Kidney International, Vol. 62 (2002), pp. 1700–1706

L-Arginine transport is augmented through up-regulation of tubular CAT-2 mRNA in ischemic acute renal failure in rats

IDIT F. SCHWARTZ, DORON SCHWARTZ, MARINA TRASKONOV, TAMARA CHERNICHOVSKY, YORAM WOLLMAN, EHUD GNESSIN, IAN TOPILSKY, YORAM LEVO, and ADRIAN IAINA

The Tel Aviv Sourasky Medical Center, Tel Aviv, Israel

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Background. Ischemic acute renal failure (iARF) is associated with increased nitric oxide (NO) production during the reperfusion period, as endothelial nitric oxide synthase (eNOS) is maximally activated, and renal tubular inducible NOS (iNOS) is stimulated. Increased NO production leads to augmented tubular injury, probably through the formation of peroxynitrite. L-Arginine (L-Arg), the only precursor for NO, is transported into cells by cationic amino acid transporters, CAT-1 and CAT-2. We hypothesized that the increased NO production observed in iARF may result from increased L-Arg uptake, which would be reflected in the augmented expression of L-Arg transporter(s).

Methods. Ischemic acute renal failure was induced in rats by right nephrectomy + left renal artery clamping for 60 minutes. L-Arg uptake was examined in freshly harvested glomeruli and tubuli from control, sham operated, and animals subjected to 15, 30, and 60 minutes, and 24 hours of reperfusion, following 60 minutes of ischemia. Using RT-PCR, renal tissues were examined further for the expression of iNOS, CAT-1, CAT-2, arginase I and arginase II.

Results. Tubular expression of iNOS mRNA was initiated by ischemia, continued to increase after 60 minutes of reperfusion, and decreased after 24 hours. L-Arg transport into glomeruli was similar in all experimental groups. L-Arg uptake into tubuli was markedly augmented following the 60-minute reperfusion, while it moderately increased after 24 hours of reperfusion. This was accompanied by a parallel, preferential increase in tubular CAT-2 mRNA expression at 60 minutes of reperfusion. CAT-1 mRNA expression was unchanged, as detected by RT-PCR. In addition, the expression of arginase II and arginase I mRNA was attenuated by 30 minutes and one hour of reperfusion, and returned to baseline values after 24 hours of reperfusion.

Conclusions. Ischemic ARF is associated with augmented tubular CAT-2 mRNA expression, which leads to enhanced L-Arg transport and increased NO production. This may contribute to the renal injury exhibited in iARF.

Key words: arginine transport, cationic amino acid transporter, ischemia/reperfusion injury, vasoconstriction, nitric oxide.

Received for publication August 15, 2001
and in revised form April 17, 2002
Accepted for publication May 28, 2002

Ischemia/reperfusion (I/R) is a leading cause of damage to vital organs in diverse clinical circumstances. Several mechanisms have been proposed to explain it, including anoxia, release of oxygen-derived radicals during reperfusion, neutrophil accumulation, and release of lytic enzymes [1, 2]. A general feature of ischemic injury is an augmented sensitivity to vasoconstrictor stimuli, which in turn increase the vulnerability of the affected organ to additional ischemic insult. In recent years, considerable information has been gathered on the role of L-arginine metabolite, nitric oxide (NO) in circulatory regulation. A commonly held explanation for the increased constrictor agonist sensitivity of the vasculature has been an ischemia-induced attenuation of vascular NO generation by endothelial nitric oxide synthase (eNOS). This was supported by intact response to endothelium-independent vasodilators but blunted response to endothelium-dependent vasodilators, such as acetylcholine, bradykinin, serotonin, and histamine following ischemic or hypoxic injury [3–6]. In the kidney, NO causes relaxation of preglomerular arteries, improving renal blood flow and oxygenation [7, 8]. Interestingly, in post-ischemic acute renal failure (ARF), Conger et al demonstrated that eNOS activity, rather than being reduced is maximal and, therefore, could not be further stimulated by endothelium-dependent dilators [9]. Although NO has been shown to be beneficial in the vasculature by attenuating leukocyte-endothelial cell adhesion during I/R injury, it may play a damaging role in renal tubular epithelium by decreasing tubular cell adhesion, contributing to tubular obstruction [10]. (A similar effect has been observed in mesangial cell–matrix adhesion [11]). We have previously reported increased NO metabolites in urine and plasma in ischemic (iARF) as opposed to nephrotoxic ARF [12–14]. By using a model of hypoxia/reoxygenation in freshly isolated tubules, Yu et al demonstrated an augmented production of NO by tubular epithelial inducible NOS (iNOS), while its inhibition protected against tubular injury [15]. Furthermore, Persleni et al reported that utilizing antisense oligodeoxynucleotides to iNOS rescued tu-

