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L-Arginine deficiency and supplementation in experimental acute renal failure and in human kidney transplantation

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L-Arginine deficiency and supplementation in experimental acute renal failure and in human kidney transplant recipients.

Background. The "L-arginine paradox" refers to situations where L-arginine (L-Arg) supplementation stimulates nitric oxide (NO) synthesis, despite saturating intracellular concentrations. This paradox is frequently observed in acute renal failure (ARF). First, the effects of L-Arg on renal function of rats with ARF were studied. Based on the promising results from these initial studies, the second part of our study searched for a form of ARF in humans that could be studied easily under conditions with little variance and yet was linked with endothelial dysfunction. Thus, we investigated the effects of L-Arg supplementation immediately after kidney transplantation in 54 patients.

Methods. In uranyl nitrate-induced ARF in rats the effects of L-Arg and L-NNA (inhibitor of nitric oxide synthase; NOS) on glomerular filtration rate (GFR), renal plasma flow (RPF), blood pressure (BP) and NO_x (NO_2^- + NO_3^-) excretion were examined. Tissue L-Arg levels, NOS activities, immunodetection of NOS and superoxide dismutase (SOD), activities of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase, and nitrotyrosine immunoreactive protein (NT-IR) were determined and compared to sham operated animals. Secondly, in a randomized, double-blind study, the effects of L-Arg on GFR and RPF were investigated in 54 kidney transplant recipients, receiving IV L-Arg for three days. GFR and RPF were measured on days 1, 3, 5 and 10 by scintigraphy.

Results. In experimental ARF, decreased RPF and GFR were associated with reduced tissue L-Arg levels, endothelial NOS-III expression, NO formation and NO_x excretion. Reduction in GFR, RPF and NO_x excretion were reversed upon administration of exogenous L-Arg. There also was a loss of Cu,Zn-SOD, a key enzyme against oxidative stress, and an elevation of NT-IR, an indicator of nitrosative stress and suggested marker for pathological actions of NO. However, NT-IR was not dependent on de novo NO synthesis and not related to the functional effects of L-Arg administration. In kidney transplant recipients receiving organs with a short cold ischemia time (CIT) and from young donors, that is, those with a higher likelihood of a functional endothelium, early administration of L-Arg improved renal function.

Conclusion. Both experimental and clinical data show that L-Arg deficiency and endothelial dysfunction are pathomechanistically relevant in ARF. The data suggest a therapeutic potential for the administration of L-Arg in ARF and kidney transplantation, at least in patients receiving kidneys with shorter CIT and from younger donors.

Key words: glomerular filtration rate, renal plasma flow, acute renal failure, peroxynitrite, superoxide, nitric oxide, L-arginine, kidney transplantation.

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In acute renal failure (ARF), mostly resulting from renal cell injury after a toxic or ischemic insult to the kidney, a defect in the L-arginine:nitric oxide (L-Arg:NO) pathway has been proposed to play an important pathogenetic role [1–3]. Administration of exogenous L-Arg to support NO synthesis has been shown to protect the kidney against toxic or ischemic injury [4–10]. However, the molecular mechanism of this effect and its clinical and possible therapeutic relevance are unclear. NO is an important messenger molecule with numerous physiological and pathological functions [11–13]. In the kidney, NO is involved in the homeostatic regulation of glomerular, vascular and tubular functions [3, 14–17]. NO is synthesized from the amino acid L-arginine (L-Arg) by the enzyme NO synthase (NOS). There are three isoforms of NOS, neuronal (NOS-I), inducible (NOS-II) and endothelial (NOS-III) [11, 12], all of which are expressed in the kidney [15].

Numerous experimental data suggest that the availability or biological activity of NO is reduced in ARF [1, 2, 6–8, 17]. In vivo, the activity of the L-Arg:NO pathway depends on a balance between synthesis and inactivation of NO. A deficiency in the synthesis could be due to a number of factors, in particular reduced avail-

ability of the substrate L-Arg, as suggested in hypercholesterolemia and diabetes mellitus [18, 19]. Alternatively, the reduction in biological activity of NO could be due to increased inactivation of NO. In certain disease states, the bioactivity of NO is markedly reduced by its reaction with superoxide anions (O_2^-) yielding peroxynitrite ($ONOO^-$) [20]. $ONOO^-$, unlike NO, is highly reactive and, as an in vivo footprint [20], nitrates protein tyrosine moieties. This nitrosative stress response [21, 22] has been suggested to be the cause of cytotoxicity in a number of toxic, ischemic and inflammatory disease models [20]. However, it is unclear whether $ONOO^-$ is the cause or a consequence of these diseases. Endothelial cells are capable of producing both NO and O_2^-. Important cellular sources of O_2^- are xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [23], and NOS, which at sub-saturating levels of L-Arg also can generate O_2^- [24]. Notwithstanding, cells have evolved antioxidant defense mechanisms against O_2^-, among them superoxide dismutases (SODs) [25, 26].

Although the use of exogenous L-Arg as a substrate for NOS in experimental ARF seems promising, little is known about its adverse potential since excess production of NO has been suggested to be associated with cytotoxicity [17, 27, 28] involving the formation of $ONOO^-$ [20]. However, with respect to the suitability of NT-IR as an in vivo marker for $ONOO^-$, it was demonstrated recently that tyrosine nitration may not necessarily require $ONOO^-$ and de novo NO formation. Indeed, myeloperoxidase (MPO) can catalyze tyrosine nitration from nitrite independently of $ONOO^-$ [29, 30]. To our knowledge, none of the previously mentioned parameters of oxidative and nitrosative stress have been investigated in ARF.

Beyond experimental ARF, in the early phase of kidney transplantation, that is, during organ harvesting, preservation and implantation, ischemia can result in impaired renal function, too. The use of the immunosuppressant, cyclosporine A (CsA), enhances graft survival but this effect is compromised by hypertension and nephrotoxicity, suggesting endothelial dysfunction [31]. Since the previously mentioned effects of L-Arg in experimental ARF are likely to be associated with endothelial dysfunction, we hypothesized that the early administration of L-Arg prior to and immediately after human kidney transplantation could exert equally beneficial effects. This hypothesis is supported by the observation that addition of L-Arg to preservation solutions enhances graft survival [32]. Moreover, a recent animal study showed beneficial effects of L-Arg supplementation on renal function after kidney transplantation [33]. Data from clinical studies with L-Arg in renal transplantation also showed that L-Arg improved renal hemodynamics but only in patients not receiving cyclosporine. These studies, however, are confounded by the small number of patients and were conducted many months after surgery [34, 35].

