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# Short communication: Ischemia increases cortical $Na^+,K^+$-ATPase activity (K-Pase) in a model of kidney auto-transplantation in the large white pig

M. Fares[1], C. François[1] and J. M. Maixent[1,2]

[1] Inserm U927, Université de Poitiers, Ischémie-reperfusion en transplantation rénale, CHU la Miléterie BP 5772, rue de la Miléterie, Poitiers Cedex 86022. France
[2] EB2M-Protée, Université du Sud Toulon-Var, BP 20132, Avenue de l'Université 83957 La Garde, France

**Corresponding author:** Pr.. J. M. Maixent, EB2M-Protée, Université du Sud Toulon-Var, BP 20132, Avenue de l'Université 83957 La Garde, France. Tel: +33 6 63 95 28 44, Fax: +33 5 49 45 36 41, Email: jmmaixent@gmail.com

**Abstract**

Acute renal failure (ARF) induced by Ischemia-Reperfusion (I-R) is associated with a significant impairment of tubular sodium reabsorption. $Na^+,K^+$-ATPase has a crucial role in tubular sodium reabsorption and maintenance of water and ion homeostasis. In this study, we examined whether renal I-R affects $Na^+,K^+$-ATPase activity in a renal auto graft model (Large White Pig) with controlateral nephrectomy. Kidney samples were obtained from medulla (n = 4) and cortex (n = 3) after one hour of warm ischemia in autotransplanted models. These experimental groups were compared with a controlateral nephrectomy group (Control). $Na^+,K^+$-ATPase activity in purified membrane fractions was measured as ouabain-sensitive $K^+$-stimulated paranitrophenyl phosphatase (pNPPase) activity. The $Na^+,K^+$-ATPase activities of control, ischemia and ischemia –reperfusion were significantly higher in the medulla than in the cortex, irrespective of the experimental group ($P<0.05$). In cortices, $Na^+,K^+$-ATPase activity increased significantly ($P<0.05$) by 35% after ischemia compared to control group. In medullae, there was only a trend toward an activation of $Na^+,K^+$-ATPase activity after Ischemia (increased by 25%) and after Ischemia-Reperfusion (increased by 30%) compared to control group, and these differences did not reach statistical significance. These data suggest that renal ischemia induces a significant alteration of $Na^+,K^+$-ATPase by increasing its ouabain-sensitive $K^+$-stimulated paranitrophenyl phosphatase (pNPPase) activity in isolated membrane from cortical renal tissues after one hour of ischemia. This change of activity seems to be a new regulation mechanism of an important membrane ion transporter during renal I-R in the large pig model of renal transplantation.

***Key words:*** Renal transplantation, Ischemia-Reperfusion, Large White pig, $Na^+,K^+$-ATPase activity.

## Introduction

Kidney transplantation is a therapeutic approach that improves the quality of life of patients by avoiding renal dialysis. However, during transplantation, grafts suffer injuries of various degrees of severity induced by ischemia and reperfusion (1). Ischemia-reperfusion injury is responsible for the delayed recovery of organ function that increases the risk of rejection after transplantation, compromising the long-term fate of the graft (2-3).

Acute renal failure (ARF) induced by Ischemia-Reperfusion (I-R) is associated with significant impairment of tubular sodium reabsorption (1). This reabsorption function needs the energy-dependent transport of sodium against its electrochemical gradient by $Na^+,K^+$-ATPase, which has a crucial role in tubular sodium reabsorption and in the maintenance of water and ions homeostasis (4, 5). A loss in cell polarity, and the presence of $Na^+,K^+$-ATPase in the apical pole, is associated to organ diseases (6-10).

Data of enzyme $Na^+,K^+$-ATPase activity during ischemia and reperfusion are controversial. Indeed, various animal models, as well as different lengths of ischemia and reperfusion have been studied, and the results of these studies have shown different trends of $Na^+,K^+$-ATPase activity. No changes of $Na^+,K^+$-ATPase activity was observed after 50 minutes of ischemia in the rat kidney (11) and Spiegel *et al.* reported an increased $Na^+,K^+$-ATPase activity for a similar duration of ischemia (12). Other studies by Coux *et al.* showed a decreased $Na^+,K^+$-ATPase activity on plasma membrane fraction after 40 minutes of renal ischemia (13) and normalized level after 40 minutes of ischemia followed by 1 hour of reperfusion (14). In our previous works on cardiac and cerebral ischemia, we evidenced a common decreased $Na^+,K^+$-ATPase activity after periods of ischemia (15, 16).