bular epithelial cells from oxidative stress injury [16], and it has been hypothesized that the cytotoxic sequelae of NO production depends on the redox state of the cell and its ability to generate peroxynitrite anion ($ONOO^-$) [17]. The activity of iNOS appears to be regulated at the transcriptional level, but is strictly dependent on the presence of extracellular arginine. Arginine availability determines the cellular rate of NO production [18, 19]. In the kidney, L-arginine transport is mediated by the y+ system carrier [20]. Several transporters have been identified, and two transporters, termed cationic amino acid transporter-1 and -2 (CAT-1 and CAT-2), exhibiting the y+ system properties have been cloned [21–23]. CAT-1 is ubiquitously expressed and appears to have a greater capacity than CAT-2 for *trans*-stimulation [24]. CAT-2B has been detected in activated murine macrophages and lymphocytes. CAT-2A, an alternately spliced transcript of CAT-2, encodes a kinetically distinct low affinity transporter, and is expressed exclusively in liver of adult rodents [25]. Alternatively, L-arginine can be metabolized to ornithine and urea by arginase (types I and II), thus limiting its utilization by NOS [26], and activated iNOS, by liberating N^G-OH-L-arginine can exert inhibitory effects on arginase [27]. The present knowledge about the regulation of arginase in renal I/R is rather fragmentary. In the present study, we aimed to confirm that iARF injury is accompanied by altered arginine transport and expression of CAT mRNA and activity. We also characterized the effects of ischemia/reperfusion on the expression of iNOS and arginase, as intracellular L-arginine availability is critical to determine the extent of renal injury.

METHODS

Ischemic ARF model

All animal experiments were conducted in accord with the protocol approved by the institutional committee on ethics in animal experiments. Studies were performed using male Wistar rats weighing 200 to 250 g. Ischemic acute renal failure was induced under general anesthesia with Ketalar 8 mg/100 g body weight (BW), and Xylazine, 0.25 mg/100 g BW, intraperitoneally. Immediately after right nephrectomy, the left renal artery was clamped for 60 minutes, and the rats were allocated to the different experimental groups:

Group 1: Control, untreated rats ($N = 5$)
Group 2: Sham, right nephrectomy without ischemia ($N = 5$)
Group 3: Ischemia (I) 60 minutes with no reperfusion (R)
Group 4: I/R 15 minutes, I 60 minutes and R 15 minutes ($N = 5$)
Group 5: I/R 30 minutes ($N = 5$)
Group 6: I/R 60 minutes ($N = 6$)
Group 7: I/R 24 hours ($N = 6$)

Creatinine clearance was measured in groups 2 and 7 ($N = 6$) as previously described [12].

Isolation of glomeruli and tubuli

At each experimental condition studied, kidneys were harvested, decapsulated and bisected. The cortex was carefully dissected free. Glomeruli and tubuli were isolated using a sieving technique. Cortices were minced to a fine paste with a razor blade and gently pressed through a 106-μm stainless steel sieve. The resulting material was suspended in HEPES buffer (KCl 5 mmol/L, $CaCl_2$ 0.9 mmol/L, $MgCl_2$ 1 mmol/L, D-glucose 5.6 mmol/L, HEPES 25 mmol/L, NaCl 140 mmol/L), at 4°C, pH 7.4. The suspension was forced through a 20 gauge needle to decapsulate the glomeruli and then passed through a 75 μm sieve. Glomeruli trapped on the sieve were washed and pelleted by centrifugation at 1000 rpm for one minute, three times. This fraction consisted of more than 95% glomeruli, the majority of which were decapsulated, while the fraction that was washed through the sieve consisted primarily of tubuli. The freshly isolated glomeruli and tubuli were further used for RNA extraction and for arginine uptake measurements

L-Arginine uptake

Arginine uptake was determined essentially as described by Gazolla et al [28]. Glomerular and tubular suspensions from the various experimental groups were incubated and shaken for 10 minutes in HEPES buffer at 37°C, pH 7.4.