The aim of the present study was to investigate the basic molecular mechanisms underlying the deficiency of the L-Arg:NO pathway in experimental ARF, and to determine whether L-Arg administration under these conditions can improve renal function. In addition, in a double-blind, randomized clinical study, we correlated these findings to a form of ARF in humans that could be analyzed easily under conditions with little variance and is linked with endothelial dysfunction. Immediately after kidney transplantation, we studied the effects of L-Arg on renal function and tried to identify a possible subset of patients who would benefit from L-Arg treatment.

METHODS

Experimental animals

To examine the role of the L-Arg:NO pathway in ARF, an established model of ARF was used that induces endothelial and tubular damage [36]. Female Sprague-Dawley rats ($N = 21$) weighing 264 ± 7.0 g were anesthetized with thiobutabarbital (Inactin®, 100 mg/kg, IP) and placed on a thermoregulated heating board to maintain body temperature at 37.5°C. Tracheotomy was performed and an endotracheal tube was inserted to allow for spontaneous breathing. The left femoral vein was cannulated with a catheter for infusion of saline (0.9% NaCl) at 1.5 mL/h to replace fluid and to compensate for electrolyte losses. For blood sampling and measurement of arterial blood pressure, the femoral artery was cannulated and attached to a blood pressure transducer, the output of which was recorded on a polygraph. Following suprapubic incision the bladder was cannulated for measurement of urine flow and urine sampling. After surgery, a period of 60 minutes was allowed for equilibration before further experimental intervention. ARF was induced by a single injection of uranyl nitrate (UN; 25 mg/kg). Animals were divided into four experimental groups. Control animals received saline ($N = 5$); ARF animals received uranyl nitrate for induction of ARF either alone (ARF, $N = 4$) or in combination with L-Arg (200 mg/kg/h; ARF+L-Arg, $N = 6$) or N^{ω}-nitro-L-arginine (L-NNA, 240 µg/kg/h; ARF + L-NNA, $N = 6$).

At the beginning of the experiment, mean arterial pressure (MAP) was approximately 120 mm Hg in all groups. For the estimation of GFR, inulin clearance was determined by infusing saline (0.5 mL/60 min) containing 2.5% inulin (Inutest®, Laevosan, Austria). GFR was calculated according to equation 1:

$$GFR = (I_U \cdot V_U)/I_P \qquad \text{(Eq. 1)}$$

where I_U is the concentration of inulin in urine, V_U is the urine volume, and I_P is the concentration of inulin in plasma. For estimation of renal plasma flow (RPF), in-

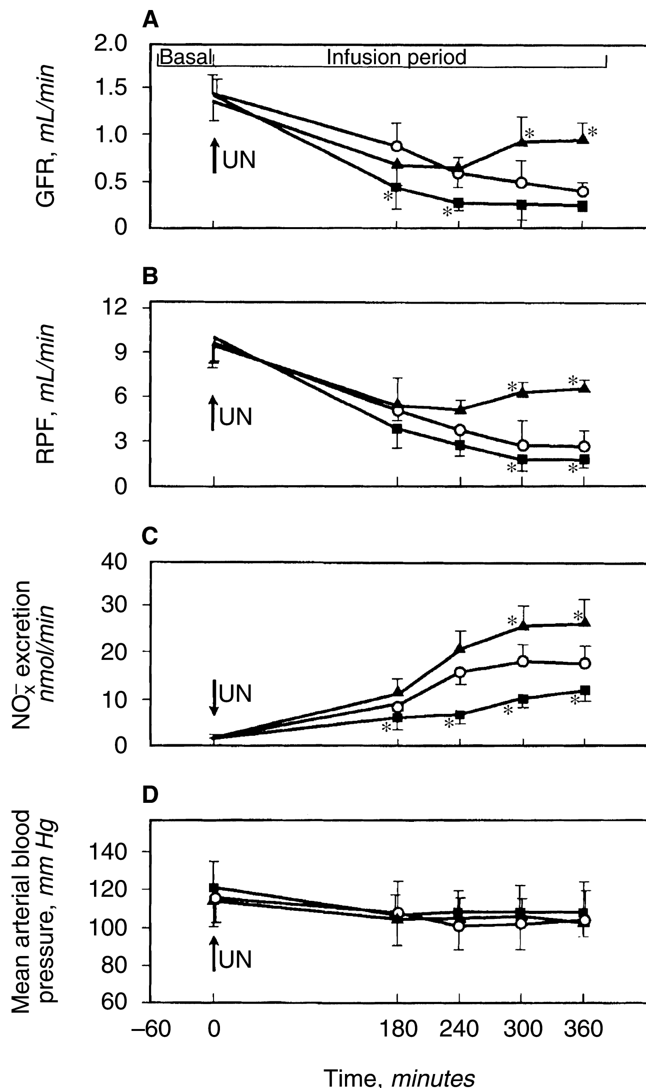

Fig. 1. Renal hemodynamics in experimental acute renal failure (ARF). Data represent mean ± SEM of the glomerular filtration rate (GFR), renal plasma flow (RPF), nitric oxide (NO_x) excretion and mean arterial blood pressure (MAP) in (N = 4 to 6) animals with ARF receiving no additional treatment (○), or receiving either L-Arg (▲) or L-NNA (■). L-Arg or L-NNA was administered at the time of ARF induction (indicated by arrows). Statistical analysis was performed by ANOVA (repeated measures); $^*P < 0.05$ vs. ARF and was accepted as level of significance.

fusion of 1% (wt/vol) sodium *para*-aminohippurate (PAH; Merck, Darmstadt, Germany) was used. RPF was calculated according to equation 2:

$$RPF = (U_{PAH} \cdot V_U)/P_{PAH} \qquad \text{(Eq. 2)}$$

where U_{PAH} is the concentration of PAH in urine and P_{PAH} is the concentration of PAH in plasma. A priming dose of 3 mg inulin and 2 mg PAH was used prior to the infusion of inulin. At the indicated time points (Fig. 1), urine samples for determination of inulin and PAH were collected and a serum sample was taken in the midpoint of each urine collection, to ensure steady state conditions.

Nitrite/nitrate measurement

Aliquots of each plasma or urine sample were diluted as necessary. Nitrate was then converted to nitrite by nitrite reductase and assayed by a modification of the Griess method [37].

