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Ischemia/reperfusion injury : a metabolic meltdown

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Succinate accumulation and ischemia/reperfusion injury: of mice but not men.

A study in renal
ischemia/reperfusion

American Journal of Transplantation

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ABSTRACT

A recent seminal paper implicated ischemia-related succinate accumulation followed by succinate-driven reactive oxygen species formation as a key driver of ischemia/reperfusion injury. Although the data show that the mechanism is universal for all organs tested (kidney, liver, heart, and brain), a remaining question is to what extent these observations in mice translate to humans. We showed in this study that succinate accumulation is not a universal event during ischemia and does not occur during renal graft procurement; in fact, tissue succinate content progressively decreased with increasing graft ischemia time ($P < 0.007$). Contrasting responses were also found with respect to mitochondrial susceptibility toward ischemia and reperfusion, with rodent mitochondria robustly resistant toward warm ischemia but human and pig mitochondria highly susceptible to warm ischemia ($P < 0.05$). These observations suggest that succinate-driven reactive oxygen formation does not occur in the context of kidney transplantation. Moreover, absent allantoin release from the reperfused grafts suggests minimal oxidative stress during clinical reperfusion.



INTRODUCTION

Ischemia reperfusion (I/R) injury is the primary cause of organ dysfunction after events such as myocardial infarction, cerebrovascular accident and organ transplantation.¹ Despite decades of intensive research and an abundance of promising preclinical results, no interventions emerged that prevent or reduce clinical I/R injury.^{2,3} A recent study in mice proposed a new mechanistic concept⁴: that progressive succinate accumulation during ischemia drives excessive post-reperfusion reactive oxygen species (ROS) formation as a result of reverse electron transport through mitochondrial complex I.⁴ This excess ROS formation is suggested to drive I/R injury.

Despite a wealth of rodent studies in support of a role for ROS in I/R injury^{5,6}, human studies consistently fail to show a benefit with ROS scavenging (antioxidant) therapy.⁷⁻⁹ Moreover, although clinical studies confirm increased redox stress during I/R, this is not followed by biomarkers of oxidative damage.¹⁰ Consequently, a pivotal role for oxidative damage as driving force of I/R injury⁴ and thus a role for succinate accumulation is unclear for clinical I/R injury. In this respect, it was noted that the proposed central role for succinate accumulation necessarily requires confirmation in the human setting.¹¹

MATERIALS & METHODS

PATIENT GROUPS

Local ethics committees approved all experiments. Renal biopsies of 24 human kidney transplants were taken after written informed consent.¹⁰ Patients were classified (n=6 per group) based on duration of cold ischemia: short (2-6 h), intermediate (6-12 h) and prolonged (12-24 h). Non-ischemic controls were biopsies taken from functioning living donor kidneys. Renal allograft transplantations were performed according to the local standardized protocol. All grafts were preserved by static cold storage. The immunosuppressive regimen was based on induction therapy with basiliximab on day 0 and 4, and tacrolimus or cyclosporine A in addition to mycophenolate mofetil and steroids as maintenance therapy. Postoperative course was uneventful in all patients. One-year patient and graft survival was 100%. Patients in the short ischemia group did not develop clinical I/R injury (delayed graft function), whereas 14% of the intermediated ischemia group and 71% of the prolonged ischemia group developed delayed graft function.

The influence of warm and cold ischemia on murine kidney succinate content was tested in kidneys from healthy surplus male mice (C57bl background). Kidneys



were removed immediately upon sacrificing. Reference (control) kidneys (n=6) were directly snap-frozen. Two series of 6 kidneys were kept on melting ice or in a warm water bath (37°C) for 45 min in order to simulate cold and warm ischemia, respectively. Another 6 kidneys were kept on melting ice for 20 h before they were snap frozen, in order to simulate prolonged cold ischemia.