[^{3}H] L-arginine and L-arginine, in a final concentration of 1 mmol/L, were added to a total volume of 1 mL for additional four minutes. The duration of four minutes was chosen because our preliminary studies demonstrated that transport of 1 mmol/L of [^{3}H] L-Arg by both tubuli and glomeruli increased over time, and was linear at approximately four minutes (data not shown). Transport activity was terminated by a rapid aspiration of medium and washing the cells with ice-cold phosphate-buffered saline (PBS; 4 times, 2 mL/tube). The glomeruli/tubuli were then dried and solubilized by 1 mL of 0.5% sodium dodecyl sulfate (SDS) in 0.5 N NaOH. Seven hundred microliters of the extract were used to monitor radioactivity, using liquid scintillation spectrometry (Betamatic; Kontron, Munich, Germany), and the remaining 300 μL for protein content determination, by Lowry method. To correct for non-specific uptake or cell membrane binding, glomeruli were incubated with 10 mmol/L unlabeled arginine in HEPES buffer, and the associated radioactivity was subtracted from each data point. The results are expressed as mean ± SE of at least five different experiments.

Efflux studies

Renal tubuli were preincubated in HEPES buffer (37°C, 10 min). They were then loaded with L-[^{3}H] argi-

nine (4 min) at 1 mmol/L. Tubuli were put briefly on ice (<5 seconds), and subsequently washed four times (1 min, 2 mL) with HEPES buffer. Then, 1 mL of buffer was added for the times indicated before it was removed by aspiration. Efflux was terminated by placing the tubes immediately on ice, and the total radioactivity associated was measured using liquid scintillation spectrometry. The tubuli were dried and solubilized by 1 mL 0.5% SDS in 0.5 N NaOH. Cell lysate (300 μL) was used for protein quantification as described previously.

Detection of lactate dehydrogenase in the urine

Different rats were used exclusively to study lactate dehydrogenase (LDH) in urine for each group ($N = 4$). After reperfusion, urine was collected through a catheter placed in the urinary bladder for two hours, and samples were kept at −80°C. LDH was measured using a commercially available cytotoxicity detection kit (Bayer, Tarrytown, NY, USA). The assay operates on the principle that LDH released from damaged cells reduces nicotinamide adenine dinucleotide (NAD) to NADH and H^+ by oxidation of lactate to pyruvate. The rate of oxidation that was proportional to LDH activity was monitored by measuring the decrease in absorbance at 340 nm.

Measurement of cytotoxicity

Tubular cell death in each experimental condition was determined by measuring the LDH released into the incubation medium due to the loss of membrane integrity. One milliliter of tubule suspension was centrifuged (30 seconds, 4°C, 1100 rpm) and LDH activity was measured in the supernatant.

Analysis of mRNA levels for iNOS, CAT-1, CAT-2, arginase I and arginase II by RT-PCR

Total cellular RNA was extracted from glomeruli/tubuli, following the method described by Chomczynski and Sacchi [29]. Reverse transcription (RT) was carried out for 1.5 hours at 42°C, followed by polymerase chain reaction (PCR) in 1 × PCR buffer, for a total of 35 cycles, each at 94°C for one minute, 60°C for one minute, 72°C for two minutes, and seven minutes (final cycle). The first pair of primers was designed to bind to a portion of the rat CAT-1 gene: forward 21-mer, 5′-GCC ATC GTC ATC TCC TTC CTG-3′, and reverse 21-mer, 5′-CCC TCC CTC ACC GTA TTT CAC-3′ [30]. The second pair of primers, which hybridize to a sequence common to both CAT-2 and CAT-2a were: forward 21-mer, 5′-AAC GTG CTT TTA TGC CTT TGT-3′, and reverse 21-mer, 5′-GGT GAC CTG GGA CTC GCT CTT-3′ [31]. The following pairs of primers were used for arginase I: forward 25-mer, 5′-AAA GCC CAT AGA GAT TAT CGG AGC G-3′, and reverse 22-mer, 5′-AGA CAA GGT CAA CGG CAC TGC C-3′. For arginase II: forward 23-mer, 5′-TTA GTA GAG CTG TGT CAG GTG GC-3′, and reverse 24-mer, 5′-ACT TGA AGC AAT CAC ATC CAC TGC-3′. The primers used for iNOS were: forward 21-mer, 5′-GTG TTC CAC CAG GAG ATG TTG-3′, and reverse 21-mer, 5-′CTC CTG CCC ACT GAG TTC GTC-3′. To exclude the possibility of contamination by genomic DNA amplification, and to assess the adequacy of cDNA, experiments were carried out in the absence of reverse transcriptase, and amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed, respectively. PCR products were electrophoresed on a 1.6% agarose gel and visualized by ultraviolet (UV) induced fluorescence. All PCR reactions resulted in the amplification of a single product of the predicted size for iNOS, CAT-1, CAT-2, arginase I, arginase II, and GAPDH.