Protein immunoblotting

At the end of each experiment, kidneys were removed and snap-frozen in liquid nitrogen. For protein extraction, the frozen tissue was crushed in a liquid nitrogen-cooled stainless steel mortar. The resulting powder was homogenized in buffer A [tetraethylammonium (TEA) 50 mmol/L, ethylenediaminetetraacetic acid (EDTA) 0.5 mmol/L, leupeptin 1 μmol/L, pepstatin A 1 μmol/L, phenylmethylsulfonyl fluoride (PMSF) 200 μmol/L and glutathione sulfhydryl (GSH) 7 mmol/L, pH 7.5] and then centrifuged (30 min, 10,000 × g, 4°C) to remove cell debris. The supernatant fractions (S10) and resuspended pellets were stored at −70°C. Total protein concentration was determined according to Bradford [38]. For protein immunoblotting, samples were solubilized in Laemmli buffer (95°C, 5 min). About 15 μg of proteins were loaded onto 7.5% (for NOS), 12.5% (for SOD) and 10% (for NT-IR) SDS-polyacrylamide gels. After electrophoresis, all proteins were transferred onto nitrocellulose membranes (Hybond ECL; Amersham, Buckinghamshire, UK) with 2.5 mA/cm^2 for 60 minutes in 48 mmol/L Tris buffer (pH 7.5), 39 mmol/L glycine, 0.04% SDS and 10% methanol. Quantitative protein and even transfer in each lane was verified by reversible protein staining of the membranes with 0.1% Ponceau S in 5% acetic acid, and absence of proteins in the remaining gel by Coomassie blue staining. Membranes were first blocked with 3% non-fat dry milk in TBS buffer (10 mmol/L Tris, pH 7.5; 150 mmol/L NaCl). They were then incubated overnight at 4°C using the respective primary antibodies dissolved in 3% non-fat dry milk in TBS. The anti-NOS-I (rabbit; Transduction Labs, Kentucky, USA), anti-NOS-II (rabbit; Transduction Lab, Lexington, KY, USA), anti-NOS-III (rabbit; Calbiochem, San Diego, CA, USA), anti-NT (monoclonal; rabbit; Biozol, New York) [20], and anti-Cu/Zn-SOD (sheep; Calbiochem) were used in dilutions of 1:1000, 2500, 2500, 4000, and 500, respectively. This was followed by repeated washing in TBS and incubation for 60 minutes at room temperature with peroxidase-conjugated anti-rabbit IgG from donkey in a dilution of 1:1000, or peroxidase-conjugated anti-mouse IgG from sheep in a dilution of 1:2000 (both Amersham), or with horseradish peroxidase-conjugated anti-sheep IgG from donkey in a dilution of 1:1000 (Sigma, St. Louis, MO, USA). All antibodies were dissolved in TBS containing 3% non-fat dry milk. Membranes were again washed twice for 15 minutes with TBST buffer (10 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween 20; pH 7.5). Immunoreactive proteins were detected

using an enhanced chemiluminescence kit from Amersham (Braunschweig, Germany). All samples were analyzed in duplicate.

Protein nitrotyrosine dot blot analysis

Proteins were denatured by heating to 95°C for five minutes in 1% sodium dodecyl sulfate (SDS) in the absence of mercaptoethanol. Approximately 25 μg protein was loaded into each well of a Dot Blot template (Bio-Dot™; Biorad, Melville, NY, USA) according to the manufacturer's instructions and allowed to be absorbed onto the membrane at 4°C overnight. Primary anti-NT (monoclonal anti-NT-BSA) were added to each well (dilution 1:4000) and allowed to equilibrate for one hour at 4°C. After repeated washing, peroxidase-conjugated anti-mouse IgG in a dilution of 1:2000 (Amersham) was added to the wells and allowed to equilibrate for one hour at room temperature. All samples were analyzed in triplicate.

Determination of NOS activity

Catalytic activities of NOS in kidney homogenate and supernatant fractions were assayed by the standard conversion of tritiated L-Arg to L-citrulline [37]. Tissue homogenates (50 μL) were incubated under V_{max} conditions, that is, for 15 minutes at 37°C in a total volume of 0.1 mL of 50 mmol/L Tris buffer (pH 7.0) containing 50 nmol/L calmodulin, 250 μmol/L cyclophosphamide, hexamethylmelamine, (3-[(3-cholamidoproyl)dimethylammonio]-l-propane-sulfonate) (CHAPS), 5 μmol/L flavin adenine dinucleotide (FAD), 5 μmol/L flavin mononucleotide (FMN), 1 mmol/L NADPH, 0.7 μmol/L $CaCl_2$, 7 mmol/L GSH, 30 μmol/L L-Arg and 10 μmol/L H_4biopterin. For detection of NOS-II activity, $CaCl_2$ was omitted from the assay buffer and replaced by 0.5 mmol/L EDTA. All samples were analyzed in triplicate.

Superoxide production

NADPH oxidase and xanthine oxidase activities in kidney homogenate were measured at 37°C in a final volume of 1 mL by lucigenin-induced, SOD inhibitable luminescence in 50 mmol/L phosphate buffer (pH 7.0) containing 1 mmol/L EDTA, 150 mmol/L sucrose, 500 μmol/L lucigenin, in the absence or presence of either 1 mmol/L NADPH or xanthine. SOD inhibitors were not included. The reaction was started by the addition of 100 μg of protein (per 100 μL homogenate) to 900 μL of the abovementioned freshly prepared reagent mixture. Luminescence was monitored every 15 seconds for 15 minutes in a luminometer (Lumat LB 9501; Berthold, München, Germany). No activity could be measured in the absence of NADPH, or was beyond detection limit with lucigenin concentrations <500 μmol/L.

Determination of L-Arg levels by HPLC

L-Arginine measurements in S10 fractions of rat kidney were performed by reversed-phase high-pressure liquid chromatography (HPLC) analysis as described previously [39].

Clinical study

In a randomized, double-blind exploratory study, the effects of intravenious L-Arg on GFR and RPF in 54 kidney transplant recipients were investigated. The experimental protocol was approved by the commission for ethics of the Medical University Clinics of Würzburg. Immunosuppressive therapy consisted of cyclosporine A and corticosteroids. Recipients were randomly allocated to the control (saline, $N = 26$) or L-Arg group ($N = 28$). Patients in the L-Arg group received 0.5 g L-Arginine hydrochloride/kg body weight over 60 minutes, starting two hours prior to surgery (renal allograft transplantation). Additionally, L-Arg was infused continuously at a dose of 0.75 g/kg body wt/day for the following three days in the treatment group. Control patients received corresponding volumes of saline. GFR [di-ethylene-triaminpentaacetate (DTPA) clearance] and RPF (iodohippuric acid clearance) were measured on days 1, 3, 5 and 10 after kidney transplantation by scintigraphy. For determination of GFR and RPF, 200 MBq [^{99m}Tc]-DTPA and 2 MBq [^{131}I]-ortho-iodohippuric acid were administered as a bolus injection with subsequent registration of radioactivity after 44 and 170 minutes by single sample algorithms. RPF was calculated by the standard method of Tauxe et al [40], GFR by that of Russell et al [41].

Statistical analysis

All results are expressed as means ± standard error of the mean (SEM). For the animal study, differences between groups were analyzed using analysis of variance (ANOVA, repeated measure). Point by point analyses were performed using the Student *t* test (paired or unpaired). For the clinical study, differences between the groups were analyzed using Mann-Whitney Rank Sum test or Student *t* test as appropriate. A probability value $P < 0.05$ was considered statistically significant.

