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Renal function and cortical (Na^++K^+)-ATPase activity, abundance and distribution after ischaemia–reperfusion in rats

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Abstract

The effects of ischaemic injury and reperfusion on renal function, cortical ATP content, alkaline phosphatase activity and (Na^++K^+)-ATPase activity and abundance in cortical homogenates and isolated basolateral and apical membranes were examined. Rats were submitted to 5 or 40 min of right renal artery occlusion and 60 min of reperfusion. Renal function of the ischaemic–reperfused kidney was studied by conventional clearance techniques. Our results show that 1 h of reperfusion after a short period of renal ischaemia (5 min) allows the complete restoration of the biochemical features of cortical cells and functional properties of the injured kidney. A longer period of ischaemia, such as 40 min, followed by 1 h of reperfusion showed functional and biochemical alterations. ATP recovered from 26% after 40 min of ischaemia to 50% of control values after 1 h reperfusion. However, renal function was strongly impaired. Brush border integrity was compromised, as suggested by AP excretion and actin appearance in urine. Although total cortical (Na^++K^+)-ATPase activity was not different from controls, its distribution in isolated apical and basolateral membranes was abnormal. Remarkably, we detected an increase in α-subunit protein abundance that may suggest that (Na^++K^+)-ATPase synthesis is promoted by ischaemia–reperfusion. This increase may play an important role in the pathophysiology of ischaemic acute renal failure.

Keywords: Rat kidney; Renal function; (Na^++K^+)-ATPase; Ischemia–reperfusion; Acute renal failure; Basolateral membrane; Apical membrane

1. Introduction

Acute renal failure (ARF) is defined as a syndrome characterised by a sudden, sustained decline in glomerular filtration rate (GFR), usually associated with azotaemia and a fall in urine output [1]. Complete occlusion of the renal artery for various time periods is a generally well accepted model of ARF [2]. Renal ischaemia results in profound alterations in cell function [3], metabolism [4] and structural integrity [5] of the proximal tubule. In addition to these direct effects of ischaemia, tubular cells undergo dynamic transformations during reflow that may contribute to renal dysfunction [4].

The proximal tubule is the site of reabsorption of approximately two-thirds of the Na^+ that enters the

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tubular fluid by glomerular filtration. Na^+ is internalised across the apical membrane, down its electrochemical gradient, via several specific transporters providing the energy for the secretion or reabsorption of glucose, amino acids and Krebs cycle intermediates and other solutes [4]. Completion of the reabsorptive process requires the energy-dependent transport of Na^+ up its electrochemical gradient via (Na^++K^+)-adenosine triphosphatase (ATPase), which is localised to the basolateral membrane. The enzyme is composed of two non-covalently attached subunits, an α-subunit (molecular mass 96–116 kDa) possessing the catalytic activity and a glycosylated β-subunit (molecular mass 40–60 kDa) involved in maturation and intracellular routing of the protein [6]. The normal localisation of (Na^++K^+)-ATPase to the basolateral membrane is regulated by direct interactions with membrane-associated cytoskeletal proteins [7]. Following an ischaemic insult proximal tubular cells exhibit a disruption of the actin-based cytoskeleton [8,9]. This reorganisation of the actin cytoskeleton is believed to be important in the surface membrane structural, biochemical and functional alterations that occur in proximal tubular cell injury [10]. Of particular importance is the involvement of apical microvillar membrane damage, including membrane internalisation into the cytoplasm and fragmentation into the urinary lumen [5]. Another important event is the decrease in the association of (Na^++K^+)-ATPase with the underlying cytoskeletal proteins [11]. Fish and Molitoris [12] have proposed that (Na^++K^+)-ATPase redistributes from its normal location to the apical membrane. Other works [13,14] using renal tissue from transplanted kidneys have reported that (Na^++K^+)-ATPase was dislocated from the basolateral membrane to the cytoplasm. These authors have suggested that this feature would effectively result in depolarisation of the tubule cell and could well serve to inhibit sodium reabsorption and enhance the delivery of this ion to the macula densa, activating the tubuloglomerular feedback mechanism.

In a previous work [15], we studied the effects of different renal ischaemic periods without reperfusion on (Na^++K^+)-ATPase activity, abundance and distribution in plasma membranes isolated by a Percoll density gradient. We reported that after a brief period of ischaemia (5 min) (Na^++K^+)-ATPase activity was increased in fractions of a higher density than those typically enriched in basolateral membranes. Forty minutes of ischaemia promoted a diminution of (Na^++K^+)-ATPase activity in all the fractions of the gradient. At each time studied (Na^++K^+)-ATPase abundance was increased in cortical homogenates and in the isolated membranes.