METABOLIC PROFILING

Metabolic profiling (hypoxanthine, succinate, and fumarate) of the tissue biopsies was performed by High-Resolution Magic-Angle-Spinning (HR MAS) Nuclear Magnetic Resonance (NMR) spectroscopy. Human tissue samples were snap frozen in liquid nitrogen and stored at -80°C. Samples were prepared on ice and fitted in a leak-proof insert (30 µL, Bruker: Kel-F, Bruker, Delft, The Netherlands) used in a zirconium HR-MAS rotor (4 mm). The insert was filled with 3 µL cold (4°C) phosphate-buffered saline (PBS in D₂O) containing 4.5 mM TSP-d₄ (trimethylsilyl-tetraduteropropionic acid) and 25 mM sodium formate (CHNaO₂) as internal standards. NMR-spectrometry was used for murine kidney succinate content, for which metabolites were extracted and dried. The dried material was reconstituted with 0.3 mL phosphate buffer solution (pH 7.4; 150 mM K₂HPO₄, Sigma-Aldrich, Germany) in deuterated water containing also 0.2 mM of bacteriostatic NaN₃ and 0.4 mM sodium TSP-d₄ (Cambridge Isotope Laboratories Inc., Buchem, Apeldoorn, The Netherlands) as chemical shift reference and 0.19 mL of each sample was transferred to 3 mm NMR tubes (Bruker Biospin, GmbH, Germany) for NMR analysis. HR-MAS spectra were recorded on a 14.1 T (600MHz, ¹H resonance frequency) Bruker Avance III NMR spectrometer equipped with a TCI cryoprobe. All experiments were acquired at 4°C while spinning the samples at a rate of 5 kHz. Metabolite signals were quantified using the BATMAN R package.¹² Spectra were normalized based on the tissue weight used.

HIGH RESOLUTION RESPIROMETRY

Effect of ischemia-reoxygenation on mitochondrial efficacy was analysed by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) and compared with different species: humans (N=7), pigs (N=10), rats (N=6) and mice (N=6). Kidney biopsies from a healthy kidney segment were taken immediately prior to clamping of the renal vasculature and stored on ice. Warm ischemia was simulated by maintaining biopsies in a warm-water bath (37°C) for 60 min.

On simulating warm ischemia, the tissue was washed and permeabilized in saponin for 30 min at 4°C. Subsequently, the tissue was washed in mitochondrial respiration solution (MiRo5, Oroboros Instruments), containing 0.5 mM ethylene glycol tetraacetic acid, 3 mM magnesium chloride, 60 mM K-lactobionate, 20



mM taurine, 10 mM potassium dihydrogen phosphate, 20 mM HEPES, 110 mM sucrose and 1 g.L⁻¹ fatty acid-free bovine serum albumin (pH 7.1). Tissue was then weighed and placed in the measurement chamber of the high-resolution respirometer and incubated at 37°C. To avoid oxygen diffusion limitation, oxygen concentration was increased to 400 μ M and maintained above 270 μ M throughout the experiment by adding pure oxygen.

The integrity of the outer-mitochondrial membrane was tested by adding 10 μ M cytochrome C; samples with a >15% increase in respiratory rate were excluded from further analysis. Leak respiration was assessed by adding the Krebs cycle intermediates sodium glutamate (10 mM), sodium malate (2 mM), and sodium pyruvate (5 mM). Adenosine diphosphate (ADP)-stimulated respiration was measured in 2.5 mM ADP. Maximal respiration, with simultaneous input of electrons through complex I and II, was measured through addition of 10 mM succinate. Maximal uncoupled respiration was measured after stepwise addition of 0.01 μ M carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone. Subsequently, complex I was blocked by rotenone (0.5 μ M). Finally, antimycin A was added to inhibit complex III, and residual oxygen consumption measured (nonmitochondrial respiration), and subtracted from all values. All measurements were performed simultaneously and subsequently averaged. Values were mass normalized, and reported as pmol O₂s⁻¹.mg⁻¹. The contribution of complex I function to the uncoupled maximal respiration was assessed by the relative decline of respiration after adding rotenone.

