Hypothermic renal perfusion during aortic surgery reduces the presence of lipocalin-2 and preserves renal extraction of dimethylarginines in rats

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Hypothermic renal perfusion during aortic surgery reduces the presence of lipocalin-2 and preserves renal extraction of dimethylarginines in rats. Am J Physiol Renal Physiol 301: F1231–F1241, 2011. First published September 5, 2011; doi:10.1152/ajprenal.00736.2010.—Cold perfusion through the renal arteries during renal ischemia has been suggested to diminish postoperative renal damage after juxtarenal aortic aneurysm repair. As the kidneys play a key role in dimethylarginine metabolism, which in turn is associated with renal hemodynamics, we hypothesized that the protective effect of cold perfusion is associated with a preserved renal extraction of dimethylarginines. Renal ischemia was induced in three groups of anesthetized Wistar rats (n = 7/group), which underwent suprarenal aortic clamping (45 min) with no perfusion (group 1), renal perfusion with 37°C saline (group 2), or renal perfusion with 4°C saline (group 3), respectively, followed by 90 min of renal reperfusion in all groups. The sham group had no clamping. In group 3 (renal ischemia with cold perfusion), postoperative serum creatinine levels as well as the presence of luminal lipocalin-2 and its associated brush-border damage were lower compared with groups 1 and 2 (P < 0.05). Also, renal extraction of asymmetrical (ADMA) and symmetrical (SDMA) dimethylarginine as well as the arginine/ADMA ratio, which defines the bioavailability of nitric oxide, remained intact in group 3 only (P < 0.04). The arginine/ADMA ratio correlated with cortical flow, lipocalin-2, and creatinine rises. Warm and cold renal perfusion (groups 2 and 3) during ischemia were similarly effective in lowering protein nitrosylation levels, renal leukocyte accumulation, neutrophil gelatinase-associated lipocalin (NGAL) expression in distal tubules, and urine NGAL (P < 0.05). These data support the use of cold renal perfusion during renal ischemia in situations where renal ischemia is inevitable, as it reduces tubular damage and preserves renal extraction of dimethylarginines. Renal perfusion with saline per se during renal ischemia is effective in diminishing renal leukocyte accumulation and oxidative stress.

JUXTARENAL ABDOMINAL AORTIC aneurysms (JAA)s originate close to the renal arteries, which in the case of repair necessitates the use of a suprarenal aortic cross-clamp that causes renal ischemia-reperfusion (I/R) injury (8, 24, 37). Postoperative renal failure is a known and feared complication of this type of surgery. The literature reports frequent azotemia as well as a postoperative new onset of dialysis of between 0 and 13% and a mortality rate between 0 and 18% (3, 15, 36, 37), especially when the renal ischemic period exceeds 45 min (15, 44, 45). We have previously reported in two retrospective pilot studies that continuous renal perfusion with a 4°C NaCl solution during the ischemic period in both elective and acute JAA repair could prevent creatinine rises, which resulted in better survival (46, 47). The use of normothermic venous blood during suprarenal clamping has also been suggested to reduce postoperative renal failure (29). Organ perfusion seems to improve the outcome during thoracoabdominal aneurysm repair (12–14, 19). In this study, we wanted to investigate whether perfusion per se has protective effects and whether hypothermia is needed to preserve renal function, which would make it easier for the vascular surgeon if any temperature could be used.

High asymmetrical dimethylarginine (ADMA) levels are known as an independent risk factor for mortality in critically ill patients (26). ADMA is an endogenously inhibitor of nitric oxide synthase (30). Normally, dimethylarginines are eliminated by the kidneys to control ADMA levels (25). An increase in ADMA levels influences systemic hemodynamics and reduces renal flow by inhibiting nitric oxide (NO) synthesis (30). We studied the potential protective effects of renal cooling or perfusion per se during renal ischemia on the preservation of the dimethylarginine metabolism, but we also investigated other parameters such as nitrosylation of renal proteins, leukocyte accumulation, and the microscopic localization of lipocalin-2 and its presence in the urine, which is an early marker of renal damage.

We hypothesized that cold perfusion during renal ischemia leads to less renal damage compared with warm perfusion or no perfusion, as well as to a preserved renal extraction of dimethylarginines and less expression of lipocalin-2 in the early onset of renal I/R injury. The outcome of this study could support the decision of the surgeon to use normothermic or hypothermic renal perfusion in situations when renal ischemia is inevitable.

MATERIALS AND METHODS

Experimental Protocol

All animal surgery and care were performed according to established guidelines and approved by the Animal Ethical Commission, VU University, Amsterdam, The Netherlands. Twenty-eight adult male Wistar rats (350 g; Harlan) were used to form four groups of seven rats (see below).

