Renal hyaluronan accumulation and hyaluronan synthase expression after ischaemia-reperfusion injury in the rat

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Abstract

Background. Hyaluronan (HA) is a connective tissue component with unique water binding and pro-inflammatory properties. It has been suggested that HA is involved in normal renal water handling but also in several pathological conditions such as organ rejection and ischaemia-reperfusion (IR) injury.

Methods. In anaesthetized normal rats we investigated if renal cortical HA accumulation and the intrarenal distribution and expression of HA synthases (Has 1, 2 and 3) correlate with renal dysfunction after renal IR injury. After 20, 30 or 45 min of unilateral renal ischaemia and 72 h of reperfusion, renal function and cortical HA content were measured. Has 1, 2 and 3 mRNA were determined in control and IR kidneys subjected to 45 min ischaemia and 72 h reperfusion.

Results. IR kidneys had reduced urine concentrating ability, potassium excretion, glomerular filtration rate (GFR) and renal blood flow. On average, IR kidneys had more than 10 times higher amounts of cortical HA than the contralateral control kidney and their water content was elevated while medullary HA was largely unaffected. Has 2 expression in the cortex was heavily up-regulated in IR kidneys while Has 3 remained at control levels. Has 1 could never be detected. There was a direct correlation between the amount of cortical HA and the time period of ischaemia and also between the cortical amount of HA and depression of functional parameters.

Conclusions. IR injury depresses parameters of renal function, which coincides with an elevated cortical HA content and Has 2 expression. The enhanced Has 2 expression indicates that the cortical HA accumulation is primarily dependent on increased HA synthesis and not impaired degradation/elimination. The water binding and pro-inflammatory properties of HA may contribute to renal dysfunction after IR.

Keywords: hyaluronic acid; ischaemia; kidney; kidney failure

Introduction

Hyaluronan (HA) is a negatively charged linear glycosaminoglycan with unique water binding capacity. It is an important connective tissue component with a variety of biological functions such as the maintenance of water and protein homeostasis, modulation of the inflammatory reaction [1–4] and stabilization of the loose connective tissue [5]. HA is also involved in several pathological conditions and accumulates in rejecting organs [6].

The healthy kidney has a heterogenous distribution of HA, with an excessive accumulation in the inner medulla and almost negligible amounts found in the cortex [3,6]. This distribution is altered in certain conditions. An accumulation of HA in the renal cortex occurs during renal transplant rejection, tubulo-interstitial inflammation and fibrosis and in renal ischaemia-reperfusion (IR) injury [6–8]. The accumulation of HA is paralleled with an increase in the cortical water content, probably due to the extreme water binding properties of HA [6]. This accumulation of HA as a result of IR injury can, partly, be responsible for the interstitial oedema known to occur in the early post-transplantation period which, in turn, influences vascular resistance and tubular function.

In the present study, our primary aim was to elucidate if an altered HA distribution correlates with alterations in kidney function during IR injury. A further aim was to follow the intrarenal distribution...
and expression of HA syntheses (Has 1, 2 and 3 mRNA) in an effort to elucidate the underlying causes to the normal heterogenous intrarenal distribution of HA and if changes in Has activity can explain changes in intrarenal HA levels during IR injury.

Subjects and methods

An official ethics committee in Uppsala approved all experiments on animals. Twenty-nine male Sprague-Dawley rats weighing 250–300 g were obtained from M&B (Skensved, Denmark) and allowed to settle for a week prior to surgery. The animals had free access to standard rat pellet chow (R3, Ewos, Södertälje, Sweden) and tap water before and during the entire experimental period. All surgical procedures were performed under gas anaesthesia with isoflurane (Forene®, Abbott Scandinavia AB, Kista, Sweden) mixed in 40% O₂ and 60% air. The gas was delivered either through a breathing mask (during implantation of chronic catheter and induction of ischaemia, day 0) or with a ventilator (Model 683, Harvard Apparatus Inc., MA, USA) connected to a tracheal tube (renal function studies, day 3).