These previous results provided evidence that ischaemia alone induced alterations on (Na^++K^+)-ATPase abundance not coordinated with (Na^++K^+)-ATPase activity. These alterations depended on the duration of the ischaemic period. The present study was undertaken to evaluate the effects of reflow that may contribute to renal dysfunction in addition to the alterations imposed by the ischaemic phase. Renal functional and biochemical parameters were studied to evaluate the relative contribution of a 1 h reperfusion period to the renal injury that resulted after 5 or 40 min of ischaemia.

2. Materials and methods

2.1. Animals

Male Wistar rats (3 months, 250–350 g body wt.) were used in all experiments. The animals were housed in rooms with controlled temperature (21–23°C), and regular light cycles (12 h). They were allowed free access to a standard diet and tap water until used.

All experiments were performed in conformity with the guide for the care and use of laboratory animals promulgated by the National Institute of Health (National Center for Research Resources), Bethesda, MD, USA.

2.2. Renal function studies after ischaemia–reperfusion

Rat renal function was assayed by clearance techniques as previously reported [16]. Animals were anaesthetised with sodium thiopental (70 mg/kg b.wt., i.p.). The femoral vein and femoral artery were cannulated. The right renal artery and ureter were exposed by a flank incision and a non-traumatic vascular clamp was placed around the right renal artery for 0, 5 and 40 min. Total ischaemia was con-

firmed by observing blanching of the entire kidney surface. The animals were placed on a heating pad to keep a constant temperature (37°C). After the ischaemic period the clamp was removed and reperfusion was allowed for 60 min. The kidneys were observed for 2–5 min to ensure colour change indicating blood reflow. Simultaneously with the removal of the clamp, a saline solution containing inulin (0.9 g%) and *p*-aminohippuric acid (PAH) (0.3 g%) was infused through the venous catheter at a rate of 5 ml/h employing a constant infusion pump (Harvard Apparatus). During the first 15 min of the reperfusion period the right ureter was cannulated for direct collection of urine. A 60 min equilibration period elapsed before clearance measurements started. Urine collection was obtained during a 20 min period and blood from the femoral artery was obtained at the midpoint of the period. Sham-operated rats were used as controls.

GFR was estimated from inulin clearance and renal plasma flow (RPF) from PAH clearance. Fractional excretion of water (FE_{H2O}), sodium (FE_{Na+}) and potassium (FE_{K+}) were calculated by conventional formulae for each animal.

2.3. Isolation of renal plasma membrane fractions

Another group of animals was employed to isolate proximal tubular membranes under physiological condition (C) or after 5 and 40 min of ischaemia followed by 60 min of reperfusion (I5R60 and I40R60, respectively), as previously reported by our group [15,17]. Briefly, both control and right-reperfused kidneys were decapsulated and placed on a buffered sucrose medium (0.25 M sucrose, 10 mM Tris–HCl, pH 7.2). All subsequent steps were performed at 4°C. The cortex was dissected out and minced. Sham-operated rats were used as control kidney donors. The kidney cortex was homogenised in 0.25 M sucrose–10 mM Tris–HCl, pH 7.2 (5 ml/g of tissue) in a Dounce homogeniser (five strokes) and then in a motor-driven Teflon glass Potter homogeniser (10 strokes at 800 rpm). The homogenates obtained after 0, 5 and 40 min of artery clamping and 60 min of reperfusion were centrifuged according to Boumendil-Podevin [18]. The partially purified plasma membrane fraction obtained consisted of a mixture of basolateral and brush border membrane vesicles ('crude fraction'). Separation of the membranes was obtained by a self-oriented Percoll gradient (Pharmacia, Uppsala, Sweden). The spontaneously formed Percoll gradient was fractionated from the top by pumping a 60% (w/v) sucrose solution to the bottom of the centrifuge tube via a steel cannula with a peristaltic pump [19]. Ten fractions of 1 ml were collected. Aliquots of each fraction were frozen in liquid nitrogen and stored immediately at −70°C until use within days. Three preparations were made for each of the studied groups: C, I5R60 and I40R60. Each preparation represented cortical tissue of eight kidneys.

2.4. Cortical ATP content studies

Cortical ATP content was measured in homogenates from control animals, I5R60, I40R60 and, in addition, in homogenates from animals submitted to 5 min (I5) and 40 min (I40) of ischaemia but not to reperfusion. ATP was extracted with 6% perchloric acid (PCA) from cortical slices. The PCA extract was then neutralised with 5 M K_2CO_3. ATP was determined in aliquots of this neutralised fraction using hexokinase and glucose-6-phosphate dehydrogenase according to Lampretch and Trautschold [20].

