# Effect of ischemia reperfusion on sodium-dependent phosphate transport in renal brush border membranes

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## Abstract

The effect of ischemia induced acute renal failure (ARF) on the transport of phosphate (Pi) after early (15–30 min) and prolonged (60 min) ischemia in the brush border membrane vesicles (BBMV) from rat renal cortex was studied. Sodium-dependent transport of Pi declined significantly and progressively due to ischemia. Western blot analysis of BBM from ischemic rats showed decreased expression of NaPi-2. A compensatory increase was observed in Pi uptake in BBMV from contralateral kidneys. There was no significant difference in NaPi-2 expression between BBMV from sham and contralateral kidneys. Early blood reperfusion for 15 min after 30 min ischemia caused further decline in Pi uptake. Prolonged reperfusion for 120 min caused partial reversal of transport activities in 30-min ischemic rats. However, no improvement in the transport of Pi was observed in 60-min ischemic rats after 120 min of blood reperfusion. Kinetic studies showed that the effect of ischemia and blood reperfusion was dependent on the $V_{max}$ of the Na-Pi transporter. Western blot analysis showed increased expression of NaPi-2 in the BBMs from ischemia–reperfusion animals. Further, a shift in the association of Na ions to transport one molecule of Pi was observed under different extracellular Na concentrations [Na]$_o$. Feeding rats with low Pi diet and/or treatment with thyroid hormone (T3) prior to ischemia resulted in increased basal Pi transport. Ischemia caused similar decline in Pi transport in BBM from LPD and/or T3 animals. However, recovery in these animals was faster than the normal Pi diet fed (NPD) animals. The study suggests a change in the intrinsic properties of the Na-Pi transporter in rat kidneys due to ischemia. The study also indicates that treatment with T3 and feeding LPD prior to ischemia caused faster recovery of phosphate uptake due to ischemia–reperfusion injury.
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Keywords: Acute renal failure; Ischemia; Pi transport; L-Proline transport; Thyroid hormone; Low phosphorus diet

## 1. Introduction

Renal ischemia and toxic insults to the kidney result in profound alterations in the structure and excretory functions of the kidney, and depending on the severity of damage caused, lead to reversible or irreversible acute renal failure (ARF) [1–3]. Morphological studies have shown that ischemic or toxic ARF causes damage to the renal proximal tubular cells, especially its brush border membrane (BBM) [1,4,5]. The effect of ischemia on renal proximal tubules is manifested in rats by reversible disappearance of brush border microvilli [1,4,6,7], or irreversible interiorization of the BBM and loss into lumen [3] leading to cell necrosis [8]. We have previously demonstrated that the activities of the BBM marker enzymes [9] and carbohydrate metabolism enzymes [10] are reversibly and variably decreased with ischemia in various sub-populations of the renal tissue. Earlier studies have shown that ischemia causes loss of membrane polarity, changes in membrane fluidity, membrane phospholipids [11,12], loss of selective permeability [6] and decrease in the proximal tubular fluid [13], sodium [14], glucose [15], and cation [16] reabsorption. However, the transport of phosphate (Pi) which

is involved in the maintenance of energy for many renal functions [17,18] was not studied under reversible and relatively irreversible ischemic conditions. Silverman et al. [19] has reported a decline in the transport of Pi after 45–60 min ischemia, which was attenuated by the administration of nitrendepine to ischemic rats. Recently, Xiao et al. [20] and Kwon et al. [21,22] have shown a decrease in the renal expression of rat type II sodium phosphate cotransporter (NaPi-2) and several other sodium coupled transporters including Na-K ATPase and NHE-3 after ischemia and reperfusion injury. Based on these studies, we hypothesized that 30 min ischemia will result in reversible loss of phosphate transport while 60 min ischemia will result in irreversible injury to the kidney functions.

To address this hypothesis, we measured in detail the transport of Pi in rats subjected to ischemia and reperfusion. The results demonstrate that ischemia caused decrease in Pi uptake and expression of NaPi-2. Reperfusion of blood to 30 min ischemic kidneys resulted in partial recovery while reperfusion to 60 min ischemic kidneys caused irreversible loss of Pi uptake.