Induction of renal ischaemia

The left kidney was exposed through a midline incision and totally freed from surrounding tissue. To ensure complete ischaemia, the ureter, renal vein and artery were clamped with microvascular clips thereby blocking inflow and outflow to the kidney. After 20 (n = 7), 30 (n = 8) or 45 min (n = 8), the clamps were removed and the abdomen closed with ligatures. Sham operated rats (n = 6) underwent the same surgical procedure with the exception of clamping the renal vessels and the ureter. Three days (72 h) after induction of ischaemia, the rats were anaesthetized and renal function studies were performed. This model of IR injury has been used in our previous investigation allowing direct comparison of results.

Renal function studies at 72 h of reperfusion

The rats were anaesthetized with isoflurane and placed on a servo-controlled heating pad, which kept the body temperature constant (37.5–38.5°C) using a rectal probe. The left femoral vein was cannulated for infusion of saline [1.5 mL/kg body weight (bw)-h] containing [3H]inulin (1.75 μCi bolus followed by 0.5 μCi/100 g bw-h; NEN, Boston, MA, USA) and para-aminohippuric acid (PAH, 4-aminohippuric acid, 2 mg bolus followed by 0.6 mg/100 g bw-h; Merck, Darmstadt, Germany). The right femoral artery was cannulated for continuous monitoring of mean arterial blood pressure (MAP) through a pressure transducer, and for blood sampling. The left and right ureters were cannulated for urine sampling. Since the rats were under gas anaesthesia delivered by a ventilator, blood samples were taken 2–3 times during the experiments to follow acid-base parameters. The protocol consisted of four consecutive 30 min urine sampling periods (U₁–U₄). After the experiments the kidneys were excised, weighed and sectioned into specimens of cortex, outer medulla and papilla for further analyses. The animals were killed with an i.v. injection of saturated KCl.

Urine and plasma analysis

Urine osmolality was measured from the depression of the freezing point (Model 3MO; Radiometer, Copenhagen, Denmark) and urinary K⁺ concentrations were measured by flame photometry (FLM3; Radiometer, Copenhagen, Denmark). [3H]Inulin in samples of plasma and urine was detected using a liquid scintillation counter (PW 4700, Philips, Holland) and PAH through a chemical spectrophotometric assay (Lambda 2; Perkin-Elmer & Co GmbH, Überlingen, Germany). The acid–base status was determined from arterial plasma samples (AVL Compact 3; AVL LIST GmbH, Graz, Austria). Glomerular filtration rate (GFR) and renal plasma flow (RPF) were estimated from the clearance of [3H]inulin and PAH, respectively, according to:

\[
\text{Clearance of } s = \frac{(C_{urine} \times V)}{C_{plasma}}
\]

where \(C_{urine}\) and \(C_{plasma}\) are the urine and plasma concentrations of [3H]inulin or PAH, respectively, and \(V\) represents urine flow rate. RBF was calculated according to:

\[
\text{RBF} = \text{RPF} / (1 - \text{Hct}),
\]

where Hct is the systemic haematocrit. Since the extraction of PAH is not complete, an underestimation of RBF will be obtained if not corrected for. Therefore, measurements of the arterio-venous (a-v) difference for PAH ([aPAH – vPAH]/aPAH) were performed in three anaesthetized normal rats. The renal artery and vein were cannulated and blood samples (100 μl) were obtained after a 60 min equilibration period for PAH analysis. The calculated average a-v difference was 0.44 suggesting that the measurement procedure using clearance of PAH is underestimated by an average of 66%. All calculated RBF values (equation 2) were therefore divided by 0.44. It is known that vascular permeability and transport mechanisms are changed in IR injury. It is therefore questionable whether the a-v difference used for correction is valid for all groups of animals. The extraction may, furthermore, vary between the different ischaemic periods tested. The same line of reasoning can be put forward regarding the use of inulin and PAH as such for the estimation of GFR and RPF in models of renal damage. However, the calculated GFR and RPF values should provide indications of the functional status of the kidney.