All rats were anesthetized by fentanyl (0.03 ml) intramuscularly and isoflurane (4.0%). They were then intubated and ventilated (Merlin Small Animal Ventilator, Ventronic services, Devon, UK) with an ~50% oxygen-air mixture, which could be adjusted depending on values found in blood. Anesthesia was maintained with continuous isoflurane 1.5–2.0%. Rats were placed on a heated platform with their body temperature continuously monitored by a rectal probe.
probe. Body temperature was maintained ~36.0°C, also during renal ischemia by adjusting the heated platform and covering the animals with warm blankets. Another temperature probe (type AMA Ad 15 th, Amarell, Kreuzwertheim, Germany) was placed on the kidney surface to measure the temperature at the start and end of the experiment, as well as during renal ischemia. The right jugular vein and left carotid artery were cannulated, heparinized (100 U/kg), and connected to a Ringer lactate pump (2 ml/h) and blood pressure analyzer, respectively. After a midline incision, the intestines were moved aside to free the abdominal aorta. A stabilizing control period of 30 min was introduced in all groups. Clamp times, perfusion times, and type of perfusion fluid were chosen based on earlier patient studies (46, 47).

At the start and at the end of the experiment in all groups, arterial blood samples (0.3 ml) were taken from the carotid artery. The same amount of fluid as a colloid suspension (Voluven, Fresenius Kabi, Hertogenbosch, The Netherlands) was infused after each blood sample. At the end of the experiment, besides arterial blood samples from the carotid artery, venous samples were taken from the renal vein at the entry point into the inferior vena cava, and thereafter the kidneys were excised. Samples from the renal vein were not taken at the beginning of the experiment because of technical difficulties. Blood was centrifuged at 7,000 rpm for 15 min, and thereafter blood plasma was stored at −80°C until analyzed. Kidneys were gathered in liquid nitrogen and also stored at −80°C until analyzed. We measured the temperature and the oxygen saturation of the perfusate with a radiometer (ABL 50 meter, Copenhagen, Denmark) at the start of the perfusion period and after 45 min at the end of the perfusion period.

Renal I/R groups. In group 1 (without perfusion during renal ischemia), we simulated JAA repair in rats. A proximal clamp was placed above the renal arteries on the suprarenal aorta for 45 min (renal ischemia time of 45 min includes the complex aortic repair with renal reconstruction in patients) (46, 47). We also placed a distal clamp on the aortic bifurcation (this is needed in patients for reconstructing the aneurysm without backbleeding). After 45 min of renal ischemia, the suprarenal aortic clamp was replaced on the infrarenal aorta under the renal arteries to start renal reperfusion. After 20 min of infrarenal aortic clamping (mean clinical time to perform the distal anastomosis of the graft in patients) (46, 47), the infrarenal aortic clamp and distal clamp on the aortic bifurcation were removed and reperfusion continued for 70 min, which is in total 90 min of renal reperfusion. All rats in this group received 6 ml saline intravenously during aortic clamping to standardize total volume as in groups 2 and 3, while blood pressures were carefully monitored.

In group 2 (warm perfusion during renal ischemia), the aorta was opened directly after placement of the proximal suprarenal aortic clamp and distal clamp on the aortic bifurcation (see group 1) to insert a small catheter just near the opening of both renal arteries for perfusion of both kidneys with 37°C NaCl (0.9%) during the 45 min of renal ischemia. First, a bolus of 2 ml was given to instantly perfuse the kidneys, and then a perfusion pump (5 ml/h) was connected to the catheter (amount of fluid is chosen in the same proportion as in the patient studies) (46, 47). The pump and catheter were isolated with aluminum and polystyrene to preserve the temperature as well as possible, although at the end of the perfusion period the fluid temperature had dropped to 29°C. After 45 min of renal ischemia, the perfusion catheter was removed and the suprarenal aortic clamp was replaced on the infrarenal aorta under the renal arteries to restore renal blood flow. During these 20 min of infrarenal aortic clamping, the insertion opening of the perfusion catheter in the aorta was restored with 9-0 Ethilon (which is also the mean clinical time to perform the distal anastomosis of the graft in patients) (46, 47). Thereafter, the infrarenal aortic clamp and distal clamp on the aortic bifurcation were removed and 70 min of lower body reperfusion was then started, which is in total 90 min of renal reperfusion.

In group 3 (cold perfusion during renal ischemia), the same operation was performed as in group 2, but during 45 min of renal ischemia the kidneys were perfused with 4°C NaCl (0.9%). At the end of the perfusion period, the fluid temperature had risen to 12°C.

Group without renal I/R injury. In the sham (control) group, the abdomen was opened by midline incision and the aorta with their renal arteries and kidneys were exposed as in the I/R groups, but without aortic clamping. The same measurements were performed and the experiments were of equal duration.

Renal Hemodynamics

Renal artery flow. A flowmeter (Transonic Systems, Ithaca, NY) was secured around the right renal artery to measure renal blood flow (ml/min). Because of spatial limitations, renal artery flow could not be measured during clamping and in the first minutes of early reperfusion.