## 2. Methods

### 2.1. Materials

Wistar rats were purchased from All India Institute of Medical Sciences (New Delhi, India). Standard rat pellet diet was purchased from Amrut Laboratories (Pune, India). Smooth surface $1.5 \times 10$ mm stainless steel microaneurysm clips were purchased from Asian Surgicals (Hyderabad, India). $^{32}$Pi, L-$^3$H-proline, and D-$^3$[H]-Glucose were purchased from Bhabha Atomic Research Center, (Mumbai, India). Scintillation fluid (Cocktail-T) was purchased from Scientific Research Laboratories (Mumbai, India). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

### 2.2. Animal protocol

Animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by US National Institute of Health (NIH Publication No. 85–23, revised 1985). Young male Wistar rats weighing 150–200 g, fed a standard rat diet and water ad libitum, were used in the study. On the day of the experiment, the rats were anesthetized by an injection of pentobarbital sodium (50 mg/kg-body wt, ip). The abdomen was opened by a left flank incision, and the left renal artery was separated from the renal vein. Ischemia was produced by clamping the left renal artery for the required time, as specified in Result, with smooth surface $1.5 \times 10$ mm stainless steel microaneurysm clip (Asian Surgicals, Hyderabad, India). Ischemia was confirmed by observing blanching of the kidney surface. During ischemia and reperfusion, the wound was temporarily closed to maintain body temperature. Sham-operated rats, subjected to the same surgical procedure except that the renal artery was not clamped, were used as controls. For reperfusion studies, renal artery was declamped and the blood was allowed to reperfuse for variable time periods (15–120 min). After the designated time of ischemia and/or blood reperfusion, the kidneys were removed, decapsulated, and kept in ice-cold buffered saline. The cortex was carefully separated from the medulla to get whole cortex as described earlier [9].

### 2.3. Brush border membrane preparation

Brush-border membrane vesicles (BBMV) were prepared at 4 °C, using MgCl₂ precipitation method, exactly as previously described [9]. Briefly, freshly minced cortical slices were homogenized in 50 mM mannitol and 5 mM Tris–HEPES buffer pH 7.0 (20 ml/g), in a glass Teflon homogenizer with four complete strokes. The homogenate was then subjected to high-speed [20,500 revolutions/min (rpm)] homogenization in an Ultra Kunkel homogenizer for three strokes of 15 s each with an interval of 15 s between each stroke. MgCl₂ was added to the homogenate to a final concentration of 10 mM and slowly stirred for 20 min. The homogenate was spun at $2000 \times g$ in J2-21 Beckman centrifuge using JA-17 rotor. The supernatant was centrifuged at $35,000 \times g$ for 30 min using JA-20 rotor. The pellet was resuspended in 300 mM mannitol and 5 mM Tris–HEPES, pH 7.4, with four passes by a loose fitting Dounce homogenizer (Wheaton, IL) and centrifuged at $35,000 \times g$ for 20 min in 15 ml corex tubes, using JA-20 rotor. The outer fluffy pellet was resuspended carefully in a small volume of buffered 300 mM mannitol. Aliquots of homogenates were also saved to check the membrane purity by enzyme analysis. Protein concentration in the homogenates and membrane preparations was determined by the modified method of Lowry et al. [23] as described by Yusufi et al. [24].

The purity of BBM(s) was confirmed by analyzing the activities of alkaline phosphatase, γ-glutamyl transpeptidase (BBM marker enzymes), Na$^+$–K$^+$ ATPase (basolateral membrane enzyme) and acid phosphatase (lysosomal enzyme). The activities of alkaline phosphatase and γ-glutamyl transpeptidase were 7- to 9-fold higher in BBM fractions, and Na$^+$–K$^+$ ATPase and acid phosphatase activities were deenriched in the BBM (Table 1).