Analysis of tissue HA and determination of water content

The kidney sections were put on filter paper for 3 min and then weighed (wet weight). The sections were frozen and stored at −70°C until analysed for HA content. The specimens were lyophilized overnight and then weighed again (dry weight). After grinding, HA was extracted from the tissue with 0.5 M NaCl for 16 h, and after centrifugation at 2700 g for 15 min at 4°C, the supernatants were analysed for HA, using a radiometric assay (Pharmacia Diagnostics, Uppsala, Sweden). The technique is based on the binding of HA to specific HA binding proteins. Briefly, 100 μl of sample or standard was mixed with 200 μl 125I-labeled HA binding protein with specific affinity for HA, and incubated for 60 min at 4–7°C. 100 μl HA-Sepharose was added and then the tubes were incubated for an additional 45 min at the same temperature. Two millilitres of washing solution were added and the HA-Sepharose was recovered after centrifugation.
at 2000 g for 10 min. Bound radioactivity in the pellet was measured with a gamma counter. A standard curve was constructed by using known amounts of HA and the radioactivity was plotted as a function of HA concentration. Every sample was measured in duplicate. The variability was <10%. The relative water content was calculated as 100 (wt wt – dry wt)/wt wt.

**Histochemical analyses**

To visualize the distribution of HA, histochemical stainings were performed. Specimens for this staining were obtained 3 days (72 h) after 45 min of left renal ischaemia. The left (ischaemic) and right (control) kidneys were fixed in buffered 4% formalin, pH 7.3 with 1% cetylpyridinium-chloride and stored in room temperature until dehydrated, embedded in paraffin and sectioned in 4 μm thick slices. The analysis was performed as described earlier [7,8]. In short, the sections were incubated with bovine serum albumin (10 mg/ml, Fraction V, Sigma Chemical Co., St Louis, MO, USA) to block non-specific binding sites and thereafter, the sections were incubated in 3% H2O2 in PBS to inhibit endogenous peroxidase. After incubation for 2 h with biontylated HA binding proteins (a kind gift from Prof. Bengt Gerdin, Uppsala), the sections were incubated with ABC Vectastain Reagent (Vector Laboratories, Burlingame, CA, USA) for 1 h. Finally, H2O2 as a substrate and 3-amino-9-ethylcarbazole (AEC) as electron donor were added. The sections were counterstained with Mayer’s haematoxylin. Sections were also stained with Mayer’s haematoxylin and eosin for morphological evaluation.

To determine the identity of infiltrating cells and examine the distribution of the HA binding receptor CD44, immunohistochemical analyses were also performed. The following monoclonal antibodies were used: R73, ED1, OX6 and OX50 (Serotec, Oxford, UK). R73 detects α/β-receptor expressing T-lymphocytes, ED1 detects macrophages, OX6 detects MHC class II-expressing cells and OX50 detects CD44-expressing cells. Specimens for this staining were obtained 3 days (72 h) after 45 min of left renal ischaemia. The kidneys were excised and immediately frozen in liquid nitrogen and stored at –70°C until cryosectioned. Sections (6 μm thick) were cut on a cryostat at –22°C and air-dried. The sections were fixed in 100% acetone and, thereafter, incubated in 0.3% H2O2 in PBS to inhibit endogenous peroxidase. Non-specific binding was blocked with goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and then the primary antibody was added and the sections incubated for 30 min. A secondary antibody, goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), was added in excess. Then the sections were incubated with horseradish peroxidase-mouse antiperoxidase (Dakopatts, Glostrup, Denmark). Finally, H2O2 as a substrate and AEC as electron donor were added to react with the horseradish peroxidase. The sections were counterstained with Mayer’s haematoxylin. Negative controls were obtained by omitting the primary antibody. All slides were evaluated blindly.