2.5. Analytical methods

Plasma and urinary inulin concentration were determined by Roe's procedure [21] and PAH concentrations in the same samples were determined by Brun's method modified by Waugh and Beall [22]. Sodium and potassium were measured by flame photometry. The volume of urine was measured gravimetrically. Urinary alkaline phosphatase (AP) activity was measured employing a commercial kit (Fosfatest, Wiener Lab., Rosario, Argentina) by use of *p*-nitrophenyl phosphate as substrate. AP activity is expressed in units (U) defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol of *p*-nitrophenyl phosphate per min at 37°C.

(Na^++K^+)-ATPase and AP activities and protein content were determined in homogenates and plasma membrane enriched fractions. AP activity was measured as mentioned above. (Na^++K^+)-ATPase activity was determined as the difference between the amounts of inorganic phosphate released by total

ATPase and the activity remaining after the addition of ouabain (1 mM) to the reaction mix in the absence of potassium. Reaction time was 10 min. The incubation medium was the same as described by Koshier et al. [23]. The inorganic phosphate was determined by the method of Summer [24].

Protein content was measured by the method of Sedmak and Grossberg [25], with bovine serum albumin as standard.

2.6. Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Western blot

For (Na^++K^+)-ATPase detection samples from homogenates and isolated membranes (10 μg protein) were subjected to SDS–PAGE on polyacrylamide gels (8%) [26]. For each gel, an identical gel was run in parallel and subjected to Coomassie staining to ensure identical loading. The amount of protein was chosen after the linearity of detection had been verified. Samples were boiled 3 min in the presence of 5% 2-mercaptoethanol and 1% SDS. 5 μl of prestained molecular weight standards were also loaded onto the gel. The separated proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) in Tris-glycine transfer buffer with 20% methanol in a mini-blotter (Sigma-Aldrich) [27]. Uniform blotting across the gel was verified by Coomassie brilliant blue staining of the post blot gel. Nitrocellulose membranes were incubated for 1 h at room temperature with 5% (w/v) non-fat dry milk in phosphate-buffered saline (PBS), followed by a 1 h (room temperature) incubation with a goat polyclonal antibody to the rabbit kidney (Na^++K^+)-ATPase (1/3000 dilution, Calbiochem-Novabiochem, La Jolla, CA, USA). After washing, membranes were incubated for 1 h with alkaline phosphatase-conjugated anti-goat antibody (1/3000 dilution, Calbiochem-Novabiochem). After washing, bound alkaline phosphatase was detected with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Promega, Madison, WI, USA). The primary antibody only recognised a double band that migrates near the 116 kDa marker. This double band appeared whether the samples were boiled or incubated at 37°C. Other authors noted narrower α-subunit bands when samples were incubated at 37°C. However, they did not find differences in the subunit abundance between samples incubated at each temperature [28]. The split of the kidney (Na^++K^+)-ATPase α-subunit into two bands has been reported previously [29]. The samples compared were run on the same gel and comparisons of band intensities were performed within one membrane. The most intense band of each membrane was chosen and its density was designated as 100. Blots were digitised using a desktop computer Scanner (HP II Scan Jet). The signal produced was quantified using the Adobe PhotoShop (4.0) computer program.

For actin detection in urine, 20 μg protein samples from control, I5R60 and I40R60 were boiled and subjected to SDS–PAGE on polyacrylamide gels (8%) as above. The gel was transferred to nitrocellulose membranes. The membrane was incubated with primary antibody to actin (1/200 dilution, Sigma-Aldrich) and detection was achieved using a commercial kit (Amplified Alkaline Phosphatase Immun-Blot Assay kit, Bio-Rad).

2.7. Statistical analysis

Results are expressed as mean ± standard error of the mean. Data were analysed using the one-way analysis of variance followed by Newman–Keuls contrasts. The 0.05 level of probability was used as the criterion of significance in all cases.

3. Results

3.1. Effect of ischaemia and reperfusion on ATP content

Cortical ATP content in controls and after ischaemia and reperfusion was studied. Cortical ATP declined rapidly to 62% of control levels in the first 5 min of ischaemia. One hour of reperfusion after this period of ischaemia allowed the restoration of the cortical ATP content to the normal levels. After 40 min of ischaemia, the ATP content was 26% of control. In this case, 1 h of reperfusion only allowed the partial recovery to 51% of the control values ($C = 0.53 \pm 0.03$, $I5 = 0.33 \pm 0.04$**, $I5R60 = 0.63 \pm 0.07$, $I40 = 0.14 \pm 0.02$**, $I40R60 = 0.27 \pm 0.02$** μmol ATP/g wet tissue; $^{**}P < 0.01$, $n = 4$).