### 2.4. Transport

Measurement of $^{32}$Pi (Bhabha Atomic Research Center, India), uptake in BBMV(s) was carried out at 25 °C by rapid filtration technique as described by Yusufi et al. [24] either in the presence or absence of Na-gradient. Uptake was initiated by addition of 30 μl of incubation medium containing 100 mM mannitol, NaCl/KCl 100 mM, 5 mM Tris–HEPES, pH 7.5, 0.1 mM K₂H$^{32}$PO₄ to 15 μl BBM suspension (50–100 μg protein) and incubated for the desired time intervals (see Results). The uptake was stopped by the addition of 3 ml ice cold stop solution (containing 135 mM NaCl, 5 mM Tris–HEPES and 10 mM sodium arsenate, pH 7.5) and filtered immediately through 0.45 μm DAWP Millipore filters (Millipore, USA) and washed three times with the stop solution using a Cornwall type syringe (Wheaton, IL). Correction for non-specific binding to filters was made by subtracting from all data the value of corresponding blank obtained by filtration of the incubation buffer without vesicles. The radioactivity of dried filters was measured by liquid scintillation

Table 1
Effect of ischemia–reperfusion on enrichment of BBM enzyme activities

| | Alkaline phosphatase* | γ-Glutamyl transferase | Na-K ATPase | Acid phosphatase |
|---|---|---|---|---|
| Sham control | 11.02 | 12.81 | 0.55 | 0.27 |
| Ischemia | | | | |
| 15 min | 8.13 | 9.91 | 0.43 | 0.39 |
| 30 min | 6.41 | 6.93 | 0.31 | 0.29 |
| 60 min | 6.37 | 7.34 | 0.29 | 0.43 |
| Contralateral | | | | |
| 15 min | 9.27 | 11.01 | 0.27 | 0.33 |
| 30 min | 9.76 | 10.75 | 0.44 | 0.25 |
| 60 min | 10.90 | 13.48 | 0.65 | 0.48 |
| Ischemia 30 min Reperfusion | | | | |
| 2 min | 7.65 | 6.37 | 0.38 | 0.30 |
| 15 min | 7.49 | 5.55 | 0.30 | 0.44 |
| 60 min | 9.67 | 7.01 | 0.33 | 0.31 |
| 120 min | 11.24 | 10.16 | 0.47 | 0.43 |

Activities of alkaline phosphatase, γ-glutamyl transferase, Na-K ATPase, and acid phosphatase were measured in the BBM and cortical homogenates as described previously (9). *Activity is represented as the ratio of specific activity (μmole p-nitrophenol, p-nitroaniline, or inorganic phosphate released/mg protein/h) in BBM to the specific activity in the homogenate. Values are mean from two independent BBM preparations.

counting (Reckbeta, LKB, Wallac) with 10 ml scintillation fluid (Cocktail T, SRL, India). Preliminary experiments showed that Na-dependent uptake of $^{32}Pi$ was not different in the BBMV(s) isolated from left or right kidney of sham-operated control rats and therefore BBMV(s) were prepared by pooling both the kidneys in the sham-operated controls.

## 2.5. Immunoblotting

BBM(s) were prepared from the control and ischemic rat kidneys, the proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with antiserum to rat type IIa Na-dependent Pi transporter (NaPi-2).

## 2.6. Preparation of low phosphate diet (LPD)

The LPD was prepared as described by Kempson et al. [25] in the laboratory under sterile conditions by mixing (in gm%) 66% sucrose, 20% casein, 4% salt mixture (1.02% NaCl, 0.53% KCl, 0.305% $MgSO_4$, 0.103% iron citrate, 1.02% $CaCO_3$, and 0.014% cupric sulphate), 0.4% vitamin mixture (Glaxo, India) and 10% vegetable oil and making the pellets using water. The normal phosphate diet (NPD) was prepared by supplementing LPD with a mixture of sodium and potassium phosphates (ratio of monobasic to dibasic salt was 1:4) to a final concentration of 0.7% w/w phosphorus.

## 2.7. Preparation of thyroid hormone (T3)

4.37 mg T3 (Sigma, St. Louis, MO) was dissolved in 10 ml of 10 mM NaOH. 10 ml Tris (5 mM) in 1.8% NaCl was added to T3 solution. The pH was slowly adjusted to $9.4\pm0.1$ with 0.1 N HCl. The solution was diluted to 25 ml with a mixture of vehicle (10 ml of 10 N NaOH, 10 ml 5 mM Tris in 1.8% NaCl pH $9.4\pm0.1$).