**Determination of Has expression using RT–PCR**

Isolation of total RNA from cortex and papillary tissues was performed according to manufacturer’s instructions (Rneasy Midi kit, Qiagen, USA). Kidney tissues were freshly taken from rats subjected to 45 min of ischaemia and 72 h reperfusion and snap frozen in liquid nitrogen. Each frozen tissue was homogenized using a mortal and pestle and thawed in lysis buffer. To investigate the effect of IR injury on Has 1, 2 and 3 expression at the mRNA level, a semi-quantitative RT–PCR was performed using the Ready-To-Go Beads (Amersham Biosciences). The primer pair for each Has isoform used are: Has 1 (forward: 5'-AGG ACC TCT ACA TGG TCG ACA T-3'; reverse: 5'-AGT GGA AGT AGC TCT GAC AAC-3'), Has 2 (forward: 5'-GAA TTA CCC AGT CCT GCC TT-3'; reverse: 5'-TCA GTA AGG CAC TTA GAT CG-3') and Has 3 (forward: 5'-CCT ACT TTG GCT GTG TGC AA-3'; reverse: 5'-TCG GTA GCT TCC ATG GAT CA-3'). In order to normalize samples for variations in the amount of cDNA, β-actin primer were used (forward: 5'-GAC CCA GAT CAT GTT TGA GAC C-3'; reverse: 5'-ATC TCC TTC TGC ATC CTG TCA G-3'). RT reactions (200 ng RNA reactions mixture) were carried out at 42°C for 30 min for the first strand cDNA synthesis. Reverse transcriptase inactivation and RNA cDNA primer denaturation occurred by heating at 95°C for 5 min in a PTC-100 Thermal Controller (MJ Research, SDS, Sweden) followed by 38 cycles (for Has 1, 2 and 3) and 15 cycles (β-actin) 95°C for 1 min, 55°C for 1 min and 72°C for 1 min. A final extension step at 72°C for 10 min was performed. PCR products were separated on 1.2% agarose gel and counterstained with ethidium bromide. The PCR products were quantified by a gel Doc 200 scanner and associated software (Bio-Rad Laboratories).

**Statistical analysis**

Data are given as mean values ± SEM. The comparison between groups was evaluated with ANOVA followed by Fisher’s PLSD post-hoc test and the correlation between HA and GFR by linear regression. A P-value of <0.05 was considered statistically significant.

**Results**

**HA and water content**

The HA and water contents in the kidney sections are shown in Tables 1 and 2, respectively. In the normal control kidney the HA distribution is heterogenous with large amounts in the papilla while the cortex is almost void of HA. The water content is, as expected, higher in the papilla than in the cortex. The left kidneys exposed to isothermic ischaemia showed an increased amount of cortical HA, which correlated to the times of ischaemia (Figure 1). On average, the cortical HA content in IR kidneys was 10 times larger compared with the right, healthy kidney and there was also an increased water content. The papillary content remained largely unaffected after ischaemia while the outer medullary HA content increased.

**Inulin clearance (GFR)**

GFR in the animals of the different groups is shown in Table 3 and Figure 2. Longer time periods of ischaemia,
i.e. 45 min, did not produce enough urine to enable GFR measurements. GFR was, as expected, lower in the left ischaemic kidneys as compared with the right control ones (Figure 2). The GFR was inversely correlated to the time of ischaemia, i.e. the GFR in the 20 min IR group was greater than that of the 30 min IR group. GFR in the IR kidneys subjected to 20 min of ischaemia was 37% of the contralateral control kidney (Figure 2). The corresponding value in the 30 min ischaemia group was 13%. The HA content was inversely correlated to the GFR in both the 20 min \( (r = 0.89) \) and the 30 min \( (r = 0.99) \) IR group (Figure 3).

**Has mRNA expression**

The intrarenal distribution and expression levels of Has isoform mRNAs before and after the IR injury is summarized in Table 4. Has 1 mRNA was undetectable in kidneys under all condition studied. However, Has 2 mRNA was detected in low amounts in the cortex but was strongly expressed in the papilla of normal kidneys. Interestingly, Has 2 mRNA expression in the cortex of ischaemic kidneys was considerably up-regulated while the papillary expression was not affected. Under the same condition, Has 3 transcript levels were low in cortex and high in the papilla, and were not affected by ischaemia. Thus, it is likely that the IR injury influences Has 2 gene induction.

**RBF**

RBF (Table 3) in the kidneys subjected to 30 min of ischaemia was only 40% of the RBF in the contralateral control kidney. Measurements of RBF were not performed in the group subjected to 20 min of ischaemia.