Statistical analysis

Data are presented as the means ± SE. Data from the various experiments were subjected to analysis of variance (ANOVA), and P values <0.05 were considered to be statistically significant.

RESULTS

The first set of experiments was performed to confirm the establishment of acute renal failure in the above model. Exposing the animals to 60 minutes of ischemia and 24 hours of reperfusion resulted in a significant decrease in glomerular filtration rate (GFR) compared to sham operated rats (140 ± 20 vs. 380 ± 35 μL/min/100 g BW, $P < 0.05$, $N = 6$).

Effect of I/R on iNOS expression

Nitric oxide produced in excess was previously incriminated as a mediator in I/R injury in several tissues, including the proximal tubuli. Therefore, we first studied the effect of I/R on iNOS mRNA expression in both tubuli and glomeruli. When tubuli were subjected to ischemia, the expression of iNOS mRNA was initiated, as compared to control, continued to increase after 60 minutes of reperfusion, and then decreased after 24 hours (Fig. 1). Under these conditions, no effect was observed on glomerular iNOS mRNA levels (data not shown).

Effects of I/R on arginine transport

To characterize the kinetics of L-Arg transport in freshly harvested tubules, saturable uptake of L-Arg (0 to 1 mmol/L) was measured. The plot of L-Arg uptake as a function of the extracellular L-Arg concentration is shown in Figure 2. A high affinity transporter was present (K_m 110 μmol/L; V_{max} 4.2 nmol arginine/μg protein/4 min). Based on previous reports, the kinetic properties of the arginine transport system in tubules resemble those of system y^+, CAT-1 and CAT-2.

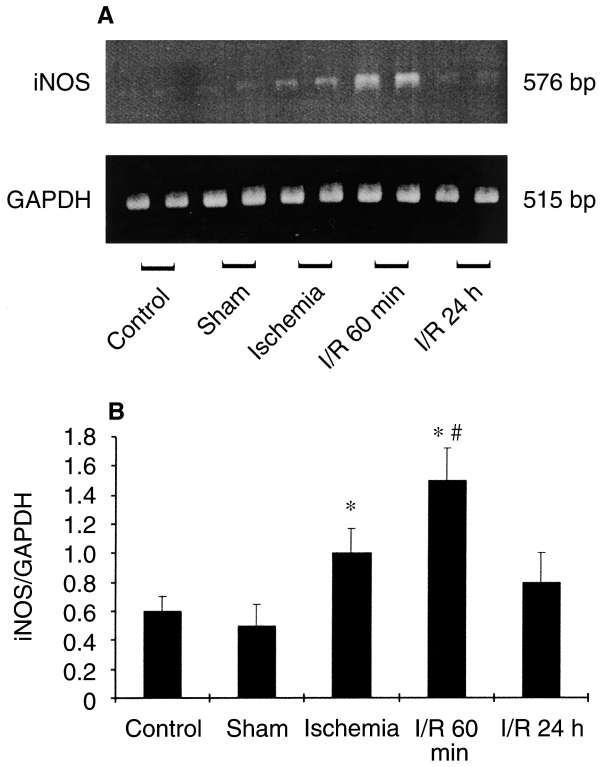

Fig. 1. (*A*) Ethidium-stained agarose electrophoresis gel showing polymerase chain reaction (PCR)-amplified inducible nitric oxide synthase (iNOS) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA from freshly harvested tubuli of control and ischemic/reperfusion (I\R)-treated rats. (*B*) Relative amount of iNOS mRNA quantitated by densitometry and expressed as iNOS/GAPDH ratio of the same experiments shown panel A. Data are presented as the mean ± SEM of three different experiments. *$P < 0.05$ vs. control, #$P < 0.05$ vs. ischemia.