Renal cortical flux. We measured cortical flow with a laser Doppler probe (periflux 4001 master, Perimed, Jär Fellä, Sweden), which was kept in position during respiratory movements with a latex sheet (Perimed) in the middle of the right kidney. The probe measures the product of velocity and concentration of the moving red blood cells within a small (≤1 mm²) volume (expressed in arbitrary perfusion units; PU).

Measurements in Plasma Samples

ADMA, arginine, symmetrical dimethylarginine, and creatinine. These were measured with HPLC, as described previously (42). Arteriovenous difference in ADMA, symmetrical dimethylarginine (SDMA), and arginine indicated kidney uptake or release and was calculated as [A] − [RV], where [A] and [RV] denote arterial and renal vein plasma concentration, respectively. Fractional extraction was calculated as [A] − [RV]/[A] (25). Arginine divided by ADMA was called the arginine/ADMA ratio, which determines the bioavailability of NO (41). Creatinine clearance (ml/min) was estimated with the calculation [urine creatinine (mg/dl)/plasma creatinine (mg/dl)] × [total volume (ml)/time (min)].

Measurements in Urine Samples

Urine production and creatinine, urea, and sodium excretion. The urine was collected after 90 min of renal reperfusion and expressed in milliliters. The excretion of creatinine, urea, and sodium was measured in collected urine after 90 min of renal reperfusion (mg/90 min).

Quantitative Immunofluorescence Microscopy of Harvested Kidneys

Of the left kidney, 5-µm-thick sections were cut, air dried, and fixed in 4% formaldehyde. Fluorescence staining and image acquisition, processing, and analysis were done as described previously (18). Primary antibodies were dissolved in PBS. After 1 h, they were exposed to Alexa 488-labeled secondary antibody for 30-min (diluted 1:100 in PBS; Molecular Probes, Eugene, OR). Negative control sections were treated the same, but without the primary antibody. For each fluorescent channel, e.g., blue [4,6-diamidino-2-phenylindole (DAPI); i.e., cell nuclei], green (FITC; i.e., secondary antibodies), and red (Cy3; i.e., rhodamine WGA-stained-glycoalyx of cell membranes), four images were made with both a 10× and 40×-objective lens (Carl Zeiss) in the following kidney areas: cortex, corticalmedullary transition zone, loops of Henle, and inner medulla. For quantification of immunofluorescence levels, the sum intensity of all pixels in the selected area was corrected for area size. Correction for nonspecific background was done by dividing the immunofluorescence levels of the positive samples through those of the negative controls. Mean values per rat were used for statistical analysis.

Tubular morphology. Forty round, perpendicularly sectioned tubules were included to measure the tubular lumen. The lumen-to-tubule width ratio was used to define tubular dilatation. We also determined flattening of the brush border with luminal obstruction
with cell fragments. Data are expressed in the percentage of tubules with brush border damage.

Epithelial cell shedding. DAPI-stained nuclei of tubular epithelial cells were counted and corrected for the total tissue surface.

NO-related free radical damage. Nitrosylation of kidney proteins was detected with a rabbit anti-nitrotyrosine antibody (diluted 1:50, A-21285; Invitrogen).

Leukocyte sequestration. The number of CD45-positive cell nuclei (diluted 1:50, OX1, sc-53045; Santa Cruz Biotechnology) were counted and expressed per full image made with the 10×-objective lens (562,798 μm²).

ICAM-1. ICAM-1 antibody (diluted 1:50; GeneTex) was used to detect ICAM-1 in endothelial cells of the medium-to-large arteries through the whole kidney slice.

Neutrophil gelatinase-associated lipocalin or lipocalin-2. Neutrophil gelatinase-associated lipocalin (NGAL) antibody (diluted 1:50, M-145, sc-50351; Santa Cruz Biotechnology) was used to localize NGAL in 40 randomly selected tubules in each kidney section. Besides localization, we also determined the level of expression. NGAL in urine samples was measured at the beginning and end of the experiment with a Rat NGAL ELISA kit (046, BioPorto Diagnostics, Gentofte, Denmark).

Statistical Analysis

The statistical program SPSS 15.0 was used. In case of a normal distribution, one-way ANOVA with a post hoc Bonferroni test was employed to compare four groups and a t-test for 2 groups. In case of a nonnormal distribution, Kruskal-Wallis and Mann-Whitney U-tests were used, respectively. Correlations were tested with Spearman’s rank correlation (Rs). A Wilcoxon test was used for paired data comparisons. Data are expressed as median and box plots (in the figures) or means ± SD. All tests were considered statistically significant at $P \leq 0.05$.

