Low Arginine Plasma Levels do not Aggravate Renal Blood Flow after Experimental Renal Ischaemia/reperfusion

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Background: ischaemic renal dysfunction is present in many clinical settings, including cardiovascular surgery. Renal hypoperfusion seems to be the most important pathophysiologic mechanism. Arginine plasma levels are rate limiting for NO synthesis, and low arginine plasma levels are seen after major vascular surgery.

Objective: to establish the effects of low arginine plasma levels on renal blood flow after renal ischaemia/reperfusion.

Design: Wistar rats were used in this unilateral renal ischaemia/reperfusion model. After 70 min of ischaemia, the kidney was reperfused for 150 min. Arginase infusion was used to lower arginine plasma levels. Blood flow measurement was performed at the end of the experiment using radiolabelled microspheres. Additional experiments were performed for histopathology.

Results: arginase efficiently decreased arginine plasma levels to about 50% of normal. There was a lower blood flow in the ischaemic kidney than the contralateral (non-ischaemic) kidney. Lowering arginine plasma levels did not reduce renal blood flow in the ischaemic kidney. Renal histopathology was not influenced by lowered arginine plasma levels.

Conclusions: lowering arginine plasma levels did not affect blood flow or histology following renal ischaemia and reperfusion.

Key Words: Arginine; Kidney; Ischaemia; Reperfusion; Blood flow.

Introduction

Ischaemic renal dysfunction occurs in many clinical settings including shock, renal transplantation and cardiovascular surgery. Of particular importance to vascular surgeons is the renal ischaemia occurring during aortic surgery. A decrease in glomerular filtration rate and an increase in renal vascular resistance characterise the acute renal failure (ARF) observed.1,2 The important pathophysiologic mechanism in these situations is considered to be renal hypoperfusion, and various strategies aimed at improving renal blood flow have been investigated.

It is known that vascular endothelium plays an important role in the control of renal function. Nitric oxide (NO) has been shown to play an important role in the regulation of renal blood flow (RBF) under physiological conditions and during acute renal ischaemia.3 NO is a free radical, formed during the conversion of L-arginine to L-citrulline. Oxidation of the terminal guanidino nitrogen of L-arginine occurs under the control of the enzyme nitric oxide synthase (NOS), which exists in three isoforms. The constitutive enzyme is responsible for a continual basal release of NO, although this production can be upregulated. It is membrane bound and possibly linked to the cytoskeleton. This accounts, in part, for the regulatory effect on endothelial NOS of vascular shear stress and flow.

The fundamental role of the L-arginine NO pathway for maintaining the high basal RBF and GFR clearly was shown in dogs and rats in experiments with inhibitors of NOS.4–6 In toxic ARF infusion of L-arginine could exert beneficial effects on renal function,7 whereas inhibiting NOS caused deterioration of kidney function. Also in ischaemic ARF, administration of L-arginine improved renal function.8–12

In the above-mentioned investigations the beneficial effects were achieved by supplementation of L-arginine, resulting in supraphysiological plasma concentrations. However, no studies in ARF have focused on the L-arginine NO pathway in the presence of low plasma levels of arginine, as is seen in situations
associated with endotoxaemia as major trauma and sepsis.13–15

Physiological plasma levels of arginine appear to be limiting for NO synthesis in some cell types16–18 and substrate inadequacy could develop when the kidney, as the major site of endogenous arginine production,19 is severely damaged. Furthermore, it became clear that arginine transport was inadequate to fully sustain NO synthesis at sites such as wounded tissue where extracellular arginine concentrations were very low.20,21 Therefore, this study was designed to establish the effects of low arginine levels on renal blood flow during the initial phase of renal ischaemia/reperfusion.