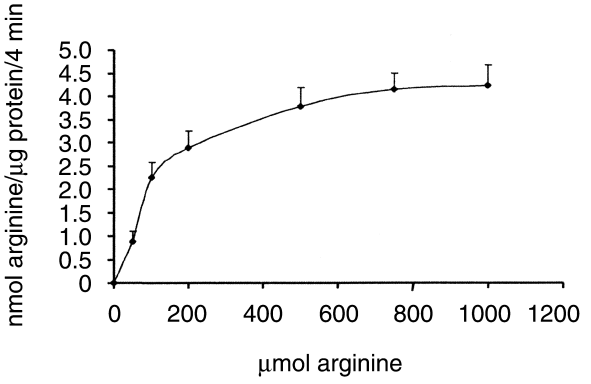

Fig. 2. Concentration dependence of L-arginine uptake by tubuli. Uptake of [^{3}H] arginine was measured for 4 minutes in freshly harvested tubuli from normal rats over a range of concentrations (0 to 1 mmol/L). Data are presented as the mean ± SEM of 4 different experiments (V_{max} = 4.2, K_m = 110 µmol/L).

Previous studies have shown that both eNOS and iNOS are maximally activated during I/R [15, 16]. This prompted us to study arginine transport in both freshly isolated tubuli and glomeruli. In the glomeruli neither ischemia nor reperfusion had an effect on arginine transport (Fig. 3A). In the tubuli, however, 60 minutes of reperfusion resulted in a maximal tubular arginine transport, while only a moderate increase was observed after 24 hours of reperfusion. Tubular arginine transport was unchanged after 15 and 30 minutes of reperfusion (Fig. 3).

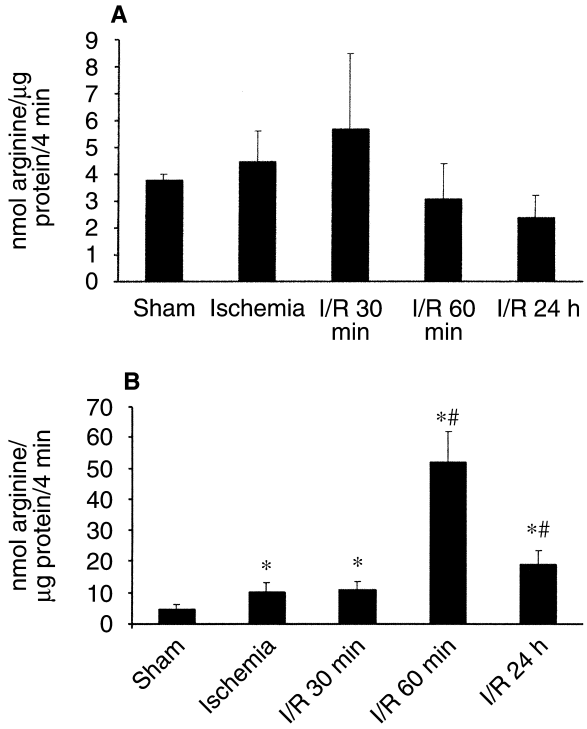

Fig. 3. Uptake of radiolabeled arginine ([^{3}H] L-arginine) by freshly harvested glomeruli and tubuli from the various experimental groups. Data are presented as the mean ± SEM of at least 5 different experiments *$P < 0.05$ vs. sham, #$P < 0.05$ vs. ischemia. (See the **Methods** section for definition of groups.)

Arginine efflux

We tested the possibility that an intracellular arginine concentration could result from changes in arginine efflux. One hour of reperfusion displayed the capacity to suppress arginine efflux, while it was mildly decreased by ischemia, and moderately decreased by 24 hours of reperfusion, as compared to control and sham groups (Fig. 4).