**RESULTS**

**Temperature of Perfusates, Renal Surface, and Body**

We succeeded in cooling the kidneys with perfusion in 4°C saline during renal ischemia to a temperature of the external surface of 30.2 ± 1.1°C, which was significantly different compared with perfusion with 37°C saline (33.0 ± 1.1°C, $P = 0.005$) or no perfusion (32.8 ± 1.2°C; $P = 0.001$). We noticed that at the end of the experiment, the renal surface temperatures of previously cooled kidneys were increased to 30.8 ± 1.5°C, but still significantly lower than in the group with warm perfusion or no perfusion, where the temperature stayed nearly the same as during renal ischemia. Body temperature had dropped from 36.6 ± 0.4 to 35.0 ± 0.8°C at the end of the experiment in all animals, with no significant differences among the groups.

The saturation with oxygen of 4°C NaCl was 95.5%. After 45 min of perfusion, the temperature of the perfusate increased to 13°C with an oxygen saturation of 92.2%. If the perfusate had a starting temperature of 37°C, the oxygen saturation was 70.7%, which was increased to 76.4% after 45 min with a temperature of 23°C.

**Renal Hemodynamics**

After placing of the proximal clamp above the renal arteries on the suprarenal aorta, renal artery flow was reduced to almost 0 ml/min and practically no flow was measured in the cortex in the group without perfusion during renal ischemia. However, if we perfused the kidneys with warm or cold saline, we measured 1.5 times higher cortical flow during renal ischemia ($P = 0.01$ and $P = 0.009$, respectively; Fig. 1). After 45 min of renal ischemia, the suprarenal aortic clamp was replaced under the renal arteries (infrarenal aortic clamping); thus the kidneys were reperfused, while we restored the insertion opening of the

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**Fig. 1.** Renal cortical laser-Doppler flow (PU = perfusion units) in the 4 groups at various time points during the experiment, i.e., sham (white boxes); group 1: 45-min renal ischemia without renal perfusion (checkered boxes); group 2: 45-min renal ischemia with warm perfusion (light dotted boxes); and group 3: 45-min renal ischemia with cold perfusion (hatched boxes). Significant differences ($P < 0.05$) between groups 1 and 2 are shown at the top of the boxes marked with 2 asterisks (***) and between groups 1 and 3 with 1 asterisk (**).
Renal Tubular Damage

**Tubular morphology. Renal ischemia without perfusion.** After 45 min of renal ischemia without perfusion and subsequently 90 min of reperfusion, the proximal tubules showed the most morphological changes. Flattening of the brush border occurred in 28% (range: 13–60) of the proximal tubules, which means that they are significantly damaged, as the sham group did not show flattening of the brush border \((P = 0.001)\). Cell fragments were found in the lumen of 15% of the proximal tubules \((\text{range:} 12.5–30)\) in the group without perfusion and in 7.5% \((\text{range:} 0–12.5)\) in the sham group \((P = 0.003)\), which also indicate significant tubular damage.

**Renal ischemia with warm or cold perfusion.** Cold perfusion during renal ischemia resulted in less tubular damage, as expressed by fewer proximal tubules with obstruction of their lumen with cell fragments (Figs. 2A and 3; \(P = 0.024\)) and two times fewer proximal tubules with flattening of the brush border compared with no perfusion \((P = 0.015)\). Warm perfusion during renal ischemia resulted also in two times fewer proximal tubules with flattening of the brush border compared with no perfusion \((P = 0.038)\); however, it did not lower the presence of cell fragments in the tubular lumen \((P = 0.95; \text{Fig. 2A})\). In all three I/R groups, the lumina of the proximal tubules were about two times more dilated compared with the sham group \((P < 0.04)\), which was not improved by perfusion.

**NGAL. Renal ischemia without perfusion.** In the rats with no perfusion, the distribution of NGAL \((\text{i.e.,} \text{lipocalin-2})\), the early marker for renal damage, was irregular with spots with higher expression \((\text{Fig. 3})\), while there was a more homogeneously distribution of NGAL in the sham group. NGAL was 1.5 times \((\text{range:} 1.3–2.4)\) more expressed after I/R in the corticomedullary tubular epithelial cells in the group without perfusion during renal ischemia compared with sham \((P = 0.004)\), especially in the distal tubule segments mostly in Henle’s loops. Remarkably, NGAL was also found in the lumen of 45% \((\text{range:} 18–80)\) of the proximal tubules in the group without perfusion during renal ischemia (Figs. 2B and 3; \(P = 0.003\)). Luminal presence of NGAL correlated with cell fragments found in the tubular lumen \((R_s = 0.66; P < 0.001)\).

The end/beginning ratio of NGAL in the urine was 6.2 ± 3.6 in the group without renal perfusion during renal ischemia, while it remained at a 1.1 ± 0.6 ratio in the sham group. Urine NGAL was correlated with the expression of NGAL in the distal tubules \((R_s = 0.47; P = 0.01)\) and also with the presence of NGAL in the lumen of the proximal tubules \((R_s = 0.60; P = 0.001)\).