ALLANTOIN RELEASE

Post reperfusion allantoin formation was quantified by establishing arteriovenous concentration differences over the transplanted kidney. For maximum sensitivity we specifically selected patients that presented with manifest I/R injury (delayed graft function). These patients were all part from a previous study.¹⁰

Prior to implantation of the graft, a 5 French umbilical vein catheter was positioned in the lumen of the renal vein through one of its side branches. At 30 s, and 3, 5, 10, 20 and 30 min after reperfusion (i.e. moment of reperfusion t=0), 10 mL blood aliquots were sampled. Paired arterial blood samples were obtained. The abdominal wall was closed about 45 min after reperfusion, and the endpoint of sampling was reached 30 min after reperfusion. Blood samples were collected in precooled containers and immediately placed on melting ice. The validity of the arteriovenous sampling method was validated earlier measuring oxygen saturation.¹⁰

Allantoin in plasma was measured using an ultraperformance liquid chromatography (UPLC)-tandem mass spectrometry method developed in-house. Briefly 30 μ L plasma was mixed with 30 μ L of a solution containing stable isotope



labelled internal standards. Samples were deproteinized with 500 μ l acetonitrile. After centrifugation (10 min, 10 000 g, 4 °C) the supernatant was evaporated under nitrogen and reconstituted in 500 μ l 50 mM ammonium formate (pH 4.00). Purine and pyrimidine metabolites were separated using a Waters Acquity UPLC system (Waters, Etten-Leur, The Netherlands) equipped with an Acquity HSS T₃ (2.1 * 100 mm, df 1.8 μ m). Separation of the compounds of interest was achieved by a 0.01 M ammonium formate (pH 4.00) / acetonitrile gradient. Compounds were quantified using a Waters XEVO TQS tandem mass spectrometer (Waters), with both negative and positive electrospray ionization using specific MRM transitions.

STATISTICAL ANALYSIS

Maximal mitochondrial respiration values were tested using analysis of variance. Paired T-tests were used to compare the baseline respiratory rate with respiratory rate after experiencing warm ischemia (SPSS 22.0).

For the plasma allantoin measurements, the area under the curve (AUC) was estimated and compared through a linear mixed-model analysis for arterial and venous measurements for the total of 30 min. The model contained the following independent variables: time as categorical, the group, and the interaction between group and time. The covariance model was specified as unstructured. The delta AUC was calculated (venous minus arterial) and the null hypothesis (AUC = 0) was tested by a Wald test based on the estimated parameters of the linear mixed model. The level of significance was set at $p < 0.05$. All data represent mean $\pm$ SEM.

RESULTS

Similar to the report for the murine setting (which also specifically tested kidney I/R)⁴, our data for the human kidney show progressive hypoxanthine accumulation during ischemia (Figure 1). Results for succinate (and fumarate) on the other hand contrast with those reported for the mouse.⁴ Fumarate concentrations remained low and stable during ischemia, whereas our clinical data showed a paradoxical decline in tissue succinate content following progressive ischemia (Figure 1A), indicating that succinate accumulation⁴ is not a universal metabolic signature of ischemia. This contrast between the reported murine data⁴ and our human data may reflect species-specific differences and relate in part to contrasting effects of warm and cold ischemia. We tested the latter by exposing murine kidneys to cold and warm ischemia. Figure 1B shows that although cold ischemia results in progressive but moderate succinate exhaustion ($p < 0.019$), tissue succinate levels remained stable during warm ischemia.