Table 1
Rat renal function after ischaemia–reperfusion

Experimental group	UFR (μl/min·g)	RPF (ml/min·g)	GFR (ml/min·g)	FF (%)	FE_{Na+} (%)	FE_{K+} (%)	FE_{H2O} (%)
Control	3.4 ± 0.5	5.5 ± 0.2	1.16 ± 0.05	19.3 ± 2.4	0.30 ± 0.04	15.8 ± 1	0.28 ± 0.04
I5R60	2.9 ± 0.5	4.2 ± 0.5	1.06 ± 0.08	26.6 ± 1.0	0.52 ± 0.06	22.5 ± 4	0.33 ± 0.04
I40R60	13.3 ± 0.1**	0.7 ± 0.5**	0.15 ± 0.09**	23.3 ± 5.9	11.93 ± 2.5**	71.6 ± 11**	11.3 ± 2.4**

Renal function was assayed in the right ischaemic–reperfused kidney. UFR, urine flow rate; RPF, renal plasma flow; GFR, glomerular filtration rate; FF, filtration fraction; FE_{Na+}, fractional excretion of sodium; FE_{K+}, fractional excretion of potassium; FE_{H2O}, fractional excretion of water. Results are expressed as means ± S.E.M. In all the groups at least $n = 4$. $^{**}P < 0.01$ vs. control.

3.2. Effect of ischaemia–reperfusion on renal function

The measured parameters of renal function in control and postischaemic kidneys are presented in Table 1. Tubular and haemodynamic functions were not modified by 5 min of ischaemia followed by 60 min of reperfusion. On the other hand, I40R60 showed a marked increase in urine flow accompanied by increases in Na^+, K^+ and water excretions. Clearances of inulin and *p*-aminohippurate decreased in parallel as confirmed by the filtration fraction (FF) constant value.

3.3. Effect of ischaemia–reperfusion on $(Na^+ + K^+)$-ATPase and AP activities in cortical homogenates

$(Na^+ + K^+)$-ATPase activity in cortical homogenates was determined under saturating conditions for sodium, potassium, magnesium and ATP. There were no statistically significant differences among the groups in $(Na^+ + K^+)$-ATPase activity (C = 8.1 ± 1.0, I5R60 = 8.3 ± 0.6, I40R60 = 10.6 ± 1.2 μmol P_i/h·mg prot.). AP activity in I5R60 and I40R60 did not differ from controls (C = 0.16 ± 0.02, I5R60 = 0.15 ± 0.01, I40R60 = 0.12 ± 0.01 U/mg prot.).

3.4. Subcellular distribution of membrane enzyme marker activities in control renal cortex

To simultaneously determine the distribution of $(Na^+ + K^+)$-ATPase and AP in the different plasma membrane domains, crude membrane preparations were fractionated by Percoll gradient centrifugation. $(Na^+ + K^+)$-ATPase activity showed the highest value in fraction 3, with a specific activity of 81.5 ± 5.0 μmol P_i/h·mg prot. and relative specific activity nor-