RESULTS

Renal function in experimental ARF

Uranyl nitrate-induced ARF resulted in a time-dependent reduction of GFR and RPF (Fig. 1). During the course of the experiment, which in total lasted about seven hours, a slight but non-significant decline in mean arterial pressure (MAP) was observed. The values for MAP were not significantly different between animals receiving either L-Arg or the NOS-inhibitor L-NNA. While NOS-inhibitors did not cause an increase in blood pressure in this study, this was probably negated by other mechanisms in ARF such as stimulation of the renin-angiotensin system, enhanced sympathetic activity and elevation of circulating catecholamines. Nevertheless, both com-

Table 1. Tissue L-arginine (L-Arg) levels in control and acute renal failure (ARF) animals with and without L-Arg administration

Treatment		Tissue L-Arg	
	N	*µmol/L*	Changes in % of control
Control	5	24.2 ± 0.4	
ARF	4	18.6 ± 0.8[a]	−23 ± 3.3
ARF + L-Arg	6	37.2 ± 5.7[ab]	+54 ± 24

Results were analysed by the Student *t* test (unpaired).
[a] Significant difference versus control ($P < 0.05$)
[b] Significant different versus ARF animals ($P < 0.05$)

pounds affected intrarenal NO metabolism, since L-Arg increased whereas L-NNA decreased NO_x excretion. Treatment with L-Arg significantly improved GFR and RPF, while treatment with L-NNA produced a further fall in these parameters. D-Arg has been shown to have no effects on these parameters in the same model of ARF (data not shown). Therefore an additional treatment group with D-Arg was avoided to keep number of experimental animals low.

Tissue L-Arg levels and NOS expression in experimental ARF

Since the previously mentioned results demonstrated a deficiency in the L-Arg:NO pathway in ARF, we attempted to elucidate further the molecular mechanism involved by investigating whether tissue L-Arg levels or NOS expression were altered. We observed that the tissue L-Arg levels in ARF animals, as measured by HPLC analysis, were indeed significantly reduced when compared to control (sham-treatment) animals. This effect was reversed by the administration of L-Arg (Table 1). While the percentage of reduction in tissue L-Arg levels appeared to be small, it was nevertheless of functional relevance or restricted to a compartment of the kidney that is essential for NO synthesis as excretory NO_x levels correlated to improvement of renal function with L-Arg (Fig. 1).

In addition, ARF caused a loss in NOS-III immunoreactivity without affecting NOS-I or NOS-II protein expression (Fig. 2). However, the total NOS activity was not significantly altered in ARF. These results suggest that NOS-III contributed only a minor part of total kidney NOS-activity, and that ARF involves a specific compartment of the kidney that is affected by both reduced NOS-III expression and L-Arg levels, presumably the vascular endothelium.

NO, O_2^- and $ONOO^-$

In addition to decreased NO synthesis, a functional defect in the L-Arg:NO pathway also may arise from increased NO breakdown. Since the reaction of NO with O_2^- represents an important mechanism herein, we investigated the enzymatic activities of two important O_2^--generating enzymes, NADPH and xanthine oxidase. NADPH oxidase activity appeared to be decreased, and

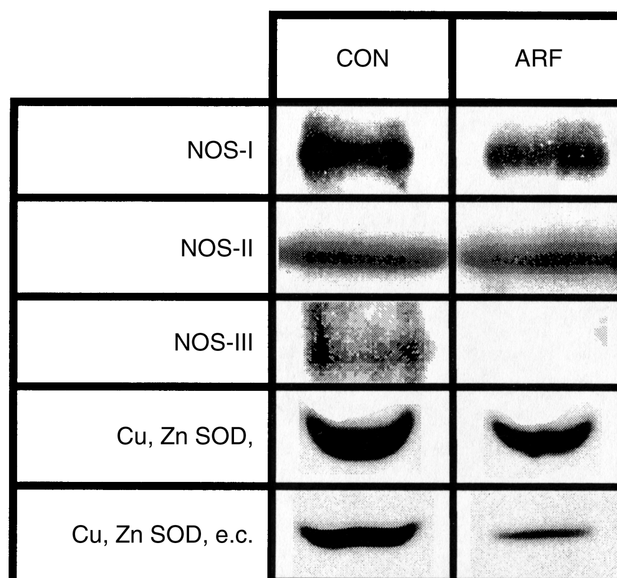

Fig. 2. Immunodetection of nitric oxide synthase (NOS) and superoxide dismutase (SOD) in control and ARF animals. Kidneys were homogenized in buffer A and centrifuged at 10,000 × *g* (S10). Proteins were separated by electrophoresis and transferred onto a nitrocellulose membrane. Membranes were incubated with primary antibodies against NOS-I, NOS-II, NOS-III and Cu,Zn SOD, followed by incubation with the respective peroxidase-conjugated or horseradish-conjugated IgG. Immunoreactive proteins were detected by enhanced chemiluminescence as described in the **Methods** section. Cytosolic Cu,Zn SOD (Cu,Zn SOD, cyt.) and extracellular Cu,Zn SOD (Cu,Zn SOD, e.c.) were detected from the pellet and S10 fractions of kidney samples, respectively. Control and ARF data for each antigen were from the same blot. Data are representative of 4 to 6 animals, each analyzed in duplicate.

xanthine oxidase activity was not different in control and ARF animals (Fig. 3). While these data argued against oxidative stress responses in ARF, NT-IR, an indicator of nitrosative stress and suggested marker for $ONOO^-$ mediated cytotoxicity [20], was increased (Fig. 4B). The consequences of this are unclear, as the protein most affected by $ONOO^-$ was serum albumin, as evidenced by immunoprecipitation analysis (Fig. 4A). Thus, tyrosine nitration may occur predominantly in the extracellular space, presumably at the intravascular lumen. Most importantly, NT-IR was not affected by the administration of L-Arg or L-NNA (Fig. 4B), suggesting that exogenous L-Arg per se did not cause an increase in nitrosative stress and its effects were not mediated by preventing the uncoupling of NO synthase. Thus, possible cytotoxic side effects of an L-Arg treatment through $ONOO^-$ formation are unlikely. Insufficient removal of O_2^-, on the other hand, may have contributed to the observed increase in NT-IR. In accordance with this hypothesis, expression of Cu,Zn-SOD, in particular that of the extracellular type, was significantly decreased in ARF as compared to control animals (Fig. 2). Similar results were obtained for total Mn-SOD-IR; however, due to considerable proteolysis of Mn-SOD these data are difficult to interpret (data not shown).