**MAP**

MAP was constant during the acute experiments and did not vary with time of the ischaemic period (Table 3).
Arterial acid–base status

Acid–base balance remained stable throughout the experiments (data not shown).

Urine parameters

Urine osmolality is a good measure of the function of the kidney, since we can assume that a poorly functioning kidney cannot form concentrated urine. The osmolalities and excretion of $K^+$ are given in Table 5. The left ischaemic kidneys showed poor concentrating ability and a reduction in the potassium secretion as compared with the contralateral control kidney. The contralateral kidney therefore increases its excretion accordingly.

Morphology

The morphology of kidneys exposed previously to 45 min of warm renal ischaemia was severely distorted with interstitial oedema, dilatation of the tubules as well as obstruction and cellular infiltration. Similar changes, although milder, were observed after 30 min of ischaemia. The group with the shortest ischaemic time, i.e. 20 min, was more heterogenous, with some kidneys showing a pattern similar to those in the 30 min group.

Table 3. MAP, GFR and RBF in the IR injured left kidney after different time periods of ischaemia

<table>
<thead>
<tr>
<th>Left ischaemic kidney</th>
<th>Urine collection periods (30 min each)</th>
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<tbody>
<tr>
<td></td>
<td>U1</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td></td>
</tr>
<tr>
<td>0 min, Sham</td>
<td>91±1</td>
</tr>
<tr>
<td>20 min</td>
<td>75±2</td>
</tr>
<tr>
<td>30 min</td>
<td>92±6</td>
</tr>
<tr>
<td>GFR (ml/min)</td>
<td></td>
</tr>
<tr>
<td>0 min, Sham</td>
<td>0.9±0.05</td>
</tr>
<tr>
<td>20 min</td>
<td>0.35±0.07a</td>
</tr>
<tr>
<td>30 min</td>
<td>0.15±0.05a</td>
</tr>
<tr>
<td>RBF, (ml/min)</td>
<td></td>
</tr>
<tr>
<td>0 min, Sham</td>
<td>6.2±0.8</td>
</tr>
<tr>
<td>30 min</td>
<td>1.2±0.4a</td>
</tr>
</tbody>
</table>

*Significantly decreased value as compared with Sham operated animals ($P<0.05$).
while others displayed a comparatively well-preserved architecture.

**Histochemical stainings**

The results of the histochemical staining for HA were in accordance with previous findings [7,8]: in the kidneys from healthy animals, HA was located to the papilla, the outer medulla and the adventitia of the vessels. Very little or no staining occurred in the interstitium of the cortex. After warm renal ischaemia and 72 h of reperfusion, HA was also found in the interstitium of the cortex. In the normal kidney, only a few proximal tubuli stained positive for the CD44 antigen, as did some vessels. The ischaemically damaged kidneys displayed strong up-regulation of the CD44 expression with positive staining of both proximal and distal tubuli. Concomitantly with the increased CD44 expression of the tubuli, there was an up-regulation of the MHC class II expression of these structures. Infiltrating macrophages were found all over the kidneys, whereas the infiltrating T lymphocytes showed a more focal distribution and were found mainly in the cortical and outer medullary regions.

**Discussion**

The aims of the present study were to investigate if changes in the intrarenal distribution of HA correlate with kidney function during IR injury and elucidate whether the length of the ischaemic period correlated with the amount of HA. A further aim was to elucidate the expression and intrarenal distribution of the HA synthases Has 1, 2 and 3 in order to give a possible mechanism to the normal heterogenous intrarenal distribution of HA and accumulation during IR injury. In essence, the results show that during IR injury, parameters of renal function are depressed, which coincides with a severe cortical accumulation of HA, interstitial oedema and up-regulation of cortical Has 2 mRNA expression. The longer the period of ischaemia the greater accumulation of HA and depression of renal function. The water binding and pro-inflammatory properties of HA may contribute to renal dysfunction after IR.