Methods

Animal model and experimental design

Thirty-six male Wistar rats (Harlan, CPB Zeist, The Netherlands) weighed 275–300 g, and were used for this study. After permission of the institutional animal care and use committee (IACUC), the animals were maintained in accordance with the “Guide for the Care and Use of Laboratory Animals” (DHEW Publication no. National Institutes of Health, 85-23, Revised 1985, Office of Science and Health Reports, DRR/National Institutes of Health, Bethesda, MD, U.S.A.). After admission, rats were allowed to adapt to the laboratory environment for 5 days. Animals had free access to food and water and were subjected to a 12:12 h light/dark cycle. On day 6, rats were randomly assigned to receive (at t = 0 min) an iv infusion of 1.5 mL NaCl 0.9% (SAL, n = 18) or 1.5 mL of an arginase (3000 IU) infusion (ASE, n = 17) over a 20-min period. ASE was obtained from Sigma Aldrich, Zwijndrecht, The Netherlands (E.C. 3.5.3.1, yellow powder, activity 130 IU/mg). This rate of infusion was used in previous studies and resulted in arginine plasma concentrations of about 50% of normal levels.8,22

Rats were anaesthetised using ketamine HCl (50 mg kg⁻¹ i.p.) and placed in the supine position on a heating pad that maintained rectal temperature at 37 °C. The trachea was intubated with a small piece of polyethylene tubing (PE-240, Fisher, Scientific, Springfield, NY, U.S.A.) to facilitate breathing. The right carotid artery and right femoral artery were cannulated using PE-50 tubing. Following these procedures, rats were allowed to stabilise for 30 min before the start (t = 0 min) of ASE or SAL infusion.

At t = 50 min, the abdomen was entered through a midline laparotomy. After heparinisation (heparin 200 U/kg), unilateral ischaemia/reperfusion (I/R kidney) was produced by clamping the renal pedicle for 70 min using a microvascular clamp. The contralateral kidney (CL-kidney) remained in situ, providing a control organ. During this 70-min ischaemic period the abdomen was lightly closed with a suture to prevent undue fluid loss. After declamping, the abdomen was closed and the kidney was reperfused for 150 min.

Haemodynamic measurements and blood sampling

Blood flow measurement was performed at the end of the experiment (t = 270 min). Blood flow was measured using radiolabelled microspheres as previously described.23 This method was chosen for its high reproducibility and enables simultaneous measurement of renal blood flow in both kidneys. The catheters in the right common carotid artery and left femoral artery were connected to P23Db Statham pressure transducers. Pressure wave monitoring was used to place the carotid catheter into the left ventricle. Mean arterial pressure (MAP) and heart rate (HR) were continuously recorded during the experiment. A thermostor was placed in the thoracic aorta via the right femoral artery and cardiac output (CO) was obtained using the thermodilution method (Cardiac Output Computer 9520A, Edwards Laboratories, Santa Ana, CA, U.S.A.). Total peripheral resistance (TPR) was calculated by dividing MAP by CO. Stroke volume (SV) was calculated by dividing CO by HR. Organ vascular resistance was calculated by dividing MAP by organ blood flow (mmHg/mL⁻¹/min/g).

At the end of the experiment (at t = 270 min) an intraventricular injection of 110Ru-labelled microspheres (approximately 1.3 × 10⁵ microspheres dissolved in 0.3 mL saline) was performed. A reference blood sample was obtained from the left femoral artery at a rate of 0.4 mL min⁻¹ over 120 s, starting 5 s before the microsphere injection.

Immediately after the microsphere procedure the abdomen was opened and blood samples were drawn from the abdominal aorta, after which the animal was killed by debleeding. Kidneys were removed and wrapped in tissue paper. The kidneys were weighed and placed in counting vials. Radioactivity was measured in a γ counter (CS 1282, Wallace Compugamma, Turku, Finland). Tissue blood flow was computed according to the “reference organ” technique using the equation F = Fa (Qo/Qa), where Fa is the reference flow, Qo is the count rate in the tissue, and Qa is the count rate in the reference blood sample. Reference blood flow was computed from the weight of blood...
Histopathological examination