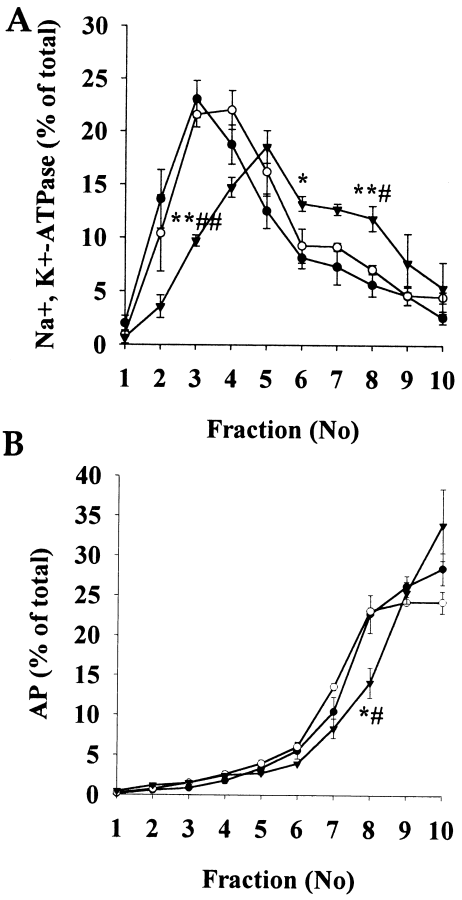

Fig. 1. Distribution pattern of enzyme activities in renal cortical membranes fractionated on a Percoll gradient after 0 (control), 5 (I5R60) and 40 (I40R60) min of artery clamping followed by 60 min of reperfusion. Renal cortices were homogenised and membranes were fractionated by density gradient centrifugation and collected as 10 fractions. (A) Distribution of $(Na^+ + K^+)$-ATPase activity, expressed as percentage of total activity in each gradient. (B) Distribution of AP activity, expressed as percentage of total activity in each gradient. Data are means ± S.E.M. In all groups $n = 3$. $^{*}P < 0.05$ vs. control preparations; $^{**}P < 0.01$ vs. control preparations; $^{\#}P < 0.05$ vs. I5R60; $^{\#\#}P < 0.01$ vs. I5R60; ●, control; ○, I5R60 preparations; ▼, I40R60 preparations.

malised to the homogenate 10.0 ± 0.6. As expected, AP reached the highest activity at higher densities. Fraction 8 was the most enriched either in enzyme activity (0.96 ± 0.14 U/mg prot.) or in relative specific activity (6.0 ± 0.9). These results are shown in Fig. 1, control curves. The distribution and the degree of purification of basolateral and brush border plasma membrane fractions are similar to those previously reported with this separation method [15,17,19].

3.5. Effect of ischaemia–reperfusion on subcellular distribution of membrane enzyme activities

Fig. 1 also summarises (Na^++K^+)-ATPase and AP activity values in the Percoll gradient after 5 and 40 min of artery clamping followed by 1 h of reperfusion. In I5R60, (Na^++K^+)-ATPase (Fig. 1A) and AP (Fig. 1B) activity distribution patterns were not significantly different when compared with controls. After 40 min of clamping and 1 h of reperfusion, (Na^++K^+)-ATPase activity was significantly decreased in fractions 2 and 3 when compared with control membrane preparations. By contrast, fraction 8 of I40R60 showed statistically significant increased (Na^++K^+)-ATPase activity. (Na^++K^+)-ATPase activity in the other membrane fractions in I40R60 was not statistically different from control or I5R60 preparations (Fig. 1A). AP activity (Fig. 1B) in I40R60 showed a generally decreased pattern, that reached statistical significance in fraction 8. The total protein distribution in the gradient of I5R60 and I40R60 did not differ from controls (data not shown).

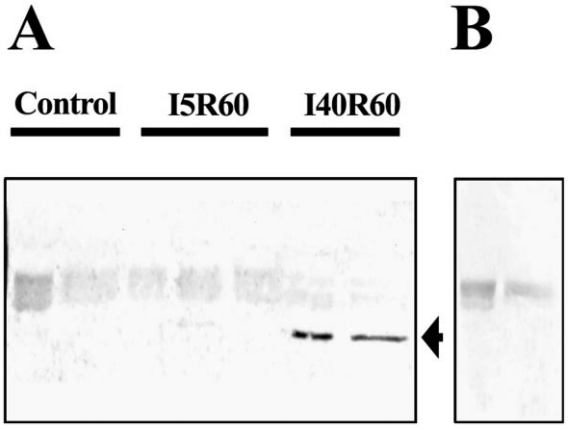

Fig. 2. Actin detection in urine. (A) Western blot analysis was used to identify actin, as described in Section 2. Actin is indicated by the arrow. (B) Control and I40R60 urine samples probed using only kit's anti-rabbit secondary antibody.