**Renal ischemia with warm or cold perfusion.** The presence of NGAL in the lumen of the proximal tubules was only reduced if the kidneys were perfused with cold saline \(22\%\), range: 12.5–32.5; \(P = 0.034\)) and not with warm perfusion \(57\%\), range: 30–92; Figs. 2B and 3; \(P = 0.44\)). However, expression of NGAL in the distal tubular epithelial cells was reduced to sham levels in both groups with warm or cold perfusion during renal ischemia \((P = 0.021; \text{Fig. 2B and 3})\).
Fig. 3). Also, both groups with warm or cold perfusion during renal ischemia decreased the end/beginning ratio of NGAL in the urine significantly to 2.1 \( \pm \) 0.9 \( (P = 0.009) \) and 1.6 \( \pm \) 0.5 \( (P = 0.004) \), respectively, compared with no perfusion.

**Oxidative Stress**

**Renal ischemia without perfusion.** After 45 min of renal ischemia without perfusion and 90 min of reperfusion, nitrosylation of renal proteins was enhanced in all kidney areas compared with sham \( (P < 0.05; \text{Fig. 4}) \).

**Renal ischemia with warm or cold perfusion.** Both groups with warm or cold perfusion of the kidneys normalized protein nitrosylation levels to the level of the sham group in all renal areas \( (P < 0.05; \text{Fig. 4}) \).

**Renal Leukocyte Accumulation**

**Renal ischemia without perfusion.** Forty-five minutes of renal ischemia without renal perfusion resulted at the end of the experiment in significant accumulation of leukocytes in Henle’s loops and the medulla (Fig. 5), with levels more than twice than those in the sham group \( (P = 0.006) \). ICAM-1 was either greatly present or absent at the luminal side of the endothelium in larger and medium renal arteries throughout the kidney slice. In the sham group, 50% of these vessels (range: 20–60%) presented ICAM-1 and in the group without renal perfusion during ischemia, 80% (range: 50–100%; \( P = 0.02 \)).

**Renal ischemia with warm or cold perfusion.** After both warm and cold renal perfusion during renal ischemia, accumulation of leukocytes at the end of the experiment was absent \( (P < 0.02; \text{Fig. 4}) \). Both groups with warm and cold perfusion
during renal ischemia had equally lower numbers of arteries with ICAM-1 than the group without renal perfusion during renal ischemia (50%, range: 0–80%; \(P < 0.05\)).

Functional Parameters

Creatinine in plasma and creatinine clearance. RENAL ISCHEMIA WITHOUT PERFUSION. The rise in creatinine levels in plasma at the end of the experiment was significantly higher in the group without renal perfusion during renal ischemia (0.46 mg/dl, range: 0.18–0.59) compared with the sham group (0.03 mg/dl, range: −0.09–0.09; \(P = 0.002\); Fig. 6). The median creatinine clearance was 0.23 ml/min (range: 0.12–0.34) in the group without renal perfusion during renal ischemia, which was significantly lower than in the sham group (1.10 ml/min, range: 0.99–1.20; \(P = 0.002\); Table 1).

RENAL ISCHEMIA WITH WARM OR COLD PERFUSION. The lowest rise in creatinine levels in plasma was seen after renal perfusion with cold saline compared with warm saline (0.14 mg/dl, range: 0.04–0.32; \(P = 0.007\); Fig. 6). Creatinine clearance was also best preserved in the group with cold perfusion during renal ischemia (0.54 ml/min, range: 0.33–0.74) compared with no perfusion (\(P = 0.004\); Table 1).

Urine production and creatinine, urea, and sodium excretion after 90 min of reperfusion. RENAL ISCHEMIA WITHOUT PERFUSION. The median total urine production after renal ischemia without perfusion and 90 min reperfusion was 1.6 ml (range: 1.00–2.00; Table 1). Urinary creatinine excretion was significantly lower in the group without renal perfusion after 90 min of reperfusion (0.23 mg, range: 0.14–0.39) compared with the sham group (0.54 mg, range: 0.27–1.64; \(P = 0.009\); Table 1). Urea and sodium excretion were not significantly different between the group without renal perfusion during renal ischemia and the sham group (\(P = 0.95\) and \(P = 0.23\), respectively; Table 1).

RENAL ISCHEMIA WITH WARM OR COLD PERFUSION. Urine production, creatinine, urea, and sodium excretion in the urine after
90-min reperfusion in the group with cold perfusion corresponded most with the values of the sham group (Table 1).

**Handling of dimethylarginines.** At the beginning of the experiment, systemic ADMA, SDMA, and arginine levels in arterial blood in all groups were nearly the same as in the sham group. Samples from the renal vein were only taken at the end of the experiment, not at the beginning because of technical difficulties.