From both groups, eight animals were used for histopathological studies instead of measuring blood flow with radioactive labelled microspheres. These animals followed the same surgical protocol as described before. After the rats were sacrificed, the kidneys were taken out and fixed in 10% buffered formaldehyde, dissected and embedded in paraffin. The sections were stained with haematoxylin and eosin and examined using light microscopy. Renal pathology associated with acute tubular necrosis was evaluated at the light microscopic level. All slides were judged in a blinded fashion by the same investigator. Histopathological grades were assigned to each kidney specimen as: 0, normal; 1, mitoses and necrosis of individual cells; 2, necrosis of all cells in adjacent proximal convoluted tubules, with survival of surrounding tubules; 3, necrosis confined to the distal third of the proximal convoluted tubule; and 4, necrosis affecting all three segments of the proximal convoluted tubule. Due to the early point in time on which kidneys were harvested all “clamped” kidneys showed grade 1 damage in the reference syringe and the duration of withdrawal assuming a whole-blood density of 1.055 g/mL. Kidney blood flow was expressed as mL min$^{-1}$ g$^{-1}$.

Blood samples were immediately placed on ice and centrifuged at 3000 rpm for 10 min at 4 °C (Sorvall GLC 2 centrifuge, Sorvall Operations, DuPont, Newton, CT, U.S.A.). For amino acid analysis, the heparinised samples were deproteinised with sulfosalicylic acid (4 mg/100 µL), immediately put in liquid nitrogen, and stored at −70°C before analysis. Amino acid analysis was determined by high-performance liquid chromatography as described previously. Blood urea nitrogen (BUN) and creatinine were determined in arterial plasma samples using an automated analyser (HITACHI 737, Hitachi, Tokyo, Japan). Glucose and lactate concentrations were determined using standard methods.

### Table 1. Histopathological examination.

<table>
<thead>
<tr>
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<th>Saline rats</th>
<th>Arginase rats</th>
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<tbody>
<tr>
<td></td>
<td>Ischaemia/reperfusion-kidney</td>
<td>Contralateral-kidney</td>
</tr>
<tr>
<td>Mitosis</td>
<td>0.58 ± 0.19</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>0.08 ± 0.08*</td>
<td>0.55 ± 0.24</td>
</tr>
<tr>
<td>Nuclear fragmentation</td>
<td>0.5 ± 0.15</td>
<td>0.08 ± 0.08*</td>
</tr>
<tr>
<td>Pyknosis</td>
<td>286 ± 18.3</td>
<td>8.91 ± 1.4*</td>
</tr>
<tr>
<td>Hyperchromasia</td>
<td>95.6 ± 12.2</td>
<td>35.25 ± 8.8*</td>
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* p<0.05 ischaemia/reperfusion vs contralateral kidney in saline rats, † p<0.05 ischaemia/reperfusion vs contralateral kidney in arginase rats.

Statistical analysis was performed using the SPSS 9.0 statistical package. Differences between the groups.
were tested using non-parametric Mann–Whitney $U$-test. Haemodynamics were recorded every 30 min during the experiment and were evaluated using General Linear Model (GLM) for repeated measurements. When appropriate intergroup differences on the individual time-points were further tested using Mann–Whitney $U$-test, whereas Wilcoxon test was used for testing the intragroup differences. Values are expressed as mean ± SEM and $p<0.05$ was considered statistically significant.

**Results**

**Plasma levels of arginine**

Infusion of arginase efficiently reduced plasma levels of arginine. At $t=270\text{ min}$, mean arginine plasma levels in ASE treated animals were $45.3\pm4.9\,\mu\text{mol}\,L^{-1}$ vs. $107.5\pm6.2\,\mu\text{mol}\,L^{-1}$ in SAL treated animals ($p<0.001$).

**Renal blood flow**

Renal blood flow is shown in Figure 1. In I/R-kidneys a significantly lower blood flow was found when compared to CL-kidneys. No statistical differences were found between SAL and ASE rats.

**Renal histopathology**

Histopathological examination of I/R- and CL-kidney was performed in both SAL an ASE treated animals at $t=270\text{ min}$. Histopathology scores are summarised in Table 1. The CL-kidneys showed a normal renal histology and no signs of injury were present. Renal injury was evident in all I/R-kidneys. No differences were found between SAL and ASE treatment for both I/R- and CL-kidneys. Figure 2 shows renal histology of representative experiments; especially pyknosis and nuclear fragmentation is clearly present in I/R-kidneys.