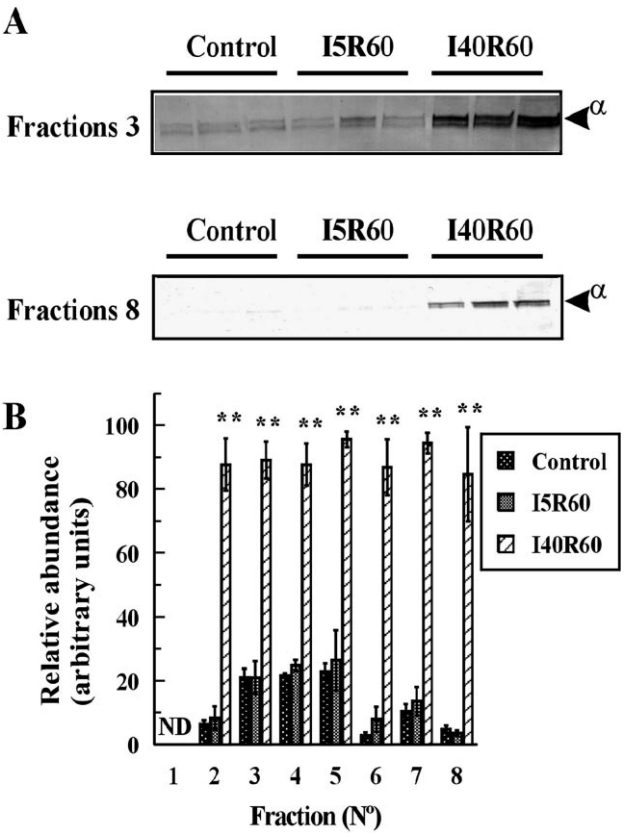

Fig. 3. (Na^++K^+)-ATPase abundance in the fractions of interest of the Percoll gradient. (A) Representative Western blots of samples of the fractions of interest in the Percoll gradient. (Na^++K^+)-ATPase α-subunit is indicated by the arrow. (B) (Na^++K^+)-ATPase expression in the fractions in arbitrary densitometry units expressed as percent of maximal (see Section 2). Gel size did not permit all fractions and periods to be run on the same gel. Group means were calculated and statistical analyses were conducted on groups of samples which were run on the same gel. Data are means ± S.E.M. In all groups $n = 3$. $^{**}P < 0.01$ vs. control values. ND, not determined.

3.6. Effect of ischaemia–reperfusion on AP excretion and urine actin detection

AP excretion was analysed in the urine obtained from control, I5R60 and I40R60 animals. AP excretion was significantly increased in I40R60 when compared to controls or to I5R60 (C = 1.0 ± 0.2, I5R60 = 2.1 ± 0.7, I40R60 = 18.1 ± 2.1 U/mg inulin**, $^{**}P < 0.01$).

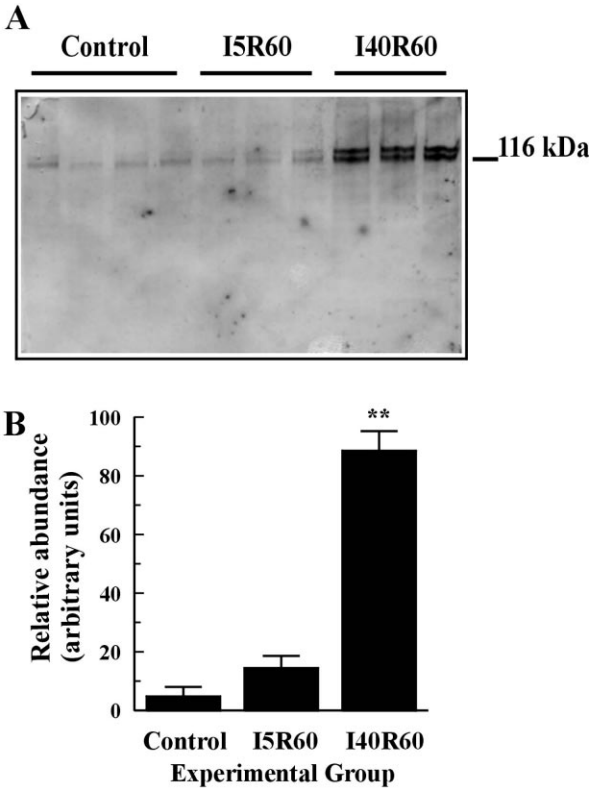

Fig. 4. $(Na^+ + K^+)$-ATPase abundance in cortical homogenates. (A) Representative Western blot of cortical homogenates after different periods of ischaemia–reperfusion. (B) $(Na^+ + K^+)$-ATPase expression in arbitrary units normalized to an internal control (see Section 2). Data are means ± S.E.M. In all groups at least $n = 3$. $^{**}P < 0.01$ vs. control values.

Tubular structural damage was evaluated by immunoblot urine actin detection. As Fig. 2A shows, actin was only detectable in I40R60 urine samples, I5R60 was indistinguishable from controls. The secondary band appearing on this Western blot above the 42 kDa area, which corresponds to actin molecular mass, was due to background binding from the secondary antibody used, as shown in Fig. 2B.

3.7. *Effect of ischaemia–reperfusion on α-subunit $(Na^+ + K^+)$-ATPase protein abundance*

Fig. 3 shows the results of the immunoblot analysis of $(Na^+ + K^+)$-ATPase α-subunit abundance in various membrane fractions of control, I5R60 and I40R60. Fig. 3A shows representative blots of fractions 3 and 8. Fig. 3B summarises the densitometric analysis. All the fractions analysed from I5R60 and control preparations were indistinguishable. On the other hand, I40R60 showed statistically significant increases in all the fractions.