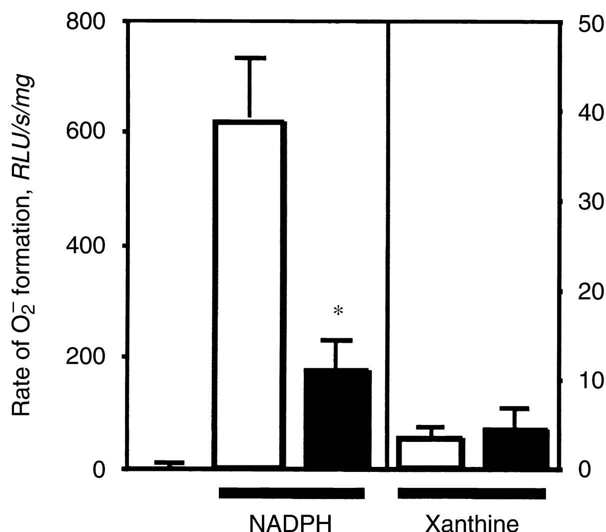

Fig. 3. NADPH oxidase and xanthine oxidase activity in ARF. Rats from control (□) and ARF animals (■) were analyzed for NADPH oxidase and xanthine oxidase activities. Data are mean ± SEM of 4 to 6 experiments, each analyzed in triplicate. There was no O_2^- production in the absence of NADPH.

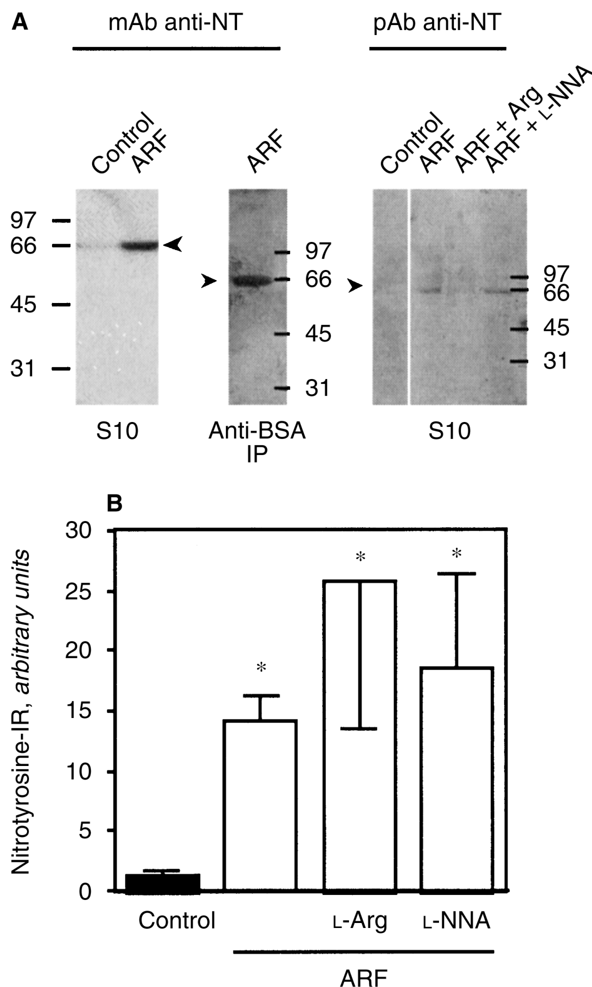

Fig. 4. Nitrotyrosine immunoreactivity (NT-IR) in control and ARF animals. (*A*) NT-IR was detected by immunoblotting using both monoclonal (mAb) and polyclonal (pAb) anti-NT antibodies [11]. In some experiments, protein was extracted by immunoprecipitation with anti-bovine serum albumin (anti-BSA IP) and then analyzed for NT-IR. (*B*) Densitometric quantification of NT-IR from control ($N = 5$) and ARF animals ($N = 4$). Quantification is given in arbitrary absorbance units (AU). S10 refers to the 10,000 × *g* supernatant fractions of total kidney homogenate. Results were analyzed by the Student *t* test (unpaired); $^*P < 0.05$ vs. control animals and was accepted as level of significance.

Clinical data

In a randomized double-blind study, 54 kidney transplant recipients were divided into two groups: one received L-Arg and the other received saline (**Methods** section). The patients' characteristics are described in Table 2. L-Arg was administered two hours prior to surgery and continuously infused for an additional three days. GFR and RPF, monitored by scintigraphy for up to 10 days after surgery, gradually improved during that time in both groups (Table 3). Moreover, GFR and RPF were better improved in the L-Arg treated group as compared to control groups (from day 1 to day 5; however, this did not reach statistical significance). These trends were reversed at day 10 post-surgery (Table 3), that is, seven days after L-Arg treatment was stopped, suggesting that L-Arg indeed improved suppressed renal function in the early phase of kidney transplantation. To analyze further whether a specific subset of patients particularly benefitted from L-Arg treatment, GFR and RPF at day 3 (after day 3 L-Arg infusion was stopped) were correlated against several parameters [NO_x, cGMP, donor sex, disease stage, HLA-mismatches, organ donor age and cold ischemia time (CIT)]. However, only organ donor age and CIT were found to affect the outcome after L-Arg administration (Fig. 5 A–D). Indeed, in patients receiving kidneys with CITs below 20 hours or from donors younger than 45 years of age L-Arg increased RPF when compared to the saline-treated control group (Fig. 5 E, F). Also, GFR on day 1 was increased for patients with CIT less than 20 hours (20 ± 3 vs. 32 ± 5 mL/min, $P < 0.05$), for donors younger than 45 years (20 ± 3 vs. 33 ± 6 mL/min) this increase did not reach statistical significance. These effects were associated with increased plasma NO_x levels at day 1 for the group with donor age <45 years (43 ± 4 vs. 78 ± 22 μmol/L) and with CIT less than 20 hours (43 ± 4 vs. 69 ± 14 μmol/L). Because of the high variance of NO_x values, however, these differences were not significant. There was no correlation between cold ischemia time and donor age in the control ($P = 0.60$; $r^2 = 0.12$) and L-Arg-group ($P = 0.58$; $r^2 = 0.14$).