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Fig. 2. Routine (H&E) histology of two representative experiments showing either I/R-kidneys (A for SAL-treatment and B for ASE treatment) and their respective CL-kidneys (C for SAL-treatment and D for ASE treatment). Both A (SAL-I/R) and B (ASE-I/R) show ischaemic damage, such as pyknosis and nuclear fragmentation ("nucl frag."), as indicated by arrows. C (SAL-CL) and D (ASE-CL) show normal renal cortex histology with normal nuclei in epithelial cells, occasionally with nucleoli. Glom = glomerulus. All photographs were taken with 63 ×-oil immersion objective and photographed with a Zeiss Photoaxioscope equipped with mounted camera. Pictures were digitalised using Kodak photoscanner and edited with Power Point.
Table 2. Blood chemistry.

<table>
<thead>
<tr>
<th></th>
<th>Saline rats</th>
<th>Arginase rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (mmol/L)</td>
<td>8.8 ± 0.5</td>
<td>9.0 ± 0.5</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>67.0 ± 2.0</td>
<td>68.9 ± 2.9</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.8 ± 0.5</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>1.9 ± 0.2</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.51 ± 0.1</td>
<td>0.50 ± 0.2</td>
</tr>
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</table>

Blood chemistry

Chemical parameters are shown in Table 2. Blood urea nitrogen and creatinine were elevated at t = 270 min in both groups. No statistical differences were found between SAL and ASE rats.

Haemodynamic parameters

Mean arterial pressure (MAP), cardiac output (CO) and total peripheral resistance (TPR) are shown in Figure 3A, B and C. At t = 30 min (just after ASE infusion), MAP was significantly higher in ASE treated animals. At the other time-points of the experiment, MAP was similar in both groups. During the entire experiment heart rate remained between 360 and 400 beats min and did not differ between the two groups (data not shown). Cardiac output fell after the release of the microvascular clamp in both groups (at 30 min after release of clamp; SAL: 54.4 ± 2.7 and ASE 62.5 ± 4.4 mL min⁻¹ (NS)). Hereafter, CO improved in both groups. During arginase infusion, a slightly higher TPR (not significant) was observed in the ASE rats. In both groups, TPR showed a striking increase after the release of the clamp (at 30 min after release of clamp; SAL: 2.1 ± 0.1 and ASE 1.9 ± 0.1 (NS)) At t = 270, TPR values had returned to baseline values in both groups. TPR was not statistically different between SAL and ASE at all time-points of the study.

Discussion

This study in rats focused on the role of plasma arginine levels with respect to the regulation of renal blood flow after ischaemia/reperfusion of the kidney. Studies in the acute model of renal ischaemia/reperfusion demonstrated a reduction in renal blood flow, which could be improved by administration of arginine. In our model a lower blood flow was found in the clamped kidney when compared to the contralateral kidney. However, administration of arginase, resulting in significant decreased arginine plasma levels, did not further reduce blood flow. Furthermore, lowering arginine plasma levels did not influence the degree of renal injury as measured by histopathological examination.

Arginase infusion at a dose of 3000 IU, as we used in this protocol and in previous studies, resulted in a 50% reduction of arginine plasma levels.⁸⁻¹²,²⁷,²⁸ These levels are comparable to the levels of arginine that are seen in patients after major surgery, sepsis and burns. They therefore represent the stressed patient who is susceptible to renal ischaemia/reperfusion injury. Recently, we confirmed the presence of very low arginine concentrations (40–50 μM) on the first postoperative day in patients undergoing thoraco-abdominal aortic surgery.¹¹ Because renal function is a very important prognostic factor in these patients, we questioned whether low arginine plasma levels could further aggravate renal injury. We designed an acute model of kidney ischaemia-reperfusion in which plasma arginine levels are lowered just before ischaemia/reperfusion. This model seems to be very suitable to mimic the patient who is undergoing major surgery and who is at risk of developing renal insufficiency.²⁷,²⁸ It is important to note this model does not allow any conclusions to be drawn regarding the patient with chronically low arginine concentrations due to malnutrition or metabolic disturbances. In that category of patients, arginine metabolism will be severely disturbed and arginine reserves will be sparse.