**RENAL ISCHEMIA WITHOUT PERFUSION.** At the end of the experiment, we measured ADMA, SDMA, and arginine levels obtained from the carotid artery and from the renal vein. They were significantly changed in the group without renal perfusion during renal ischemia compared with the sham group ($P < 0.005$). Under normal circumstances, the kidneys extract ADMA and SDMA as seen in the sham group, meaning that the arteriovenous differences and fractional extraction have positive values (Table 2). In the group without renal perfusion during renal ischemia, ADMA and SDMA were not extracted ($P < 0.001$) and release of arginine was more pronounced (Table 2). Also, the arginine/ADMA ratio in the carotid artery and renal vein, which defines the bioavailability of NO, was significantly lower than in the sham group ($P < 0.001$; Fig. 7). The arginine/ADMA ratio correlated with the flow measured in the kidney cortex ($R_s = 0.56; P = 0.002$; Fig. 7), the rise in creatinine levels ($R_s = -0.77; P < 0.001$), luminal NGAL ($R_s = -0.66; P < 0.001$), and urine NGAL ($R_s = -0.67; P < 0.001$).

**DISCUSSION**

In the present study, we showed that cold perfusion of the kidneys during renal ischemia resulted in low presence of NGAL in the proximal tubular lumen, which was homogeneously distributed, and preservation of renal function, as reflected by lower creatinine rises in the early onset of renal I/R injury. In addition, renal extraction of dimethylarginines was maintained, resulting in a higher arginine/ADMA ratio (bioavailability of NO) compared with warm perfusion or no perfusion during renal ischemia. The arginine/ADMA ratio was strongly correlated with renal cortical flow, but also with luminal NGAL, urine NGAL, and rises in creatinine levels. Continuous perfusion of saline per se through the renal arteries during renal ischemia had a beneficial effect on inflammation.

### Table 1. Urine production and excretion during 90 min of reperfusion and creatinine clearance

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Without perfusion</th>
<th>With warm perfusion</th>
<th>With cold perfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urine production, ml/90 min</strong></td>
<td>2.10 (0.95–2.40)</td>
<td>1.60 (1.00–2.00)</td>
<td>1.86 (1.00–3.00)</td>
<td>1.81 (1.40–2.00)</td>
</tr>
<tr>
<td><strong>Excretion in 90 min</strong></td>
<td></td>
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<tr>
<td>Creatinine in urine, mg/90 min</td>
<td>0.54 (0.27–1.64)</td>
<td>0.23 (0.14–0.39)</td>
<td>0.15 (0.06–0.33)</td>
<td>0.34 (0.26–0.45)</td>
</tr>
<tr>
<td>Urea in urine, mg/90 min</td>
<td>1.01 (0.46–1.15)</td>
<td>0.96 (0.54–1.32)</td>
<td>0.94 (0.48–1.62)</td>
<td>0.97 (0.67–1.30)</td>
</tr>
<tr>
<td>Sodium in urine, mg/90 min</td>
<td>7.24 (3.47–8.50)</td>
<td>5.51 (3.54–6.90)</td>
<td>6.51 (3.24–10.90)</td>
<td>6.57 (4.90–7.26)</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>1.10 (0.99–1.20)</td>
<td>0.23 (0.12–0.34)</td>
<td>0.17 (0.09–0.23)</td>
<td>0.54 (0.33–0.74)</td>
</tr>
</tbody>
</table>

Median and range are shown.

### Table 2. Endogenous nitric oxide parameters in all 4 groups

<table>
<thead>
<tr>
<th>Values at the End of the Experiment</th>
<th>Sham</th>
<th>Without Perfusion</th>
<th>With Warm Perfusion</th>
<th>With Cold Perfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADMA, μmol/l</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.782 ± 0.119</td>
<td>1.036 ± 0.158</td>
<td>0.956 ± 0.156</td>
<td>0.929 ± 0.104</td>
</tr>
<tr>
<td>RV</td>
<td>0.491 ± 0.028</td>
<td>1.183 ± 0.157</td>
<td>1.016 ± 0.136</td>
<td>0.922 ± 0.074</td>
</tr>
<tr>
<td>A – RV</td>
<td>0.291 ± 0.119</td>
<td>-0.146 ± 0.163</td>
<td>-0.060 ± 0.121</td>
<td>0.007 ± 0.152</td>
</tr>
<tr>
<td>A – RV/A (RE)</td>
<td>0.36 ± 0.09</td>
<td>-0.16 ± 0.21</td>
<td>-0.07 ± 0.14</td>
<td>0.01 ± 0.15</td>
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<tr>
<td><strong>SDMA, μmol/l</strong></td>
<td></td>
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<tr>
<td>A</td>
<td>0.373 ± 0.041</td>
<td>0.509 ± 0.080</td>
<td>0.435 ± 0.077</td>
<td>0.389 ± 0.099</td>
</tr>
<tr>
<td>RV</td>
<td>0.317 ± 0.024</td>
<td>0.546 ± 0.072</td>
<td>0.466 ± 0.099</td>
<td>0.368 ± 0.107</td>
</tr>
<tr>
<td>A – RV</td>
<td>0.056 ± 0.042</td>
<td>-0.037 ± 0.040</td>
<td>-0.031 ± 0.047</td>
<td>0.022 ± 0.018</td>
</tr>
<tr>
<td>A – RV/A (RE)</td>
<td>0.15 ± 0.09</td>
<td>-0.08 ± 0.08</td>
<td>-0.07 ± 0.12</td>
<td>0.06 ± 0.05</td>
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<tr>
<td><strong>Arginine, μmol/l</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>95.2 ± 12.0</td>
<td>34.8 ± 15.7</td>
<td>65.5 ± 23.7</td>
<td>84.6 ± 18.3</td>
</tr>
<tr>
<td>RV</td>
<td>111.0 ± 17.1</td>
<td>52.7 ± 25.3</td>
<td>71.9 ± 24.5</td>
<td>94.6 ± 28.7</td>
</tr>
<tr>
<td>A – RV</td>
<td>-15.8 ± 13.2</td>
<td>-17.9 ± 12.5</td>
<td>-6.4 ± 17.7</td>
<td>-10.0 ± 20.4</td>
</tr>
<tr>
<td>A – RV/A (RE)</td>
<td>-0.17 ± 0.13</td>
<td>-0.48 ± 0.28</td>
<td>-0.14 ± 0.24</td>
<td>-0.13 ± 0.24</td>
</tr>
</tbody>
</table>