DISCUSSION

The present study provides several molecular mechanism for the relative deficiency in the L-Arg:NO pathway

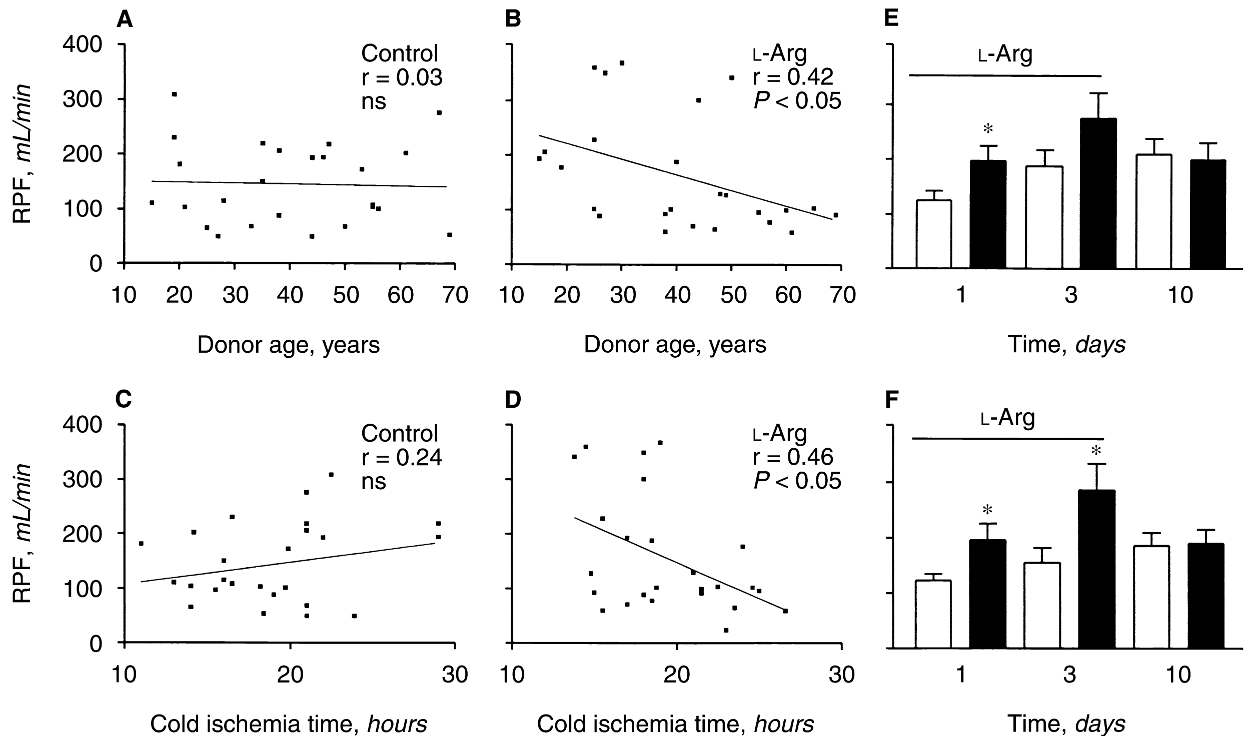

Fig. 5. Effect of L-Arg in renal transplant patients. (*A*, *B*) Linear regression analysis of the relationship between donor age and the effect of L-Arg on renal plasma flow (RPF). (*C*, *D*) Linear regression analysis of the relationship between the duration of cold ischemia time (CIT) and the effect of L-Arg on RPF. There was no significant correlation in the control group (A, C), but there was a significant negative correlation in the L-Arg treatment group (B, D). (*E*) Mean data ± SEM for RPF when including recipients with donor age <45 years in control ($N = 16$) and L-Arg treated groups ($N = 15$). (*F*) Mean data ± SEM for RPF when including recipients with duration of cold ischemia time <20 hours in the control ($N = 19$) and L-Arg treated groups ($N = 16$). Symbols are: (□) control group; (■) L-Arg treated group. L-Arg was infused from day 1 to day 3. Differences between control and L-Arg treatment were analyzed by the unpaired Student *t* test; NS indicates not significant; $*P < 0.05$ compared to control, which was accepted as level of significance.

and endothelial dysfunction in both experimental ARF and in the early stage of kidney transplantation. In experimental ARF, reduced GFR and RPF were associated with a deficiency in tissue L-Arg, reduced NOS-III expression and consequently NO_x excretion. The functional consequences of this were reversed by L-Arg administration and exacerbated by the addition of the NOS inhibitor L-NNA. The respective D-isomers are ineffective in this model of ARF (data not shown). The fall in tissue L-Arg levels is thus of pathomechanistic and therapeutic relevance. This explanation, however, is difficult to reconcile with the fact that L-Arg concentrations within entire tissue exceeds the K_m value for endothelial NOS (5 μmol/L) by far [42]. It is therefore perplexing why the administration of L-Arg augmented the formation of NO and excretion as NO_x, while a reduction in tissue L-Arg by only 23% impaired it. Although plasma levels of L-arginine could give additional information, it has been found that plasma levels can be misleading; NO formation is affected and augmented by supplying arginine despite—theoretically—saturating arginine plasma levels (the so-called "arginine-paradox"). This paradox has been explained by subcellular compartmentalization of the L-Arg pool and the caveolar localized endothelial NOS. Several other mechanisms also have been put forward. Firstly, it has been found that the expression and distribution of cationic amino Y^+ transporter (CAT), which is responsible for approximately 70% of all L-Arg transported into cells, is co-localized with NOS in caveolae [43]. At present, it is unknown whether the expression of CAT is altered in disease states. However, a decrease in CAT activity or expression could alter the rate of L-Arg transport into vascular endothelial cells. This could explain the protective effect of L-Arg administration by improving L-Arg uptake. Secondly, L-Arg administration can overcome the activity of endogenous inhibitors of NOS such as asymmetric dimethyl arginine (ADMA) and methylguanidine, which have been shown to be increased in hypercholesterolemic humans and in patients with end-stage renal failure [44, 45]. Although doubts have been raised about whether the level of ADMA within cells can become high enough to effectively compete with L-Arg for NOS, it was reported recently that dialysis-related hypotension in end-stage renal failure patients was associated with removal of ADMA from the circulation [46]. A third possibility is that L-Arg deficiency

Table 2. Clinical features of randomized kidney transplant recipients ($N = 54$)

Characteristics	Control	L-Arg
Number of patients	26	28
Sex (male = 1, female = 0)	0.65 ± 0.09	0.65 ± 0.09
Age of recipients *years*	49.4 ± 2.33	45.6 ± 1.94
Age of donor *years*	39.5 ± 2.98	40.3 ± 2.83
Body weight *kg*	72.8 ± 2.64	71 ± 2.58
Duration of warm ischemia *min*	41.2 ± 1.82	40.6 ± 1.99
Duration of cold ischemia *h*	19.4 ± 0.99	19.4 ± 0.66
Duration of dialysis *years*	3.3 ± 0.35	4.3 ± 0.52

None of the differences between control group and L-Arg group was significant.

Table 3. Effects of L-Arg on GFR and RPF in kidney transplant recipients

	Treatment	Day 1	Day 3	Day 5	Day 10
GFR *mL/min*	Control	23 ± 2	27 ± 3	31 ± 3	40 ± 4
	L-Arg	26 ± 3	31 ± 4	33 ± 4	38 ± 4
RPF *mL/min*	Control	142 ± 14	191 ± 28	188 ± 22	209 ± 24
	L-Arg	158 ± 21	222 ± 31	219 ± 29	177 ± 22

Mean data showing glomerular filtration rate (GFR) and renal plasma flow (RPF) in kidney transplant recipients from control ($N = 26$) and L-Arg treatment group ($N = 28$). L-Arg was infused from day 1 to day 3. Results were expressed as mean ± SEM. There were no significant differences between the control and L-Arg groups.

caused an uncoupling of NOS, resulting in O_2^- formation instead of NO. This could then affect not only total NO production but also, via metabolism to $ONOO^-$, its bioactivity.