Effects of I/R on CAT-1 and CAT-2

To determine whether the observed I/R-induced changes in arginine uptake are associated with similar directional changes in mRNA levels for CAT family of transporters, total RNA was analyzed by RT-PCR to amplify portions of CAT-1 and CAT-2 in freshly harvested tubuli from all experimental groups (Fig. 5). RT-PCR identified cDNA encoding CAT-1 and CAT-2 in both tubuli and glomeruli in all groups. Interestingly, 60 minutes of reperfusion markedly augmented the CAT-2 mRNA steady state, while it was only minimally increased at 24 hours of reperfusion as compared to the control

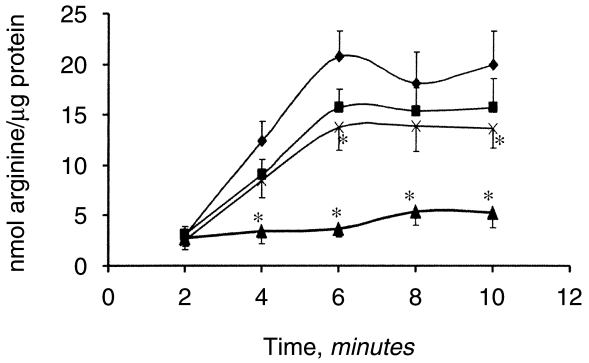

Fig. 4. Efflux of radiolabeled arginine ([^{3}H] L-arginine) by freshly harvested tubuli from the various experimental groups. Symbols are: (◆) sham-operated; (■) ischemia; (▲) I/R for 60 minutes; (×) I/R for 24 hours. Data are presented as the mean ± SEM of at least 5 different experiments. *$P < 0.05$ vs. sham. (See the **Methods** section for definition of groups.)

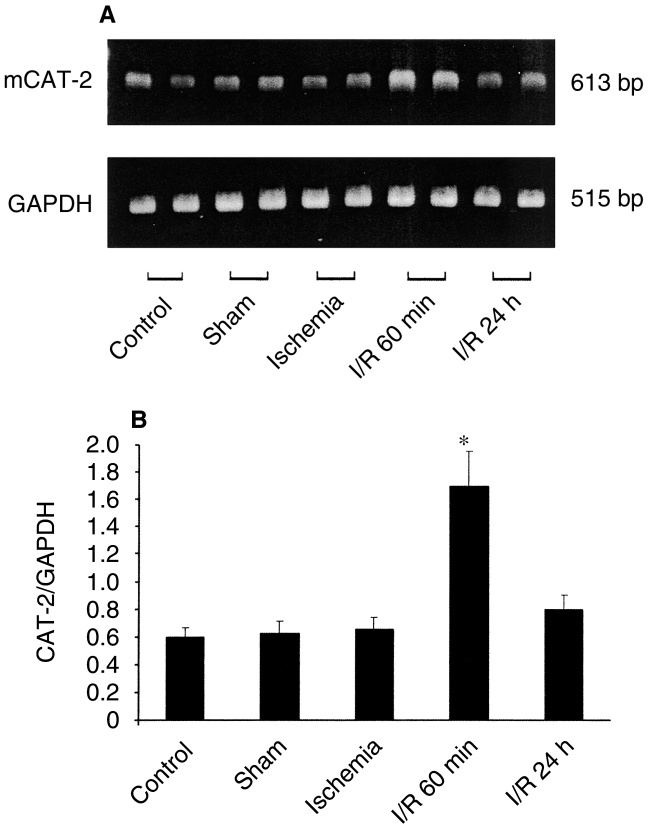

Fig. 5. (*A*) Ethidium-stained agarose electrophoresis gel showing PCR-amplified CAT-2, and GAPDH cDNA from freshly harvested tubuli of control and I/R treated rats. (*B*) Relative amount of CAT-2 mRNA quantitated by densitometry and expressed as CAT-2/GAPDH ratio of the same experiments shown in panel A. Data are presented as the mean ± SEM of three different experiments. *$P < 0.05$ vs. control.

and sham groups, when normalized to GAPDH in three different experiments. In contrast, CAT-1 mRNA expression was unaffected by reperfusion as compared to control and sham groups (data not shown). RT-PCR products were not obtained with RNA samples in the absence of RT or when cDNA was omitted from the PCR reaction.

Effect of I/R on arginase I and arginase II mRNA expression

Reverse transcription-PCR was utilized to examine the changes in arginase I and II expression that affected the intracellular arginine level at one hour of reperfusion (Fig. 6). mRNA for both isoenzymes was clearly detected in tubuli. A transient decrease in arginase I and II mRNA levels was found to be significant one hour following reperfusion as compared to controls.