Tissue metabolites upon progressive ischemia in humans

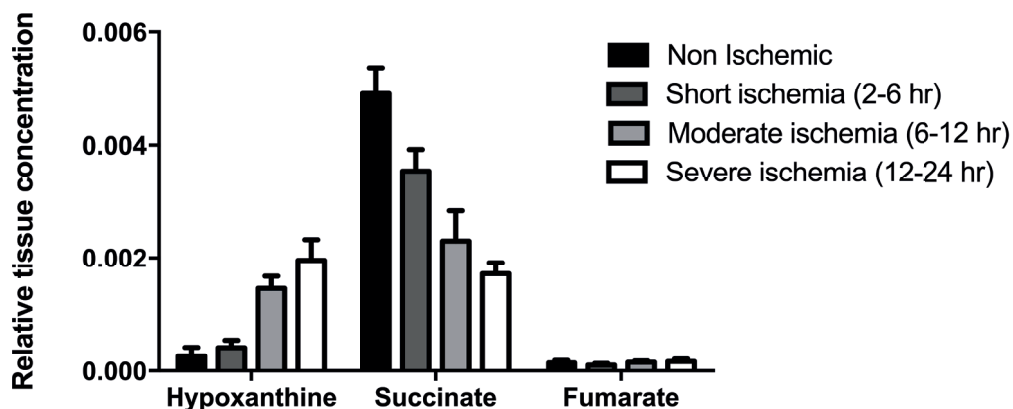


Figure 1A. Relative hypoxanthine, succinate and fumarate tissue content (HR MAS NMR) on incremental ischemia time (human kidney).

Increasing hypoxanthine ($p < 0.031$), but declining succinate levels ($p < 0.007$) during ischemia. Fumarate levels remained low and stable during ischemia. Non-ischemic control values are included as a reference.

All values were normalized on basis of tissue weight. ($n = 6$ per group, mean $\pm$ SEM).

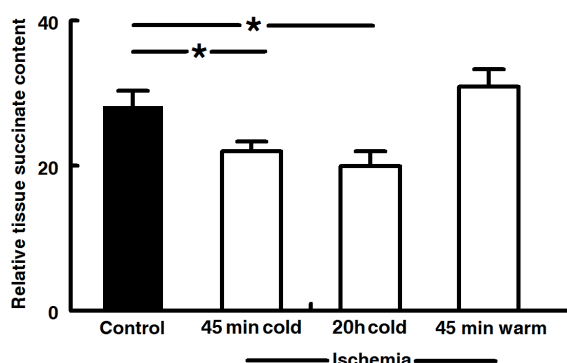


Figure 1B. Cold ischemia but not warm ischemia reduced murine kidney succinate content (ANOVA $p < 0.019$) (mean $\pm$ SEM).



Interspecies differences also exist with regard to mitochondrial respiratory efficiency and ROS production.¹³ This is further illustrated by the clear reciprocal association between species body size and overall mitochondrial respiration (Fig. 2A) and normalized activities of mitochondrial complex I and II activity (Figure 2B and C).

We tested for potential interspecies differences with regard to mitochondrial susceptibility to ischemia/reperfusion (Figure 2D-F), and in particular with respect to succinate metabolism. Figure 2D shows that while postischemic mouse mitochondria retained or even increase their ability to oxidize succinate, reperfused human mitochondria lose their ability to oxidize succinate. Results from validation experiments using rat and pig renal mitochondria parallel those for mice and humans, respectively (Figure 2D). In contrast to their rodent counterparts, postischemic human (and pig) mitochondria have a reduced capacity to oxidize succinate.

Respirometry revealed further interspecies differences in mitochondrial susceptibility. Not only are rodent mitochondria significantly more resistant to ischemia than human and pig mitochondria ($p < 0.05$ and $P < 0.03$, respectively) (Figure 2D and E), but it was observed that increases in complex II activity in rodent mitochondria compensate for loss of complex I function ($p < 0.038$)¹⁴ (Figure 2F).

These findings challenge a prominent role for succinate-driven ROS formation in the context of renal graft ischemia/reperfusion.⁴ To that end, we tested reperfusion-related ROS formation in the context of clinical graft reperfusion by quantifying allantoin release from reperfused kidneys by means of arteriovenous concentration differences (Figure 3). Absent allantoin-release in the first 30 min following reperfusion implies negligible ROS quenching during reperfusion.