Indeed, our finding that NT-IR was dramatically increased in ARF animals suggests that the bioavailability of NO was altered by scavenging through O_2^-. However, importantly, we found that increased NT-IR was not due to an increase in known prominent cellular sources of O_2^- production (xanthine and NADPH oxidase) and was independent of de novo NO synthesis, arguing against intermediate $ONOO^-$ formation as the underlying mechanism. Conversely, the increase in NT-IR in ARF was most likely due to a lack of antioxidative defense, that is, reduced SOD expression. Clearly, a more proteomic approach including all SODs, catalase, all glutathione reductases and thioredoxin would be desirable, but is beyond of the scope of this investigation. Unequivocal evidence for the functional importance of SODs in mammalian cells has been provided by studies using transgenic mice over-expressing different SOD genes [47, 48]. Transgenic mice carrying human SOD genes were more resistant to toxic oxygen exposure compared to wild-type animals [47, 48]. However, the mechanism by which the expression of SOD, in particular the extracellular type and possibly Mn-SOD, were down-regulated in this model are unknown and await further investigation. Alternatively, the increased NT-IR levels in ARF may be derived from nitrogen sources other than newly synthesized NO, such as NO_2^-. This could be the consequence of either the action of myeloperoxidase, which generates NO_2Cl from Cl^- and H_2O_2 [29], or tissue acidification, which converts NO_2^- back to NO [30].

It should be noted that in a recent study selective inhibition of inducible NOS (by antisense oligodeoxynucleotides) attenuated acute renal failure [49]. The authors suggested that after inhibition of all forms of NOS by non-selective substances, the deleterious consequences of inhibiting eNOS might prevail over the possible benefits of inhibiting inducible NOS. Thus, it cannot be excluded that with non-selective NOS inhibitors different effects might mix with each other.

Our experimental findings may be translated to the clinical situation of impaired kidney function in renal transplant patients co-medicated with CsA. Here, an endothelial dysfunction also appears to play an important pathophysiological role. L-Arg significantly improved renal function in that subset of organ recipients (young donor age and short CIT) with the highest likelihood of functional endothelium, thereby making it possible for L-Arg to augment vascular NO synthesis. This finding is at variance with previous studies showing no effect of L-Arg on renal function in kidney transplant patients on CsA treatment. However, these studies were conducted many months after kidney transplantation [34, 35]. Moreover, the number of patients engaged in these studies was too small (that is, between 6 and 10 patients per group) to perform subgroup analyses, possibly obliterating the protective effects of L-Arg. Donor age has been excluded as an important parameter of successful kidney transplantation [50], while the role of CIT is controversial [51, 52]. However, in our exploratory study, in the presence of sufficient L-Arg RPF improved both in patients with shorter CIT and with organs from young donors. Also, GFR improved, however, statistical significance was reached only in patients with a CIT less than 20 hours. This may be of clinical relevance and—in context with the experimental results from ARF—calls for a critical evaluation of the effects of L-Arg on impaired renal function in kidney transplant recipients and ARF in general.

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APPENDIX

Abbreviations used in this study are: ADMA, asymmetric $N^{\omega,\omega}$-dimethyl-L-arginine; ARF, acute renal failure; L-Arg, L-arginine; CAT, cationic amino acid transporter; CIT, cold ischemia time; CsA, cyclosporine A; GFR, glomerular filtration rate; MPO, myeloperoxidase; L-NNA, N^{ω}-nitro-L-arginine; NO, nitric oxide (nitrogen monoxide); NOS, nitric oxide synthase; NT-IR, 3-nitrotyrosine immunoreactivity; O_2^-, superoxide; $ONOO^-$, peroxynitrite; RPF, renal plasma flow; SOD, superoxide dismutase.