To independently assess whether the increase in $(Na^+ + K^+)$-ATPase α-subunit abundance described in Fig. 3 was a result of redistribution of existing pools or a general increase of pump α-subunit abundance, the cortical homogenates were assayed for the presence of the protein. The results are shown in Fig. 4. Fig. 4A shows a representative blot of the homogenates. Fig. 4B summarises the correspondent densitometric analysis. I40R60 showed a statistically significant increase in the Western blot signal correspondent to $(Na^+ + K^+)$-ATPase α-subunit, while I5R60 did not differ from controls.

4. Discussion

Maintenance of epithelial cell function and polarity is essential for efficient vectorial solute and water transport [3,4,7]. Proximal tubules are a major site where organic compounds and ions are reabsorbed and are severely affected by ischaemia [2]. We have reported previously that ischaemic cellular injury in vivo without reperfusion was associated with abnormalities in AP activity and $(Na^+ + K^+)$-ATPase activity, abundance and distribution in renal cortical homogenates and isolated plasma membranes [15]. Briefly, we reported that after 5 min of ischaemia without reperfusion $(Na^+ + K^+)$-ATPase activity presented an altered pattern of distribution in a Percoll density gradient fractionation. After 40 min of ischaemia $(Na^+ + K^+)$-ATPase and AP activities were decreased in typical basolateral and apical membranes, respectively. $(Na^+ + K^+)$-ATPase α-subunit showed increased abundance in cortical homogenates and isolated membranes [15]. This study was performed to evaluate the possible transformations induced by 1 h of reflow, in addition to the already described effects of ischaemia alone. Renal function, marker enzyme activity distributions and $(Na^+ + K^+)$-ATPase abundance were analysed under the ischaemic–reperfusion challenge.

Renal function parameters of the ischaemic–reperfused kidney were analysed. After 5 min of ischaemia followed by 1 h of reperfusion renal function parameters remained at control values. I40R60 showed renal haemodynamic parameters (GFR and Cl_{PAH}) sig-

nificantly diminished without changes in FF. Intrarenal vasoconstriction has been described as the dominant mechanism for the reduced GFR in ARF [30]. RPF, estimated by Cl_{PAH}, is decreased in our model, suggesting cortical vasoconstriction. Renal fractional excretions of Na^+, K^+ and water in I40R60 were significantly higher than in controls. FE_{Na+} evaluates total nephron Na^+ reabsorption. However, the significantly higher excretion of AP enables us to suggest proximal damage and, therefore, probable proximal impaired Na^+ reabsorption. Many authors have reported impaired proximal Na^+ reabsorption in different ischaemic ARF models [12–14]. Moreover, they have suggested an association between a loss of cell polarity of $(Na^+{+}K^+)$-ATPase and enhanced sodium delivery to the macula densa.

Microfilaments, or filamentous actin, are the primary structural components of the proximal tubule apical microvilli [31]. Kellerman and Bogusky [9] have reported progressive disruption and early decreases (within 5 min) in apical F-actin during ischaemia. They also reported that ischaemia did not affect either glomerular or distal tubular F-actin. Analysis of rat urine during reperfusion has been utilised to determine whether the blebs that formed in proximal tubules during ischaemia appeared in the urine [32]. In the present study, urine samples collected from controls or I5R60 contained no intact actin. However, urine collected from I40R60 showed the presence of actin. Actin presence in urine, together with AP excretion is consistent with detachment and exfoliation of cells and breakdown and release of microvilli described by many authors [9,32]. The integrity of the luminal surface of the proximal tubule may, in turn, influence reabsorptive capability. It has been suggested that loss of apical membrane via bleb formation results in substrate for tubular obstruction and is, in part, responsible for Na^+ wasting [33].

Changes in cellular ATP during ischaemia and reperfusion were evaluated. ATP returned rapidly to normal levels and was not statistically different from control values after 1 h of reperfusion following 5 min of ischaemia. On the other hand, ATP in I40R60 was about 50% of the control value. One of the major requirements for the initiation of the cellular repair process is the repletion of intracellular ATP [34]. Moreover, ATP is critically required for regulation and maintenance of the cell cytoskeleton [4]. Our data showing diminished ATP levels in I40R60 are consistent with the altered tubular function, AP excretion and actin presence in urine.