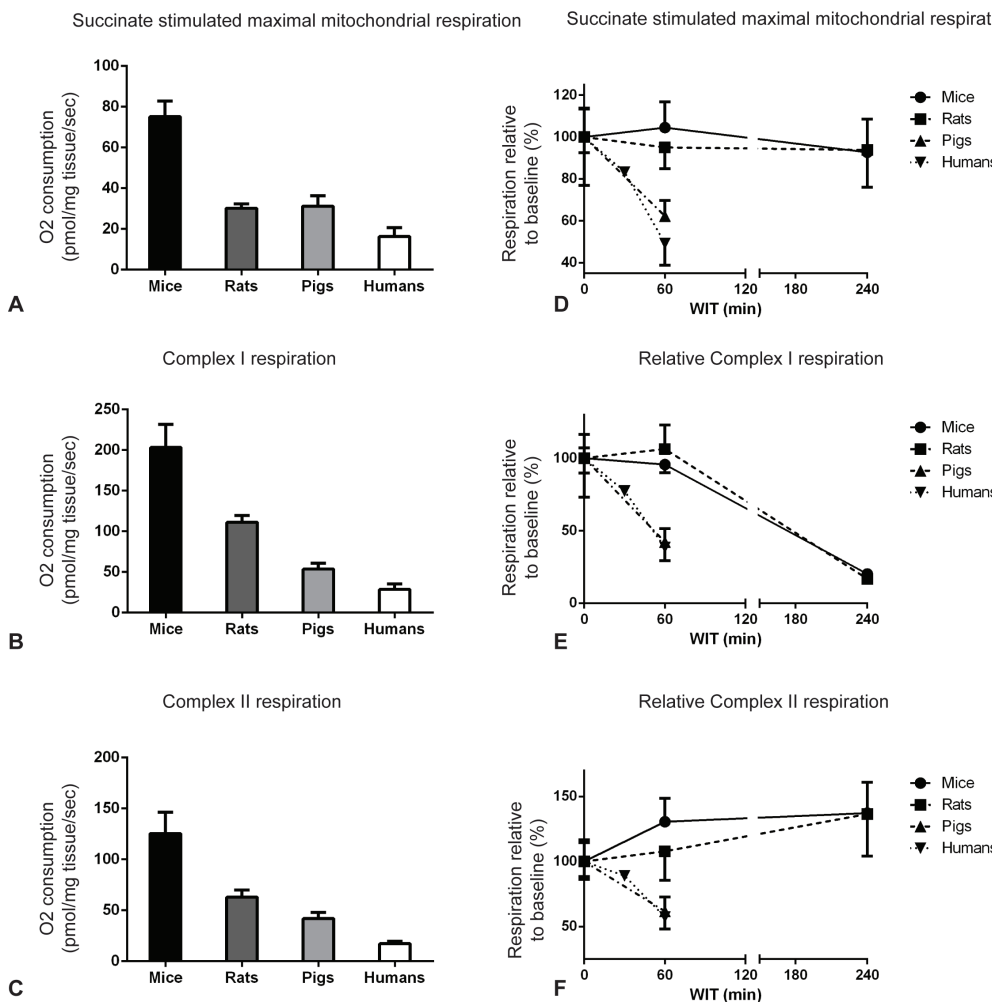


Figure 2. Interspecies differences in baseline mitochondrial respiration rates (A-C) and mitochondrial susceptibility towards ischemia and reperfusion (D-F).

A-C: Baseline aspects of mitochondrial oxidative phosphorylation (absolute values (oxygen consumption per mg tissue per second) for different species. Note the inverse associations ($p < 0.000001$) between body weight and maximal oxidative phosphorylation capacity (A), complex I activity (B), and complex II activity (C). Bars represent mean $\pm$ SEM.

D-F: Effect of warm ischemia time and re-oxygenation on the aspects of mitochondrial function for different species (values relative to baseline values (baseline= 100%), Bars represent SEM). D) Sixty minutes of warm ischemia significantly ($p < 0.05$) reduced succinate-driven mitochondrial respiration (combined complex I and II activities) in human and pig mitochondria, whereas succinate-driven respiration in rat and mice mitochondria is not influenced by 4 h of warm ischemia.