Effect of I/R on urinary LDH release

Urinary LDH release was used as a marker for tubular cell injury, and in control and sham operated rats it was minimal (15 ± 4 U/L). Reperfusion of 15 and 30 minutes resulted in similar values. However, one hour of ischemia resulted in increased LDH levels (110 ± 18 U/L), which were further dramatically augmented following one hour of reperfusion (320 ± 34 U/L; mean ± SE of 4 different experiments, $P < 0.05$).

Tubular damage by I/R

Tubular injury also was assessed by measuring the released LDH. With tubuli in the control group, the LDH release was similar to sham operated animals (18.2 ± 2.0 and 24.3 ± 1.6 U/L). Ischemia increased the LDH release fourfold (93.0 ± 4.8 U/L), while one hour of reperfusion resulted in an eightfold increase as compared to controls (117.4 ± 9.6 U/L). Reperfusion for 24 hours following ischemia only mildly decreased this value to 102 ± 5.4 U/L (mean ± SE of 4 different experiments).

DISCUSSION

Reperfusion of ischemic tissue is necessary to restore its normal function, however, paradoxically it is the culprit for more severe tissue damage. In the kidney, acute ischemic injury induces marked structural changes in epithelial cells, especially in the proximal tubule [32]. Alteration of the cytoskeleton, disruption of normal cell-matrix interaction, loss of renal epithelium polarity and shedding of the apical microvilli into the tubular lumen leads to increase in tubular pressure and impaired renal function [33]. Ischemic ARF in the rat was accompanied by an induction of renal tubular iNOS and production of NO [15, 34]. It also was suggested that NO production serves as a priming event, which in the presence of oxidative stress, results in the generation of peroxynitrite that is cytotoxic to renal tubular cells [17, 33]. Furthermore, inhibition of NOS by L-NAME prevented the hypoxia-induced increase in NO and cytotoxicity, as evaluated

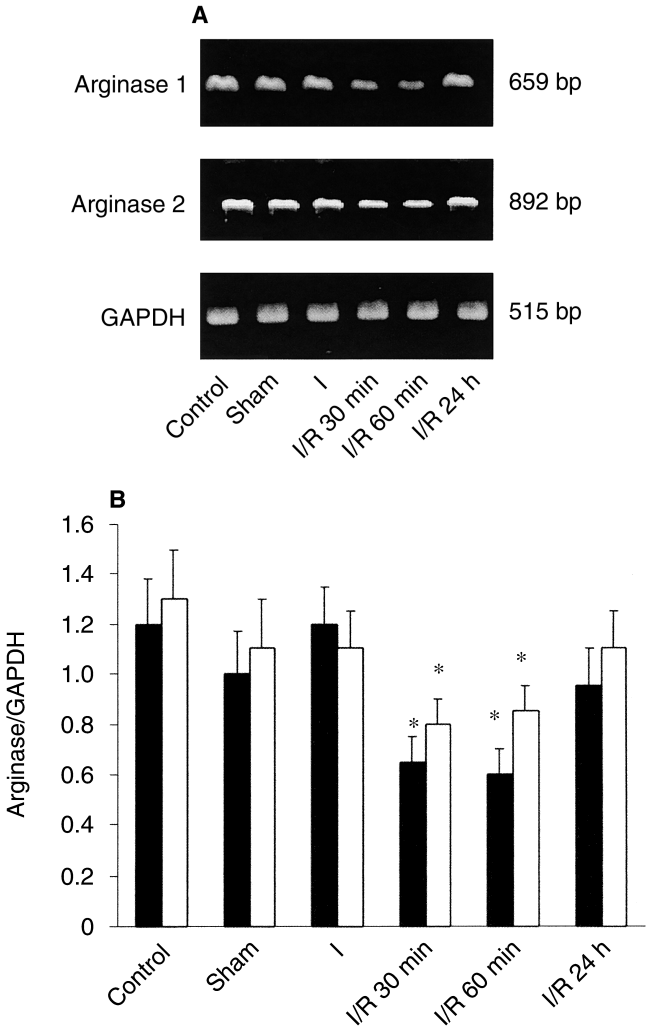

Fig. 6. (*A*) Ethidium-stained agarose electrophoresis gel showing PCR-amplified arginase, and GAPDH cDNA from freshly harvested tubuli of control and I/R treated rats. (*B*) Relative amount of arginase mRNA quantitated by densitometry and expressed as arginase/GAPDH ratio of the same experiments shown in panel A. Data are presented as the mean ± SEM of three different experiments. $*P < 0.05$ vs. control.