The activities of $(Na^+{+}K^+)$-ATPase and AP in cortical homogenates were unaltered in I5R60 as in I40R60. However, I40R60 showed altered patterns of both activities in the membranes isolated by Percoll gradient fractionation. $(Na^+{+}K^+)$-ATPase activity was decreased in the lower density fractions, corresponding to basolateral membranes, and shifted to higher density fractions, typically enriched in AP, in response to the ischaemic–reperfusion challenge. Redistribution of $(Na^+{+}K^+)$-ATPase activity in response to ischaemia–reperfusion could result from: (1) proximal tubular damage that has altered the properties of basolateral membranes so that their peak density was shifted towards higher densities, but the $(Na^+{+}K^+)$-ATPase does not leave the basolateral membranes, (2) transport of the pumps out of the basolateral membranes to internal endosomal membranes that share the same density distribution as some of the fractions of the gradient that we analysed and (3) redistribution of $(Na^+{+}K^+)$-ATPase to apical membranes. The higher $(Na^+{+}K^+)$-ATPase activity in the fraction enriched in the apical marker AP is in agreement with previous results of Molitoris [3,12]. This author suggested that ischaemia induces the redistribution of active $(Na^+{+}K^+)$-ATPase to the apical domain and the pumping of Na^+ back to the tubule lumen would contribute to the reduced Na^+ reabsorption. In our work, the appearance of increased $(Na^+{+}K^+)$-ATPase α-subunit abundance in the fraction typically enriched with apical membranes could support this hypothesis.

Our data show that 1 h after 40 min of ischaemia followed by 60 min of reperfusion there is a decrease in the activity of AP in typically brush border membranes. This may be evidence of loss of brush border. In this connection, we have shown an increase in the urinary activity of a brush border enzyme (AP) and the appearance of actin in urine.

The abundance of $(Na^+{+}K^+)$-ATPase α-subunit was analysed by Western blot. In all the fractions analysed, I5R60 was indistinguishable from control, while I40R60 showed increased Western blot signal. In I40R60, $(Na^+{+}K^+)$-ATPase α-subunit abundance was elevated in cortical homogenates; however, no

changes in total cortical activity were observed. The fact that no change in $(Na^+ + K^+)$-ATPase cortical activity occurred while there was an increase in abundance suggests inactivation of the Na pumps. Inhibition of the pump has been reported in the kidney after ischaemia–reperfusion [3,35]. Proposed mechanisms may involve lipid environment alteration [36], ATP depletion [34], damage by oxygen-derived free radicals [37,38] or cytoskeletal alterations [39] among other events described during ischaemia. The simultaneous increment in $(Na^+ + K^+)$-ATPase α-subunit in homogenates and fractions of I40R60 could suggest that ischaemia promotes $(Na^+ + K^+)$-ATPase α-subunit synthesis by an undetermined mechanism. In this connection, there are previous reports about the induction of different genes due to ischaemic–reperfusion stress: inducible nitric oxide synthase in liver [40], heat-shock proteins [41] and colonic $(H^+ + K^+)$-ATPase in renal cortex [42]. Besides, increased steady state mRNA levels in both $(Na^+ + K^+)$-ATPase subunits in Madin Darby canine kidney cells have been reported after hyperoxia, a stress condition also associated with oxidant and ionic disturbances. Increases in $[Na^+]_i$ have been implicated in the induction of $(Na^+ + K^+)$-ATPase [43] and are a well-known feature of ischaemic injury. In our experiments, the decreased rate in protein degradation may not be disregarded. Rapid transit of newly synthesised protein to the plasma membrane was also observed in different experimental conditions as in the MDCK and A6 cells [44,45]. $(Na^+ + K^+)$-ATPase β-subunit influences the transport properties of the Na^+,K pumps [46]. Furthermore, α-subunits are inactive without β-subunits [47]. Delivery of free α-subunits to the plasma membrane has been reported in studies of $(Na^+ + K^+)$-ATPase expression in Sf9 cells [48] and A6 epithelia [45]. The participation of β1-subunit in the possible missorting and lack of activity of Na^+,K pumps during ischaemia–reperfusion will be the matter of further investigations in our laboratory.

In summary, our results show that 1 h of reperfusion after a short period of renal ischaemia (5 min) allows the complete restoration of the biochemical and functional properties of cortical cells. A longer period of ischaemia, such as 40 min, followed by 1 h of reperfusion showed functional and biochemical alterations. ATP recovered from 26% to 50% of control values after 1 h reperfusion period. However, renal function was strongly impaired. Brush border integrity was compromised, as was suggested by AP excretion and actin appearance in urine. Although total cortical $(Na^+ + K^+)$-ATPase activity was not different from controls, its distribution in isolated apical and basolateral membranes was abnormal. Remarkably, we detected an increase in α-subunit protein abundance that may suggest that $(Na^+ + K^+)$-ATPase synthesis is promoted by ischaemia–reperfusion. The mechanism underlying this increase in abundance, which may be of physiopathological relevance for the maintenance of ischaemic ARF, remains to be determined.

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