## 2.8. Animal protocol for T3 treatment and LPD

Separate groups of animals were placed simultaneously on the different dietary regimens and throughout the experiment all groups were studied in parallel (Fig. 1). The animals were stabilized on NPD for seven days. After day 7, the animals were divided into four groups (12 animals in each group) and the allocation of animals was adjusted so that at this time they did not differ in body weight and urinary Pi. The rats were then fed with specified diets for seven days. Group I received NPD and 1 ml intraperitoneal (ip) injection of vehicle, Group II received NPD and 1 ml ip injection of T3 (100 μg/100 g body wt), Group III received LPD and 1 ml ip injection of vehicle and Group IV received LPD and 1 ml ip injection of T3 (100 μg/100 g body wt). After 7 days of treatment, the rats were anaesthetized and ischemia was produced for 30 min as described above followed by reperfusion of blood for 15 and 60 min. BBMVs were prepared and Na-dependent Pi uptake was measured as described above.

## 2.9. Statistical analysis

All experiments were repeated at least 3–5 times to document reproducibility. In each experiment tissue from five to six animals was pooled to prepare BBM in each group. All data are expressed as mean±S.E. Statistical evaluation was conducted by ANOVA followed by Bonferroni's analysis using Graph Pad software. A $P$ value less than 0.05 was a priori considered statistically significant.

## 3. Results

### 3.1. Effect of ischemia on the transport of Pi

To study the time course of ischemia on the sodium-dependent Pi uptake, the left renal artery was occluded for 15,
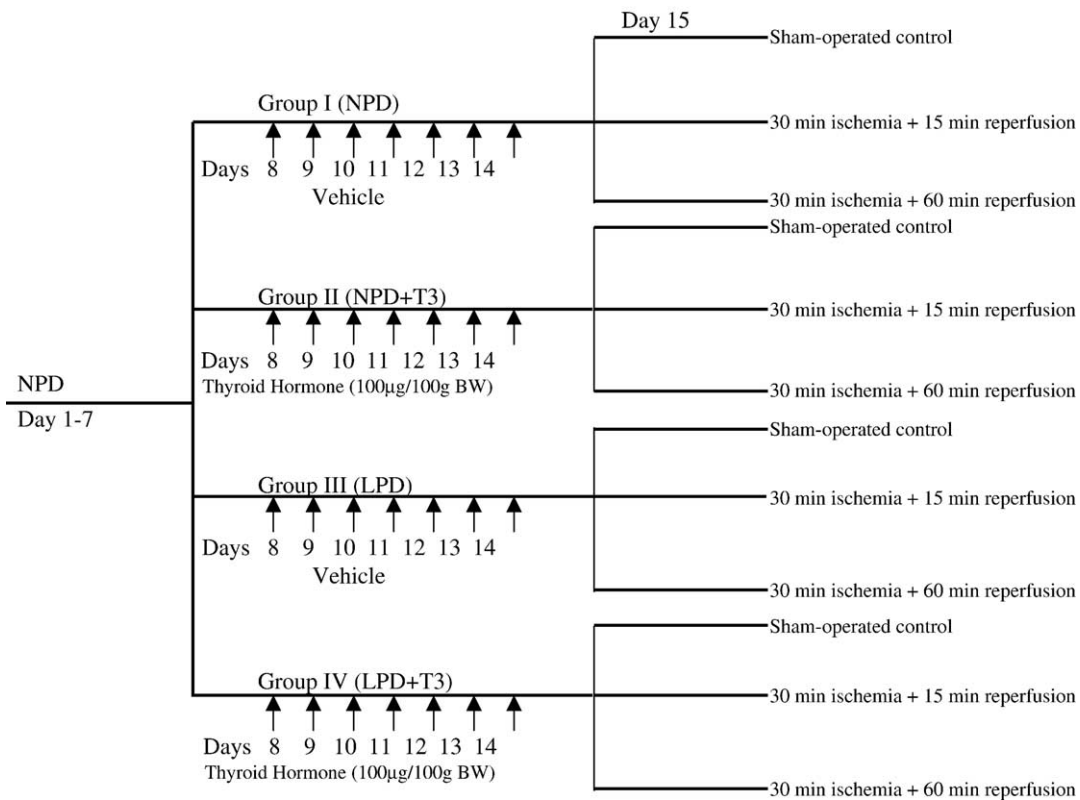