Since controversial data seem to exist for the renal $Na^+,K^+$-ATPase and, to the best of our knowledge, nothing is known on the modulation of the $Na^+,K^+$-ATPase activity following sequences of ischemia-reperfusion in the large white pig kidney model of auto-transplantation, this study was undertaken to document $Na^+,K^+$-ATPase in the cortical and medullar renal tissues in experimental ischemia reperfusion and autotransplantation in pig. The ouabain sensitive $K^+$ -stimulated paranitrophenyl phosphatase assay was used in plasma membrane enriched fraction as a representation of the $Na^+,K^+$-ATPase activity.

## Materials and methods

### *Surgical procedures and experimental groups*

Fifteen large white male pigs (INRA, GEPA, Le Magneraud, Surgères, France) weighting 30–35 kg were prepared as previously described (17). All experiments were performed according to the Institutional Animal Care and Use Committee guidelines (Ministère de l'Agriculture, Article R214-87 and R215-10 from « code rural »). The animal protocol was in accordance with INRA ethical guidelines.

In the auto-transplant pig model, the right kidney is har-

vested and submitted to warm ischemia (WI) for 60 min by clamping the right renal pedicle with a vascular non-traumatic clamp (18). Then the kidney was cold flushed with 400 ml of preservation solution (University of Wisconsin solution) and preserved for 24h at 4°C immersed in the solution before auto-transplantation. (19). In each experimental group, the left kidney was removed to mimic the reduced nephron mass in transplanted situation. A reduction in nephron mass mimics a reduction in the nephron number thus reducing sodium excretory capacity and inducing hypertension and reduced renal reserve, thereby limiting compensation for renal injury. Three groups were studied: C-control (sham-operated); ISC 60-warm 60 minutes of ischemia; ISC 60+R3- 60 minutes of warm ischemia followed by a cold conservation of 24h in University of Wisconsin (UW) solution and 3 hours of reperfusion. Medulla and cortex samples were removed from eleven animals, frozen in liquid nitrogen, and stored at -80C until use.

### *Kidney medulla and cortex plasma membrane isolation*

Frozen pieces of medulla and cortex (100-300 mg) were homogenized in ice-cold buffer containing 250 mM sucrose, 80 mM KCL, 20 mM, tetrasodium pyrophosphate, 0.1 mM phenylmethane sulfonyl fluoride, 1 mM EDTA, and 20 mM imidazol-HCl, pH 7.4, with a polytron PT 10 (5 sec, setting 5) (20). The homogenate was subfractionated by two sequential differential centrifugations at 12,000 x g for 5 min and 540,000 x g for 5 min, using a TLA100.3 rotor in the Beckman TL 100 centrifuge (Beckman Instruments; Gagny, France). The final pellet was re-suspended in 250 mM sucrose and 16 mM HEPES-HCl, pH 7.4 and stored at -80C until use. These preparations consisted of a membrane fraction highly enriched in $Na^+$,$K^+$-ATPase.

### *$K^+$-stimulated paranitrophenyl phosphatase activity*

$Na^+$,$K^+$-ATPase activity was measured as $K^+$-stimulated paranitrophenyl phosphatase (pNPPase) activity ($K^+$-Pase activity) using a modified method from Maixent *et al.* (2014) (20). In this method, the measurement of the $Na^+$,$K^+$-ATPase activity is less dependent on the vesicular nature and the presence of detergent to obtain leaky vesicles. The $Na^+$,$K^+$-ATPase activity from right side out vesicles could be fully measured by using the ouabain dependent $K^+$-Pase activity. No inside-out vesicles could be evidenced by a digitoxigenin dependent activity. The activity in triplicate was assessed in 96-wells plates. Each well contained (final volume 300 µl) 6 mM $MgCl_2$, 30 mM Imidazole, 250 mM sucrose, 8 mM pNPP (Sigma Aldrich, France) with 20 mM KCl or 20 m KCl and $10^{-3}$ M ouabain (Sigma Aldrich, France). The enzymatic reaction was initiated by the addition of protein (1 µg). As similar inhibition of the enzyme activity was obtained with $Na^+$ (20-100 mM) and ouabain, we did not choose to pre-incubate the membranes with ouabain before addition of $K^+$. Optical density was measured at 405 nm using the Wallac 1420 VICTOR³™ multilabel, multitask plate reader. $K^+$-stimulated paranitrophenyl phosphatase (pNPPase) activity was calculated as the difference in paranitrophenyl (pNP) production with KCl or KCl-ouabain-NaCl. Enzyme activities are expressed as µmol pNP/h/mg of protein. Protein content was determined by the method of Lowry et al. (21), using bovine serum albumin (Sigma) as a standard.

### *Statistical analysis*

Results are expressed as means ± SEM of three (cortex) and four (medulla) experiments. Statistical analysis was done using analysis of variance (ANOVA) followed by the Tukey's post-test (GraphPad Prism®, GraphPad Software, San Diego, CA). Values of $P<0.05$ were considered statistically significant.