Values are means ± SD in μmol/l. Arterial samples were obtained from the carotid artery (A) and venous samples from the renal vein (RV). ADMA, asymmetrical dimethylarginine; SDMA, symmetrical dimethylarginine. Arteriovenous difference of ADMA, SDMA, and arginine indicated the uptake or release by the kidney and was calculated as [A] – [RV]. RE, renal extraction. Fractional extraction rates for ADMA, SDMA, and arginine were calculated as [-] – [RV]/[A]. Negative fractional extraction indicates generation, and positive fractional extraction indicates renal extraction of ADMA, SDMA, and arginine.
parameters, such as diminished leukocyte infiltration and expression of ICAM-1 in the kidneys, low nitrosylation of renal proteins and less expression of NGAL in the distal tubules and urine. Cortical flow at the end of the experiment recovered to near baseline levels in the groups with renal cold or warm perfusion during renal ischemia. However, although urine output in both latter groups was improved, it was not significant. Besides the beneficial effects of perfusion and cooling, we found a difference in the localization of NGAL after renal I/R injury during aortic repair. Usually, NGAL is homogeneously distributed; however, in the groups with no perfusion or with warm perfusion the distribution is irregular with spots with higher expression. Also, NGAL is mostly expressed in the distal tubule segments mostly in Henle’s loops in the latter groups, and a remarkable result is that NGAL is highly present in the lumen of the proximal tubules in the latter groups. Both latter findings were correlated with urine NGAL. Our examination of the influence of warm or cold perfusion during renal ischemia could provide more insight into some aspects of the pathophysiological mechanisms that are involved in the early onset of renal I/R injury during aortic repair.

If we speculate on the cause, it has been shown before that hypothermia reduces cell metabolism, metabolic activity, and consumption of oxygen and ATP (38). Renal oxygen consumption is reduced to 40% when the renal parenchyma is cooled to 30°C, to 15% at 20°C, and to <5% at 10°C (6, 11, 20, 39, 49).

Fig. 7. The arginine/ADMA ratio, which defines nitric oxide bioavailability, measured in blood obtained from the renal vein in the 4 groups (left) and its correlation with cortical flow at the end of the experiment (right). Left: groups and boxes are defined as in the legend for Fig. 1. Right: groups are defined as in the legend for Fig. 1, but the symbols for groups are as follows: ○, sham; X, group 1; △, group 2; and ▲, group 3. The arginine/ADMA ratio correlated with the flow measured in the cortex of the kidney ($R_s = 0.56; P = 0.002$).
tective effect of perfusion and highlights the additional effect of hypothermia.

In the present study, nitrosylation of renal proteins was equally reduced in both perfusion groups, especially in the medulla, compared with the group without renal perfusion. Earlier reports have shown opposite effects of hypothermia on oxidative stress; however, we showed that warm perfusion could also reduce nitrosylation of renal proteins. Several studies investigated the role of reactive oxygen species (ROS) in reperfusion injury after cold preservation of the kidney in transplantation surgery. Peters et al. (28) suggested that cell injury is caused by ROS formation after cold renal preservation. Other studies have shown a clear protective effect of hypothermia on cells that are exposed to cold storage and showed that they could sustain mitochondrial injury and cell death (34, 35). Another study still demonstrated significant renal damage after 40 min of cold renal preservation via pulsatile perfusion with 4°C of University of Wisconsin solution, and investigators observed increased renal oxidant production assessed by nitrotyrosine immunohistology (33).