Fig. 1. Flow chart for T3 and LPD treatment: Flow diagram of the dietary regimen and T3 treatment in rats prior to inducing ischemia–reperfusion injury. Group I received normal phosphate diet (NPD) and 1 ml injection of vehicle (ip), Group II received NPD and 1 ml injection of T3 (ip), Group III received low phosphate diet (LPD) and 1 ml injection of vehicle (ip), and Group IV received LPD and 1 ml injection of T3 (ip). The rats with 30 min ischemia and 15 min reperfusion were considered ischemic rats, while rats with 30 min ischemia and 60 min reperfusion were considered recovered rats.

30 and 60 min as described in Methods, followed by 2 min reperfusion. Uptake of Pi in the presence of a Na-gradient ($Na_o > Na_i$) was measured at 5, 20, and 40 s as described in Methods (Fig. 2). As shown in Fig. 2A, phosphate uptake maximally decreased after 15 min of ischemia. The Pi uptake after 30 and 60 min ischemia was significantly higher compared to 15 min ischemic group but lower than the sham-operated controls (Fig. 2A). The uptake of $^{32}$Pi was not significantly different in BBMVs prepared from ischemic and sham-operated rats when the uptake was measured after sodium equilibration (120 min of incubation). Ischemia produced a significant and time-dependent decrease in uptake of L-[$^3$H] proline (data not shown). To confirm that the changes in $^{32}$Pi uptake were due to changes in the number of active Na-Pi cotransporters, NaPi-2 expression was determined by Western blot analysis using anti-NaPi2 (the rat type IIa NaPi cotransporter) antisera. As shown in Fig. 2B, ischemia decreased the expression of NaPi-2 cotransporter in the BBM from ischemic kidneys. A compensatory increase in uptake of $^{32}$Pi was observed in the BBMV(s) isolated from the contralateral kidneys (Fig. 2C). Western blot analysis showed no significant changes in the expression of NaPi-2 in BBM from contralateral kidneys (Fig. 2D).

To determine whether the increase in Pi uptake after 30 and 60 min ischemia compared to 15 min ischemic animals was due to an increase in Na-independent Pi uptake, the uptake of Pi was determined in the absence of sodium (NaCl replaced by KCl ($K_o > K_i$) in the incubation medium). As shown in Fig. 3, ischemia caused a significant increase in Na-independent

diffusion of Pi after 30 and 60 min ischemia. Therefore, net Na-dependent uptake (difference in uptake between $Na_o > Na_i$ and $K_o > K_i$) was calculated. The net Na-dependent uptake at 20 s decreased proportional to the duration of ischemia (Fig. 3).

## 3.2. Effect of ischemia and reperfusion on the transport of $^{32}$Pi

To study the effect of ischemia and reperfusion, ischemia was produced for 30 min or 60 min followed by reperfusion for 2, 15, 60, or 120 min. As observed in the previous section, the net sodium-dependent phosphate uptake declined significantly (35–40%) after 30 min ischemia and 2 min reperfusion. Reperfusion of blood for 15 min caused a further decrease (55%) in $^{32}$Pi uptake. Sodium-dependent $^{32}$Pi uptake recovered significantly (60–80%) after 2 h reperfusion (Fig. 4A). To confirm that the changes in uptake were due to changes in the number of active Na-Pi cotransporters, NaPi-2 expression was determined by Western blot analysis. As shown in Fig. 4C, ischemia decreased the expression of NaPi-2 cotransporter in the BBM from 30 min ischemic kidneys. The expression was restored after reperfusion of blood for 120 min. Similar results were obtained when L-$^3$H-proline and D-$^3$[H]-Glucose uptake was measured in the same BBMV preparations (data not shown).