## Results

$Na^+$,$K^+$-ATPase activity was determined using Kpase assays ($K^+$-stimulated paranitrophenyl phosphatase activity) in crude membrane preparations of kidney medulla and cortex biopsies. $K^+$-stimulated paranitrophenyl phosphatase activities were higher in the medulla than in the cortex in all conditions (control, ischemia 60 minutes and 60 minutes ischemia followed by 3 h reperfusion, $P<0.05$) (Figures 1a & 1b). In each of these renal regions, we also compared this activity in the three different experimental conditions: control, 60 minutes of ischemia and 60 minutes of ischemia followed by reperfusion for 3 hours. In the cortex (n = 3 for each condition), $K^+$-stimulated paranitrophenyl phosphatase

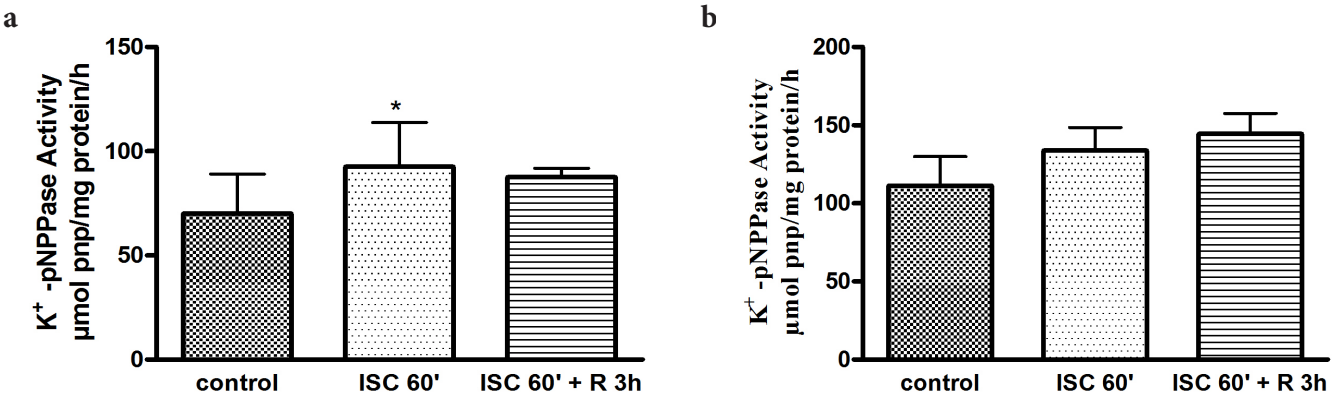


**Figure 1. (a)** $K^+$-stimulated paranitrophenyl phosphatase activity in renal cortical membranes control; ischemia 60 minutes (ISC 60'); 60 minutes of warm ischemia followed by a cold conservation of 24h in University of Wisconsin (UW) solution and 3 hours of reperfusion (ISC 60'+R3h). Data are means ± S.E.M. In all groups n=3. **P*<0.05 vs. control. **(b)** $K^+$-stimulated paranitrophenyl phosphatase activity in renal medullary membranes control; ischemia 60 minutes (ISC 60'); 60 minutes of warm ischemia followed by a cold conservation of 24h in University of Wisconsin (UW) solution and 3 hours of reperfusion (ISC 60'+R3h). Data are means ± S.E.M. In all groups n=4 (number of animals).

activity increased significantly by 35% after 60 minutes ischemia compared to control ($P < 0.05$). The increase in activity (25%) after 3 hours of reperfusion was not statistically significant (Figure 1a).

Results from the $K^+$ -stimulated paranitrophenyl phosphatase activity in the medulla ($n = 4$ for each condition), showed increased activity after 60 minutes of ischemia by 25% and 30% after 3 hours of reperfusion compared to control (Figure 1b). This difference was not significant ($P > 0.05$).

## Discussion

In this work we examined whether Ischemia-Reperfusion affects $Na^+,K^+$-ATPase activity in a renal auto graft model (Large White Pig) with controlateral nephrectomy. Renal Ischemia-Reperfusion injury is a leading cause of acute renal failure (ARF), and it is well known that the pathogenesis of ARF is multifactorial. However, alteration of renal $Na^+,K^+$-ATPase function is considered to be a crucial factor of impaired cellular sodium and water homeostasis.