Increased expression of lipocalin-2 (NGAL) has been found under various pathophysiological conditions and appears to be a powerful, early marker for renal damage (9). Its expression can be induced by ROS (32), and NGAL may ameliorate renal damage (23). NGAL binds low-molecular-weight ligands such as heme and forms a complex with iron-binding siderophores, which may facilitate the removal of excess intracellular iron, thereby limiting oxidant-mediated apoptosis of renal tubular cells (9). Roulken et al. (31) showed that NGAL may improve cell proliferation and preservation to prevent cold ischemia injury in transplantation surgery. It has been suggested that NGAL protects cells and therefore is upregulated during cold stress, when free iron and free radicals are released (31). Induction of NGAL can be observed in reperfusion injury caused by a sudden increase in ROS (31). Our data reveal that cold perfusion of the kidneys did not result in upregulation of NGAL in the tubular epithelial cells. However, this is an in situ situation (as in JAA repair) which differs from kidney transplantation. Although NGAL was present in the tubular lumen in the cold perfusion group, concentrations were significantly lower compared with the warm perfusion and no perfusion group. It is possible that fewer ROS are formed in the cold perfusion group, and therefore less NGAL is formed in light of a protective role of NGAL. We showed that warm perfusion also prevented high expression of NGAL in the distal tubules, but not the presence of NGAL in the tubular lumen. However, urine NGAL was also lowered after warm perfusion, but mostly after cold perfusion during renal ischemia compared with no perfusion. Kuwabara et al. (16) showed that expression of NGAL in the distal tubule segments is seen after post-acute renal kidney injury. However, they also showed failure of reabsorption of NGAL by the proximal tubule, resulting in altered sparse distribution, which actually is mainly seen after chronic kidney disease. Our results showed both alterations in NGAL expression in the distal tubule segments but also features of chronic kidney failure such as sparse distribution with spots of high expression and luminal obstruction in the proximal tubule. Therefore, postoperative renal failure after JAA shows beginning features of chronic kidney failure, which is prevented by renal cooling during renal ischemia. On the other hand, the changes in NGAL expression and distribution could be transient. Unfortunately, we focused only on early renal damage after 90 min of reperfusion. It would be interesting to investigate the interaction between NGAL and ROS after a longer reperfusion period in a future study. Urine output was not enormously different between the groups, which could be therefore not suitable for early detection of renal damage; since in this early stage intervention is desirable, NGAL might be a better marker.

Leukocytes have been suggested to play an important role in renal I/R injury (10). Ischemic kidneys cause neutrophil retention and activation, which are mediated by oxygen metabolites and ICAM-1. Hypothermia alone has been shown before to significantly reduce the inflammatory process in ischemic mice (4) and less neutrophil infiltration in a longer renal reperfusion period in mice with ischemia at 32°C compared with 37°C (7). We did not find this additional effect of renal hypothermia, because the leukocyte counts were already lowered by renal perfusion regardless of temperature.

High ADMA levels after renal I/R injury has been reported by Li Volti et al. (21). However, we found that cold perfusion during renal ischemia seems to reduce ADMA and SDMA levels compared with warm perfusion or no perfusion during renal ischemia. Cold perfusion during renal ischemia preserved renal function as the kidneys maintain the capacity to extract dimethylarginines. This may have prevented the uncoupling of nitric oxide synthase (NOS), which prevented the production from NO to superoxide (2, 43). It may also be the case that fewer ROS are formed after renal cooling and the enzyme dimethylarginine dimethylaminohydrolase (DDAH) is still capable of eliminating ADMA via breakdown (27). Renal cooling during ischemia is capable of maintaining low ADMA levels, which is important as it has been shown that low arginine plasma levels in combination with high ADMA levels deteriorate systemic hemodynamics (30) and high ADMA levels are associated with intensive care unit mortality (26). It is known that ADMA compromises the integrity of the glomerular filtration barrier by altering the bioavailability of NO (40). In our study, the arginine/ADMA ratio was strongly correlated with the rise in creatinine levels, the presence of NGAL in the tubular lumen, and urine NGAL. Besides ADMA, SDMA levels have also been suggested as an excellent marker for renal function (30). We found a significant increase in SDMA levels after renal I/R injury, which were decreased in the group with renal cold perfusion during renal ischemia.

In summary, the present study shows that cold perfusion during renal ischemia could preserve renal extraction of dimethylarginines, resulting in a high arginine/ADMA ratio, which is highly correlated with cortical flow. Cold perfusion during renal ischemia reduces kidney damage, as reflected by low creatinine rises and low presence of NGAL in urine, which are also correlated with the arginine/ADMA ratios. Continuous perfusion with saline per se through the renal arteries, regardless of warm or cold perfusion, has a protective effect during aortic surgery, which is reflected by reduced leukocyte accumulation in the kidneys and less nitrosylation of renal proteins, with a better recovery of cortical flow at the end of the experiment compared with no perfusion. Besides these results, this study showed that if the kidneys were not cooled, both alterations in NGAL expression in the distal tubule segments but also features of chronic kidney failure of NGAL such as

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altered distribution were present, which were highly correlated with urine NGAL. These data support the use of hypothermic renal perfusion during aortic surgery to further reduce renal damage when renal ischemia is inevitable.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