The normal kidney shows a heterogenous distribution of HA with excessive amounts in the renal papilla, while almost negligible amounts are found in the renal cortex. An important mechanism explaining this heterogenous distribution can most likely be found in the distribution and expression of Has [9,10], which is responsible for the biosynthesis of HA. In the present study Has 2 and 3 mRNA was highly expressed in the renal medulla and only weakly present in the cortex in the normal kidney. Has 1 mRNA expression could not be detected in any part of the kidney. It thus seems likely that Has 2 and 3 continuously supply the medullary interstitium with HA. Has 2 synthesize HA polymers with larger sizes than Has 3 (typically $10^6$ vs $10^5$ Da, respectively) [11], which may infer different water binding capacity for a given chain. Has 2 also demonstrates a higher HA polymerization rate ($10^5$ vs $10^4$ monosaccharides/min for Has 2 and 3, respectively) emphasizing the importance of Has 2. Furthermore, the almost complete lack of lymph drainage from the papilla as opposed to the renal cortex might contribute to the papillary accumulation and the small amounts found in the cortex. This is due to the fact that tissue HA leaves the interstitial compartment by lymph drainage and is then degraded by the regional lymph nodes before it reaches the general circulation and is taken up by the liver. Finally, HA can be

<table>
<thead>
<tr>
<th>Group</th>
<th>Left kidney</th>
<th>Right kidney</th>
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<tbody>
<tr>
<td></td>
<td>Osmolality (mOsm/kg H₂O)</td>
<td>Osmolality (mOsm/kg H₂O)</td>
</tr>
<tr>
<td></td>
<td>UₖV (µmol/min)</td>
<td>UₖV (µmol/min)</td>
</tr>
<tr>
<td>0 min, Sham</td>
<td>1661 ± 238</td>
<td>1462 ± 337</td>
</tr>
<tr>
<td>20 min</td>
<td>766 ± 143ᵇᵇ</td>
<td>2036 ± 265</td>
</tr>
<tr>
<td>30 min</td>
<td>379 ± 71ᵃᵇᵇ</td>
<td>1400 ± 242</td>
</tr>
</tbody>
</table>

ᵃSignificantly decreased value as compared with the right control kidney ($P<0.05$).
ᵇSignificantly decreased value as compared with sham operated animals ($P<0.05$).

**Table 4.** Arbitrary mean mRNA expression (RT–PCR) of HA synthases Has 1, 2 and 3 where (0) indicates no expression, (+) low expression, (+++) intermediate and (++++) strong expression

<table>
<thead>
<tr>
<th>Cortex control</th>
<th>Cortex ischaemia</th>
<th>Papilla control</th>
<th>Papilla ischaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Has 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Has 2</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Has 3</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
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Control denotes contralateral control kidney, ischaemia represents left kidneys exposed to isothermic 45 min ischaemia and 72 h reperfusion.

**Table 5.** Urinary osmolality and urinary potassium excretion (UₖV) in the left IR injured kidney and the right control kidney
degraded locally in the kidney thereby being involved in the normal turnover of HA. Hyaluronidases are the enzymes responsible for most of the degradation of HA. Several hyaluronidase isoforms exist of which three have been found in the kidney; Hyal 1–3 [12]. The hyaluronidase activity is known to be low in the cortex but increases significantly towards the tip of the papilla.

The heterogeneous distribution of HA is considered to be of importance in renal water handling. This is suggested based on the findings of increased papillary HA content during acutely induced water diuresis (i.v. water loading) and decreased HA content during antidiuresis [3] and also on the knowledge of changes in hyaluronidase excretion with changes in the water balance [13]. After renal IR injury, HA accumulation occurs in the cortex and leads to an increase in water content, probably due to HA’s extreme ability to bind water. HA is a linear, negatively charged polysaccharide that can form a unique network, which gives the system interesting properties, such as high viscosity and resistance to water flow [5]. Physico-chemical studies on HA in vitro have shown that when the concentration of high molecular HA exceeds 0.1 mg/ml, inter- and intra-molecular interactions occur such that the volume occupied by HA is increased, consequently excluding other macromolecules from their molecular environment, and effectively increasing the concentration of the HA. This phenomenon may influence water transport and osmotic activity in the intercellular matrix [4]. The reports by Wang et al. [14] that an i.p. injection of HA decreased peritoneal fluid absorption are in line with the idea that HA decreases water transport. Furthermore, it has been demonstrated that HA diminishes the water permeability of the interstitium [15]. In parallel with the effects on renal physiology, the increased tissue content of HA may also affect the inflammatory response [1].