In contrast to 30 min ischemia, 60 min ischemia produced an irreversible loss in Pi uptake. As shown in Fig. 4B, ischemia for 60 min decreased net Na-gradient-dependent transport of $^{32}$Pi. The uptake did not recover after 120 min reperfusion.
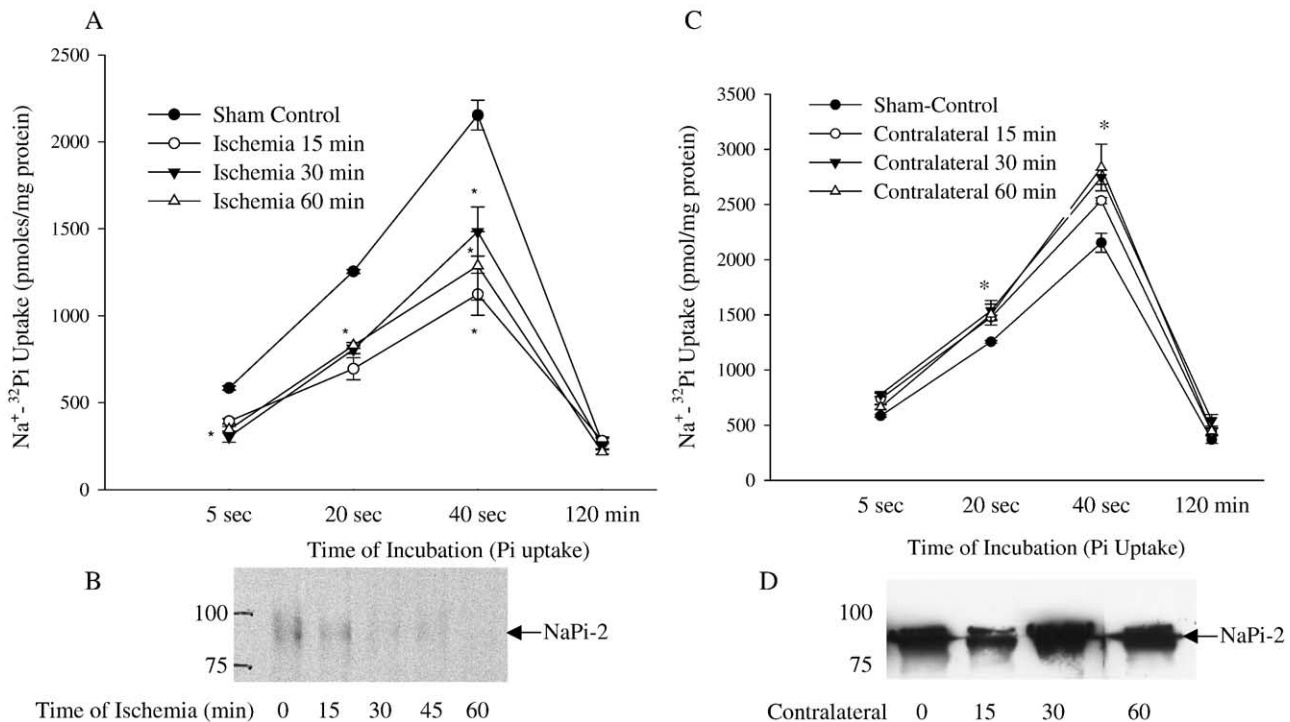