From our data, it can be concluded that a 50% decrease of arginine plasma levels did not influence renal blood flow and renal injury in this acute ischaemia/reperfusion model. It could be speculated that NO synthesis is severely impaired following 70 min of ischaemia and 150 min reperfusion, and therefore the levels of arginine were not of any influence. On the other hand, Kin et al. showed that in the kidney NO production is still maintained after similar ischaemic times.²⁷ Also other studies were able to show protective effects of arginine in severe ischaemia/reperfusion models, indication a functioning of the NO system after severe injury.

Recent studies have indicated that specific amino acid transporters on the cell membrane may play an important role in the “arginine paradox”.³⁰ The “arginine paradox” is the observation that endothelial NO production can be regulated by varying the extracellular arginine concentration, despite the fact that the reported intracellular arginine concentrations (0.1–1 mM) greatly exceed the Kₘ of endothelial NOS (eNOS) for arginine (2.9 μM). We did not investigate this specific cellular transporter and therefore cannot rule out effects of ischaemia/reperfusion on arginine
Low Arginine Plasma Levels

Fig. 3. Mean arterial pressure (MAP, Fig. 3A), mean cardiac output (CO, Fig. 3B), and mean total peripheral resistance (TPR, Fig. 3C) ± SEM, in saline (■) and arginase (○) treated rats. * p<0.001 arginase vs. saline.

Transport systems. It could be speculated, however, that there may be a depletion of local arginine around cNOS, initially due to the high production of NO observed after the onset of ischaemia. Arginine depletion may be maintained, under ischaemic conditions, by the inability of ATP-dependent argininosuccinate synthetase to resynthesise arginine from citrulline and aspartate, and by hindered mass transport of ingested arginine to the cells. In this respect, it remains unclear what is the exact mechanism.

Eur J Vasc Endovasc Surg Vol 22, September 2001
of improved NO synthesis, observed after administration of high, supraphysiological doses of arginine (10–20-fold higher than physiological plasma concentrations) following an ischaemic event. However, if concentration gradients over the cellular membrane play an important role in facilitating arginine transport, this offers an attractive explanation for the protective effects of the very high doses of arginine, whereas the plasma arginine concentrations (about 50–110 μM) in our model seem not to be of any influence on NO synthesis in the post-ischaemic kidney.

Histological examination confirmed the harmful effect of ischaemia/reperfusion on the affected kidney, but no effects were seen of lowering arginine concentration. Similarly, no differences were observed between both groups of rats in renal function as assessed by urea and creatinine concentrations.

Haemodynamics showed increased MAP levels just after infusion of arginine, an effect also observed in earlier studies using arginase infusion at the same dosage. In that studies we found that arginine plasma levels were undetectable during the infusion of arginase, and we concluded that the rise in MAP was most likely caused by the inhibition of NO synthesis due to a shortage in circulating arginine as the rate limiting precursor of NO synthesis. From t = 60 min, MAP returned to control values and remained similar in both groups. After the release of the clamp TPR increased in both groups, which could be explained by the release of vasoactive substances such as endothelin, angiotensin II, and thromboxane B2 from the ischaemically injured kidney. The rise in vascular resistance was accompanied by a change in CO, which typically fell after the release of the clamp. At the end of the experiment, TPR and CO levels returned to baseline values. No significant differences were observed between ASE and SAL treated animals.

In conclusion, the results show that lowering arginine plasma levels by arginase, hereby mimicking arginine plasma levels in patients undergoing major surgery and trauma, has no effect on renal blood flow, renal function parameters and haemodynamics in the initial phase after ischaemia/reperfusion of a single kidney.

Acknowledgement

Statistical analysis was performed using the SPSS 9.0 for Windows statistical package (SPSS Inc.1999).

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