E) Sixty minutes of warm ischemia significantly reduced complex I activity of human and pig mitochondria ($p < 0.03$). Complex I activity of mouse and rat mitochondria is not influenced by 60 minutes warm ischemia but significant drops are found after 4-hours of warm ischemia ($p < 0.001$).

F) Sixty minutes warm of ischemia significantly reduced complex II (succinate dehydrogenase) activity of human and pig mitochondria ($p < 0.008$ resp. $p < 0.04$), but increased respectively stable Complex II activity in mouse and rat mitochondria ($p = 0.038$).

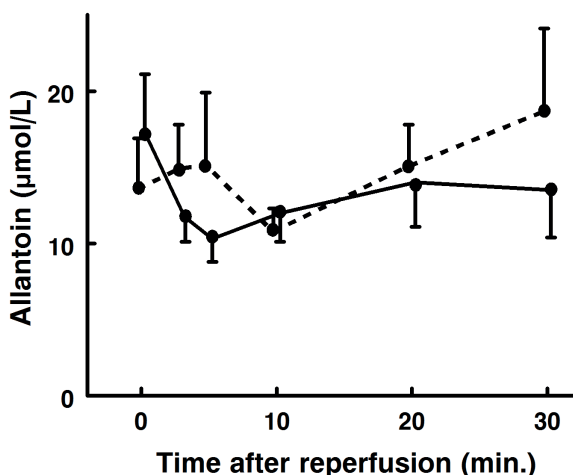


Figure 3. Absent postreperfusion uric acid ROS quenching (allantoin release from the reperfused grafts following prolonged ischemia). Dotted line represents arterial allantoin levels and solid line represents renal vein allantoin levels. (arteriovenous difference by area under the curve (AUC): $p = 0.368$).



DISCUSSION

From these data, the picture emerges of divergent metabolic and mitochondrial responses to I/R in mice and in humans, and for succinate accumulation in murine kidneys between warm and cold ischemia. It is unclear how these observations translate to the human context. Pre-reperfusion succinate content of grafts from donation after cardiac death (DCD) was actually lower than that in from donation after brain death (Supplementary Figure 2), showing that the additional period of warm ischemia in DCD donors does not translate to increased graft succinate content.

In mice, progressive succinate accumulation during ischemia and reversed transport through mitochondrial complex-I drives reperfusion-related ROS formation.⁴ In contrast, the human context is characterized by an inverse relationship between ischemia and succinate tissue levels, and ischemia-driven knockdown of mitochondrial respiration. As such, the question arises of whether clinical I/R in the context of kidney transplantation is accompanied by excess ROS formation.

As noted earlier, clinical studies consistently fail to show a beneficial effect of antioxidant therapy on I/R injury,⁷⁻⁹ and markers for oxidative damage remain low following ischemia/reperfusion.¹⁰ These observations would be consistent with a minor role for ROS in clinical I/R, but they may also indicate superior antioxidant responses, with effective ROS quenching, effectively preventing ROS-mediated damage. To test whether excess ROS is formed during clinical I/R, we quantified ROS quenching by establishing allantoin release from reperfused human kidneys. Allantoin is the stable end product of ROS quenching by the antioxidant uric acid and, as such, reflects the extent of oxidative stress. Absent allantoin release in the first 30 minutes following reperfusion implies that ROS quenching is negligible and that ROS stress during clinical I/R may be less than generally expected.

In summarizing the mechanism of succinate accumulation driving I/R injury, Heger et al.¹¹ pointed out that ‘most research in I/R injury has been proven of limited translational value’. The findings noted for I/R in the clinical setting of kidney transplantation point to fundamental mechanistic differences between findings obtained in rodent models of I/R and the actual human context. It came to our attention that the above observations (partly) explain the apparent antioxidant paradox in the context of I/R with clear advantages in murine studies, but no apparent benefit in clinical studies.^{7-9,15}

Supplementary Figure 1 and 2 are published online.



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