In numerous $Na^+,K^+$-ATPase studies, the enzyme activity is measured in the plasma membrane fractions. These membrane fractions are enriched in $Na^+,K^+$-ATPase. During the membrane preparation, sealed and leaky vesicles are formed and sealed vesicles can be right side out or inside out. Ouabain sensitive ATPase activity requires the presence of $Na^+$, $Mg^{2+}$, and ATP at the cytoplasmic face of the membrane and $K^+$ and ouabain to inhibit at the extracellular face. Ouabain and ATP poorly diffuse across the membrane and have opposite sidedness of action (22)**.** It has been proposed that detergent activation results from increased permeability of the membranes (23). Although this hypothesis has been given further support by Walter (1975) and Jones *et al.* (1977) (24, 25), the detergent activation of ligand binding complexities have led some to propose that the phenomenon reflects alteration of subunit interactions (26).

To experimentally explore $Na^+,K^+$-ATPase activity, we used the $K^+$ -stimulated paranitrophenyl phosphatase (pNPPase) activity. The associated $K^+$-pNPPase is equally distributed across the plasma membrane (27). Hence, the $K^+$-pNPPase as a step of the $Na^+,K^+$-ATPase cycle can be used as a convenient and reliable marker for the functional $Na^+,K^+$-ATPase system without denaturing conditions of $Na^+,K^+$-ATPase assay in the presence of detergents or proteases. This method can reconcile disparate results of measuring of $Na^+,K^+$-ATPase activity using different methods such as $Na^+,K^+$-ATPase, 3-O-MFP and $^3H$ ouabain binding (28).

The results obtained with the assay of $Na^+,K^+$-ATPase activity showed differences in activities between the two kidney regions studied (cortex and medulla) in control condition. These data confirms a heterogeneous distribution of $Na^+,K^+$-ATPase along the nephron. Indeed the number of $Na^+,K^+$-ATPases in the medulla at the center of sodium reabsorption is higher than in the cortex where glomerular filtration occurs (29).

Our data shows an alteration of $Na^+,K^+$-ATPase activity in cortical plasma membrane fractions after 60 minutes of ischemia. We expected a decrease of $Na^+,K^+$-ATPase activity during physiopathological conditions studied in this report according to the results of $Na^+,K^+$-ATPase activities during cardiac, cerebral or renal ischemia (15, 16, 30). These increased values are in agreement with the previous results from Spiegel *et al.* (12) describing an increased $Na^+,K^+$-ATPase activity after ischemia in the rat kidney. Previous works on renal proximal tubule cells demonstrate that ATP depletion induces a disorganization of the cytoskeleton and a loss of cell polarity. These in turn resulted in a redistribution of functional protein to the apical pole (7). Coux *et al.* (14) observed an increase of α-subunit abundance on apical membrane fractions of rat kidney after similar sequence of ischemia-reperfusion (i.e.; 40 minutes of ischemia followed by 60 minutes of reperfusion). This result suggests a new $Na^+,K^+$-ATPase protein synthesis induced by ischemia-reperfusion. The increase of $Na^+,K^+$-ATPase proteins and/or the maintainance of enzyme activity after depolarization in apical pole may explain the increased activity observed. The cellular polar distribution of $Na^+,K^+$-ATPase and its membrane activation during diseases could be investigated *in situ* using Kpase activity assay in histological preparations as we previously described in the liver (10).

This study was terminated before the target sample size could be achieved because of administrative issues. So this study is preliminary and has limitations in that the number of animals tested was too small and not powered to detect all statistical differences. A statistical difference could be evidenced for cortical enzyme activity after one hour of ischemia.

In the literature, few studies have distinguished the medulla and the cortex when renal ischemia-reperfusion was studied. Almost all studies have focused on cortical or total renal tissue. In this study, we investigated for possible $Na^+,K^+$-ATPase activity alteration in renal medullary tissue and our results showed an increased of activity after ischemia and ischemia-reperfusion by 25% and 30% respectively compared to control. A recent study demonstrated that $Na^+,K^+$-ATPase α-subunit expression in rat medullas after 40 minutes of ischemia and 24 hours of reperfusion increased and normalized to control level after 48 hours of reperfusion (31). More research is needed to support this preliminary result and our speculations on the activation of medulla $Na^+,K^+$-ATPase activity.

In summary, we examined whether renal Ischemia-Reperfusion affects $Na^+,K^+$-ATPase activity in a large experimental model of kidney transplantation physiologically similar to humans after DCD. These preliminary data suggest that renal Ischemia induces a significant alteration of $Na^+,K^+$-ATPase by increasing its ouabain-sensitive $K^+$-stimulated paranitrophenyl phosphatase (pNPPase) activity in isolated membrane from cortical renal tissues after one hour of ischemia. This change of activity seems to be a new regulatory mechanism of an important membrane $K^+$ transporter during renal Ischemia-Reperfusion in a large pig model of transplantation.

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