R, Welbourne TC: Renal acid-base metabolism after ischemia. *Kidney Int* 1986;29(5):989-994.
- 13 Schwerdt G, Freudinger R, Mildnerberger S, Silbernagl S, Gekle M: The nephrotoxin ochratoxin A induces apoptosis in cultured human proximal tubule cells. *Cell Biol Toxicol* 1999;15(6):405-415.
- 14 Canuelo A, Siles E, Martinez-Romero R, Peinado MA, Martinez-Lara E: The nitric oxide system response to hypoxia/reoxygenation in the aged cerebral cortex. *Exp Gerontol* 2007;42(12):1137-1145.
- 15 Lu H, Shi JX, Zhang DM, Shen J, Lin YX, Hang CH, Yin HX: Hemolysate-induced expression of intercellular adhesion molecule-1 and monocyte chemoattractant protein-1 expression in cultured brain microvascular endothelial cells via through ROS-dependent NF-kappaB pathways. *Cell Mol Neurobiol* 2009;29(1):87-95.
- 16 Li JH, Zhu HJ, Huang XR, Lai KN, Johnson RJ, Lan HY: Smad7 inhibits fibrotic effect of TGF-Beta on renal tubular epithelial cells by blocking Smad2 activation. *J Am Soc Nephrol* 2002;13(6):1464-1472.
- 17 Jiang W, Prokopenko O, Wong L, Inouye M, Mironchitschenko O: IRIP, a new ischemia/reperfusion-inducible protein that participates in the regulation of transporter activity. *Mol Cell Biol* 2005;25(15):6496-6508.
- 18 Ivanov AI, Pero RS, Scheck AC, Romanovsky AA: Prostaglandin E(2)-synthesizing enzymes in fever: differential transcriptional regulation. *Am J Physiol Regul Integr Comp Physiol* 2002;283:R1104-1117.
- 19 Sauvant C, Holzinger H, Gekle M: Prostaglandin E2 Inhibits Its Own Renal Transport by Downregulation of Organic Anion Transporters rOAT1 and rOAT3. *J Am Soc Nephrol* 2006;17(1):46-53.
- 20 Sauvant C, Silbernagl S, Gekle M: Exposure to ochratoxin A impairs organic anion transport in proximal- tubule-derived opossum kidney cells. *J Pharmacol Exp Ther* 1998;287(1):13-20.
- 21 Sheridan AM, Bonventre JV: Cell biology and molecular mechanisms of injury in ischemic acute renal failure. *Curr Opin Nephrol Hypertens* 2000;9(4): 427-434.
- 22 Abbate M, Brown D, Bonventre JV: Expression of NCAM recapitulates tubulogenic development in kidneys recovering from acute ischemia. *Am J Physiol* 1999; 277(3 Pt 2):F454-463.
- 23 Villanueva S, Cespedes C, Vio CP: Ischemic acute renal failure induces the expression of a wide range of nephrogenic proteins. *Am J Physiol Regul Integr Comp Physiol* 2006;290(4):R861-870.
- 24 Guan Z, Gobe G, Willgoss D, Endre ZH: Renal endothelial dysfunction and impaired autoregulation after ischemia-reperfusion injury result from excess nitric oxide. *Am J Physiol Renal Physiol* 2006;291(3):F619-628.
- 25 Chatterjee PK, Patel NS, Kvale EO, Cuzzocrea S, Brown PA, Stewart KN, Mota-Filipe H, Thiemermann C: Inhibition of inducible nitric oxide synthase reduces renal ischemia/reperfusion injury. *Kidney Int* 2002;61(3):862-871.
- 26 Furuichi K, Wada T, Iwata Y, Kitagawa K, Kobayashi K, Hashimoto H, Ishiwata Y, Asano M, Wang H, Matsushima K, Takeya M, Kuziel WA, Mukaida N, Yokoyama H: CCR2 signaling contributes to ischemia-reperfusion injury in kidney. *J Am Soc Nephrol* 2003;14(10):2503-2515.
- 27 Takaya K, Koya D, Isono M, Sugimoto T, Sugaya T, Kashiwaga A, Haneda M: Involvement of ERK pathway in albumin-induced MCP-1 expression in mouse proximal tubular cells. *Am J Physiol Renal* 2003;284(5):F1037-F1045.
- 28 Viedt C, Dechend R, Fei J, Hänsch GM, Kreuzer J, Orth SR: MCP-1 induces inflammatory activation of human tubular epithelial cells: involvement of the transcription factors, nuclear factor-kappaB and activating protein-1. *J Am Soc Nephrol* 2002;13(6):1534-1547.
- 29 Tokuyama H, Hayashi K, Matsuda H, Kubota K, Honda M, Okubo K, Takamatsu I, Ozawa Y, Saruta T: Role of nitric oxide and prostaglandin E2 in acute renal hypoperfusion. *Nephrology (Carlton)* 2003;8(2):65-71.
- 30 Myers SI, Wang L, Liu F, Bartula LL: Suprarenal aortic clamping and reperfusion decreases medullary and cortical blood flow by decreased endogenous renal nitric oxide and PGE2 synthesis. *J Vasc Surg* 2005;42(3):524-531.
- 31 Bonventre JV, Zuk A: Ischemic acute renal failure: An inflammatory disease? *Kidney Int* 2004;66(2):480-485.
- 32 Gong R, Morris DJ, Brem AS: Human renal 11beta-hydroxysteroid dehydrogenase 1 functions and co-localizes with COX-2. *Life Sci* 2008;82(11-12):631-637.
- 33 Campean V, Theilig F, Paliege A, Breyer M, Bachmann S: Key enzymes for renal prostaglandin synthesis: site-specific expression in rodent kidney (rat, mouse). *Am J Physiol Renal Physiol* 2003;285(1):F19-32.
- 34 Hoffmann U, Banas B, Krüger B, Pietrzyk M, Obed A, Segerer S, Kammerl M, Rümmele P, Riegger GA, Krämer BK: Expression of cyclooxygenase-1 and cyclooxygenase-2 in human renal allograft rejection—a prospective study. *Transpl Int* 2006;19(3):203-212.
- 35 Felder E, Jennings P, Seppi T, Pfaller W: LLC-PK(1) cells maintained in a new perfusion cell culture system exhibit an improved oxidative metabolism. *Cell Physiol Biochem* 2002;12(2-3):153-162.
- 36 Gstraunthaler G, Seppi T, Pfaller W: Impact of Culture Conditions, Culture Media Volumes, and Glucose Content on Metabolic Properties of Renal Epithelial Cell Cultures. are renal cells in tissue culture hypoxic? *Cell Physiol Biochem* 1999;9(3):150-172.
- 37 Covington MD, Burghardt RC, Parrish AR: Ischemia-induced cleavage of adherins in NRK cells requires MT1-MMP (MMP-14). *Am J Physiol Renal Physiol* 2006;290(1):F43-51.
- 38 Goligorsky MS, Brodsky SV, Noiri E: Nitric oxide in acute renal failure: NOS versus NOS. *Kidney Int* 2002; 61(3):855-861.
- 39 Du C, Guan Q, Diao H, Yin Z, Jevnikar AM: Nitric oxide induced apoptosis in renal tubular epithelial cells through activation of caspase-8. *Am J Physiol Renal Physiol* 2006;290(5):F1044-1054.
- 40 Clancy R, Varenika B, Huang W, Ballou L, Attur M, Amin AR, Abramson SB: Nitric oxide synthase/COX cross-talk: nitric oxide activates COX-1 but inhibits COX-2-derived prostaglandin production. *J Immunol* 2000;165(3):1582-1587.