As to the cellular identity of the HA synthesis, we have demonstrated previously that the fibroblast-like renomedullary interstitial cell (RMIC) produces HA and regulates the HA turnover depending on growth media osmolality [16]. Cortical fibroblasts in culture also produce HA [17] but the production is smaller in the cortical fibroblast as compared with medullary fibroblasts, which can be explained by the results of the present study of low Has mRNA expression in the renal cortex during normal physiological conditions. It is known that cytokines, which are released after renal injury, can stimulate other fibroblasts as well as tubular epithelial cells to increase HA expression [9,18]. Previously, we have demonstrated that it is not the hypoxia per se which triggers RMICs to elevate HA expression [16] and this probably is also true for the cortical fibroblasts. A possible explanation for the severely elevated cortical levels of HA in IR kidneys may thus lie in stimulation of Has 2 activity/expression by cytokines produced during IR injury. The present study demonstrates that Has 2 mRNA is severely up-regulated in the cortex of IR kidneys while Has 3 mRNA remains at low levels not different from control kidneys. The reason for the differences in response to IR injury between Has 2 and 3 mRNA expression is not known but may reside in the sensitivity to inflammatory mediators and growth hormones. In support of such an explanation are studies by Jacobson et al. [19] showing that platelet-derived growth factor BB up-regulates Has 2 whereas the expression of Has 3 remains unaffected. In any case, it is evident that in the cortical accumulation of HA that takes place in IR injury, Has 2 is more important than both Has 3 and 1. The accumulation may also be a consequence of a dysfunctional lymphatic drainage of HA but the consequences are probably larger after organ transplantation where the lymph flow is abrogated. Finally, a reduced normal breakdown of HA during IR by hyaluronidases may also be involved in the accumulation but the importance of such a change cannot be elucidated from the results in the present study.

There was a direct correlation between the amount of cortical HA and the time period of ischaemia and also between the cortical amount of HA and depression of GFR. The design of the present study does not allow us to conclude that the elevated cortical HA is causally linked to the depression of filtration. It is, however, tempting to suggest that the cortical interstitial oedema that follows the accumulation of HA can increase both the vascular and tubular resistance, which at least can contribute to the reduction in both GFR and RBF.

Infiltrating immune cells were found in the cortical areas staining positive for HA in the histochemical analysis. The reason for this co-existence could be 2-fold: (i) HA could promote the homing of inflammatory cells to the injured tissue and (ii) the inflammatory cells release factors (e.g. cytokines and other growth factors) that induce HA synthesis via stimulation of the HA synthases [18]. Furthermore, the expression of CD44 is up-regulated in kidneys subjected to ischaemia. In a recent study by Lewington et al. [8], no CD44 expression was found in the cortex of non-ischaemic kidneys but 1 day after ischaemic injury, mRNA for CD44 was evident. It was primarily demonstrated in the proximal tubules undergoing repair and was found both in the basal and lateral membranes. Several other pathological models, e.g. models of tubulointerstitial nephritis and glomerulonephritis, also show increased cortical tubular CD44 expression (see for example [20]). The reason for this distribution is not clear but might play a role in the regeneration of the tissue.

The IR model used in the present study involves clamping of the ureter. This ischaemia procedure was used in a previous publication [7], which simplified the ability to compare results and to ensure a similar type of kidney damage. It is highly unlikely that the transient obstruction of the ureter in this study would be important for the results obtained primarily because of the complete lack of filtration during the ischaemic event which will not give rise to pressure increments in the pelvic region.

In conclusion, IR injury depresses parameters of renal function which coincides with an elevated cortical HA content, interstitial oedema and Has 2 mRNA expression. Longer time periods of ischaemia cause
greater HA accumulation and a larger reduction in GFR. The water binding and pro-inflammatory properties of HA may contribute to renal dysfunction after IR.

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Conflict of interest statement. None declared.

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