by LDH release [34]. Regulation of L-Arg availability can determine the rate of NO production, as arginine is the only physiological substrate for the NOS reaction, and iNOS activity is strictly dependent on the presence of extracellular arginine. To our knowledge, this is the first report on L-Arg transport in a renal tissue following in vivo ischemia/reperfusion. The present observations provide evidence for augmented, maximal, tubular arginine uptake as early as one hour following reperfusion, which remains moderately elevated after 24 hours of reperfusion and is associated with a decrease in arginine efflux. System y+ mediates both influx and efflux of cationic amino acids across cell membranes. The ability to operate in both membrane directions is important in renal tubular cells that exhibit the capacity for de novo synthesis of arginine. An attenuation of arginine efflux one hour following reperfusion, as observed here, may provide a physiological mechanism to maintain intracellular arginine availability. Alternatively, it could simply reflect an elevated renal tubular arginine uptake and utilization. In any event, these data suggest a unidirectional augmentation of tubular arginine traffic during I/R. The mechanism causing increased L-Arg transport during I/R is not entirely clear. Analysis of freshly isolated tubules indicated that arginase I and II may be co-expressed under in vivo conditions. However, at one hour of reperfusion we noticed that mRNA for both isoenzymes substantially decreased. This indicates that the increased arginine transport at one hour of reperfusion is not related to modulated arginase expression, but rather to a different arginine axis activation. The change in arginine uptake is accompanied by an increase in both CAT-2 mRNA and iNOS mRNA levels. Indeed, the association between augmented arginine transport and iNOS activation was previously reported. In cardiac myocytes, for example, the induction of iNOS with interleukin-1 (IL-1) and interferon-γ (IFN-γ) results in the up-regulation of both CAT-1 and CAT-2, and a similar effect was observed in stimulated vascular smooth muscle cells. Kakuda et al described a more distinct regulation for CAT isotypes in different tissues, following specific stresses or activators, which resulted in decreased CAT-1 levels and elevated CAT-2 and iNOS mRNA and proteins, with parallel kinetics [35]. We have recently described that the co-administration of lipopolysaccharide (LPS) and tetrahydrobiopterin augments arginine transport in rat cardiac myocytes through selective up-regulation of CAT-2 mRNA [36]. Since previous reports suggested a modulatory role for cellular cationic amino acid concentration on L-Arg uptake and CAT expression [37], one possible explanation to our findings involves increased expression of CAT-2 mRNA that follows decreased cytosolic levels of L-Arg: When iNOS is induced and the high output pathway of NO synthesis is activated, L-Arg concentrations may become rate-limiting, favoring synthesis of O_2^- rather than NO [38]. In addition, during I/R, there is a decrease in renal de novo L-Arg synthesis from citrulline and aspartate, due to depleted adenosine 5′-triphosphate (ATP) levels, which further contributes to lower cytosolic levels of L-Arg [38, 39]. Taken together, it is tempting to hypothesize an augmented expression of CAT-2 mRNA during I/R. Future experiments to define changes CAT-2 transporter at the protein level are required to strengthen our hypothesis. Unfortunately, we were unable to localize the specific tubular segment(s) in which I/R exerts its effect on arginine transport. We can only assume that the increased arginine transport does not originate in the inner medullary collecting duct, wherein only CAT-1 mRNA is exclusively expressed [40].

In summary, by using a rat model of ischemic ARF, we

show an augmented expression of arginine transporter CAT-2, which leads to a substantial increase in tubular arginine transport. It appears likely that selective inhibition of this transporter may attenuate tubular nitric oxide/peroxynitrite production and ameliorate the renal injury exhibited in ischemic ARF.

Reprint requests to Professor Adrian Iaina, Department of Nephrology, Tel Aviv Sourasky Medical Center, 6 Weizman Street, Tel Aviv, Israel. E-mail: iaina@tasmc.health.gov.il

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