REFERENCES

1. Conger JD: NO in acute renal failure, in *Nitric Oxide and The Kidney*, edited by Goligorsky MS, Gross SS, New York, Chapman & Hall, 1997, pp 332–348
2. Schramm L, Heidbreder E, Zimmermann J, *et al*: Acute renal failure: Influence of nitric oxide on renal function. *J Nephrol* 9: 118–125, 1996
3. Goligorsky MS, Noiri E: Duality of nitric oxide in acute renal injury. *Semin Nephrol* 19:263–271, 1999
4. Chintala M, Chiu P, Vemulapalli S, *et al*: Inhibition of endothelial derived relaxing factor (EDRF) aggravates ischemic acute renal failure in anesthetized rats. *Naunyn Schmiedebergs Arch Pharmacol* 348:305–310, 1993
5. Schramm L, Heidbreder E, Lopau K, *et al*: Toxic acute renal failure in the rat: Effects of L-arginine and N-methyl-L-arginine on renal function. *Nephrol Dial Transplant* 9:88–93, 1994
6. Lopau K, Kleinert D, Erler J, *et al*: Tacrolimus in acute renal failure: Does L-arginine infusion prevent changes in renal hemodynamics? *Transplant Int* 13:436–442, 2000
7. Valdivielso JM, Lopez-Novoa JM, Eleno N, *et al*: Role of glomerular nitric oxide in glycerol-induced acute renal failure. *Can J Physiol Pharmacol* 78:476–482, 2000
8. Can C, Sen S, Boztok N, *et al*: Protective effect of oral L-arginine on gentamycin-induced renal failure in rats. *Eur J Pharmacol* 390: 327–334, 2000
9. Jerkic M, Varagic J, Jevovic D, *et al*: L-arginine reduces tubular cell injury in acute post-ischemic failure. *Nephrol Dial Transplant* 14:1398–1407, 1999
10. Schramm L, Heidbreder E, Kartenbender K, *et al*: Role of L-arginine-derived NO in ischemic acute renal failure in the rat. *Renal Fail* 16:555–569, 1994
11. Schmidt HHHW, Walter U: NO at work. *Cell* 78:919–925, 1994
12. Knowles RG, Moncada S: Nitric oxide synthesis in mammals. *Biochem J* 298, 249–258, 1994
13. Klahr S: Role of arginine in health and in renal disease. *Am J Physiol* 267:F331–F346, 1994
14. Kone BC, Baylis C: Biosynthesis and homeostatic roles of nitric oxide in the normal kidney. *Am J Physiol* 272:F561–F578, 1997
15. Bachmann S: Distribution of NOSs in the kidney, in *Nitric Oxide and The Kidney*, edited by Golisgorsky MS, Gross SS, New York, Chapman & Hall, 1997, pp 133–157
16. Yaqoob B, Edelstein CL, Schrier RW: Role of nitric oxide and superoxide balance in hypoxia-reoxygenation proximal tubular injury. *Nephrol Dial Transplant* 11:1743–1746, 1996
17. Tome LA, Yu L, de Castro I, *et al*: Beneficial and harmful effects of L-arginine on renal ischaemia. *Nephrol Dial Transplant* 14:1139–1145, 1999
18. Pieper GM, Dondlinger LA: Plasma and vascular tissue arginine are decreased in diabetes: Acute arginine supplementation restores endothelium-dependent relaxation by augmenting cGMP production. *J Pharm Exp Ther* 283:684–691, 1997
19. Drexler H, Zeiher AM, Meinzer K, *et al*: Correction of endothelial dysfunction in coronary microcirculation of hypercholesteolemic patients by L-arginine. *Lancet* 338:1546–1550, 1991
20. Beckman JS, Koppenol WH: Nitric oxide, superoxide and peroxynitrite: The good, the bad and the ugly. *Am J Physiol* 271:C1424–C1437, 1996
21. Wink DA, Cook JA, Kim SY, *et al*: Superoxide modulates the oxidation and nitrosation of thiols by nitric oxide-derived reactive intermediates. Chemical aspects involved in the balance between oxidative and nitrosative stress. *J Biol Chem* 272:11147–11151, 1997
22. Hausladen A, Privalle C-T, Keng T, *et al*: Nitrosative stress: Activation of the transcription factor OxyR. *Cell* 86:719–729, 1996
23. Wolin MS: Reactive oxygen species and vascular signal transduction mechanisms. *Microcirculation* 3:1–17, 1996
24. Xia Y, Tsai A, Berka V, *et al*: Superoxide generation from endothelial nitric oxide synthase. *J Bio Chem* 273:25804–25806, 1998
25. Fridovich I: Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 64:97–112, 1995
26. Ichikawa I, Kiyama S, Yoshioka T: Renal antioxidant enzymes: Their regulation and function. *Kidney Int* 45:1–9, 1994
27. Dawson VL, Dawson TM, London ED, *et al*: Nitric oxide mediates neurotoxicity in primary cortical cultures. *Proc Natl Acad Sci USA* 88:6368–6371, 1991
28. Millar CGM, Thiemermann C: NO in septic shock, in *Nitric Oxide and The Kidney*, edited by Goligorsky MS, Gross SS, New York, Chapman & Hall, 1997, pp 271–306
29. Eiserich JP, Hristova M, Cross CE, *et al*: Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391:393–397, 1998
30. Zweier JL, Wang P, Samouilov A, *et al*: Enzyme-independent formation of nitric oxide in biological tissues. *Nat Med* 1:804–809, 1995
31. Saunder A, Danielewicz R, Ametani M, *et al*: L-arginine in 5 days perfusion of canine kidneys. *Transplant Proc* 25:3004–3005, 1993
32. Kopp JB, Klotman PE: Cellular and molecular mechanisms of cyclosporine nephrotoxicity. *J Am Soc Nephrol* 1:162–179, 1990
33. Vos IHC, Rabelink TJ, Dorland B, *et al*: L-Arginine supplementation improves function and reduces inflammation in renal allografts. *J Am Soc Nephrol* 12:361–367, 2001
34. Vigano E, Badalamenti S, Paone G, *et al*: Renal effects of L-arginine infusion in cyclosporine-treated transplant recipients. *Transplant Proc* 26:2622–2623, 1994
35. Gaston RS, Schlessinger SD, Sanders PW, *et al*: Cyclosporine inhibits the renal response to L-arginine in human kidney transplant recipients. *J Am Soc Nephrol* 5:1426–1433, 1995
36. Avasthi PS, Evan AP, Hay D: Glomerular endothelial cells in uranyl nitrate-induced acute renal failure in rats. *J Clin Invest* 65: 121–127, 1980
37. Schmidt HHHW, Pollock JS, Nakane M, *et al*: Purification of a soluble isoform of guanylyl cyclys-activating factor synthase. *Proc Natl Acad Sci USA* 88:365–369, 1991
38. Bradford MM: A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
39. Meyer J, Richter N, Hecker M: High-performance liquid chromatography determination of nitric oxide synthase-related arginine derivates in vitro and in vivo. *Anal Biochem* 247:11–16, 1997
40. Tauxe W, Dubovsky E, Kidd T, *et al*: New formulas for the calculation effective renal plasma flow. *Eur J Nucl Med* 7:51–54, 1982
41. Russell C, Bischoff P, Kontzen F, *et al*: Measurement of glomerular filtration rate: Single injection plasma clearance method without urine collection. *J Nucl Med* 26:1243–1247, 1985
42. Arnal JF, Münzel T, Venema RC, *et al*: Interactions between L-arginine and glutamate chande endothelial NO production: An effect independent of NO synthase substrate availability. *J Clin Invest* 95, 2563–2572, 1995
43. McDonald KK, Zharikov S, Block ER, *et al*: A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox." *J Biol Chem* 272:31213–31216, 1997
44. Vallance P, Leone A, Calver A, *et al*: Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* 339:572–572, 1992
45. Boger RH, Bode-Boger SM, Kienke S, *et al*: Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. *Circulation* 95:2068–2074, 1997
46. Hand MF, Haynes WG, Webb DJ: Hemodialysis and L-arginine, but not D-arginine, correct renal failure-associated endothelial dysfunction. *Kidney Int* 53:1068–1077, 1998

47. Wang P, Chen H, Qin H, *et al*: Overexpression of human copper, zinc-superoxide dismutase (SOD1) prevents postischemic injury. *Proc Natl Acad Sci USA* 95:4556–4560, 1998
48. Kiouchi H, Epstein CJ, Mizui T, *et al*: Attenuation of focal cerebral ischemic injury in transgenic mice overexpressing Cu/Zn SOD. *Proc Natl Acad Sci USA* 88:11158–11162, 1991
49. Noiri E, Peresleni T, Miller F, Goligorsky MS: In vivo targeting of inducible NO synthase with oligodeoxynucleotides protects rat kidney against ischemia. *J Clin Invest* 97:2377–2383, 1996
50. Kumar A, Kumar RV, Scrinadh, *et al*: Should elderly donors be accepted in a live related renal transplant program? *Clin Transplant* 8:523–526, 1994
51. Koning OH, Ploeg RJ, van Block JH, *et al*: Risk factor for delayed graft function in cadaveric kidney transplantation: A prospective study of renal function and graft survival after preservation with University of Wisconsin solution in multi-organ donors. *Transplant* 63:1620–1628, 1997
52. Jordan ML, Aprile MA, Cardella CJ: The role of cold ischemia in a provincial organ-sharing program in the cyclosporine era. *Transplant* 49:367–373, 1990