Fig. 2. Time course of sodium-dependent phosphate uptake during ischemia: Ischemia was produced by occlusion of the left renal artery as described in Methods. BBMs were prepared from ischemic (Panels A and B) or contralateral (Panels C and D) kidneys. Pi uptake was measured in BBM from control (●), 15 min ischemic (○), 30 min ischemic (▼), and 60 min ischemic (△) rats as described in Methods. Sodium-dependent phosphate uptake was measured at 5 s, 20 s, 40 s, and 120 min as described in Methods. Values are expressed as mean ± S.E. from four different experiments. *Indicates significantly different from control group ($P < 0.05$) by ANOVA followed by Bonferroni's analysis. Representative Western blots for NaPi-2 in BBM from ischemic (Panel B) and contralateral kidneys (Panel D).
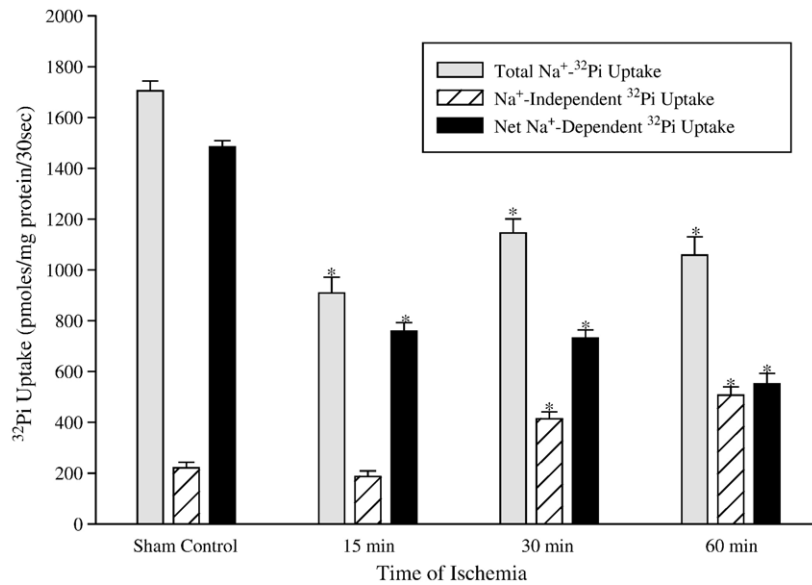
Fig. 3. Net sodium-dependent phosphate uptake during ischemia: Ischemia was produced by occlusion of the left renal artery as described in Methods. BBMs were prepared from control, 15 min ischemic, 30 min ischemic, and 60 min ischemic rat kidneys as described in Methods. Sodium-dependent and sodium-independent phosphate uptake was measured at 20 s as described in Methods. Each bar represents mean±S.E. from four different experiments. *Indicates significantly different from control group ($P < 0.05$) by ANOVA followed by Bonferroni's analysis.

## 3.3. Kinetics of $^{32}$Pi uptake during ischemia and reperfusion

To further characterize the changes in $^{32}$Pi uptake due to ischemia and reperfusion, the uptake was determined at various concentrations of $^{32}$Pi in the incubation medium. As shown in Table 2 reduction in the $^{32}$Pi uptake after 30 min of ischemia was due to decrease in the maximum rate of transport ($V_{max}$), after 15 min reperfusion. The increase in Pi uptake after 60 and 120 min of reperfusion was due to an increase in the $V_{max}$ without any significant change in the $K_m$ values (Table 2).

## 3.4. Effect of extra-vesicular Na-concentrations in the incubation medium on the transport of $^{32}$Pi during 30-min ischemia and up to 120-min blood reperfusion

In order to further characterize the effect of ischemia, $^{32}$Pi uptake was determined under Na-gradient ($Na_o > Na_i$) and non-Na-gradient ($Na_o = Na_i$) conditions. As shown in Table 3, the net Na-dependent $^{32}$Pi uptake decreased Pi uptake only when Na-gradient was present.

Pi transport was also determined at various extra-vesicular Na concentrations ($Na_o = 20$, 40, 60 and 100 mM in the incubation medium). The total osmolality and molar concentration of $Cl^-$ in the medium was held constant (300 mosM, and 100 mM) by appropriate additions of choline chloride as reported by Szczepanska-Konkel et al. [26,27]. As shown in Fig. 5, the $^{32}$Pi transport increased steadily with the increase in extra-vesicular Na concentrations in incubation medium. At all concentrations of extra-vesicular Na concentrations $^{32}$Pi uptake decreased maximally after 30-min ischemia/15 min reperfusion. Reperfusion for 60 and 120 min significantly increased the transport compared to 15 min reperfusion. The graphic analysis of the relationship between $^{32}$Pi uptake and $[Na]_o$

showed sigmoidal curve in sham-operated controls but not in ischemic/reperfused groups (Fig. 5). Hill Plot transformation of the data showed that the calculated half-maximum substrate concentrations ($[S]^{0.5}$) were not significantly different. However, $n$ values that represent the association of Na for transport of one Pi decreased significantly from the sham-operated controls (Table 4).

## 3.5. Effect of thyroid hormone (T3) and low phosphate diet (LPD) on ischemia induced inhibition of Na-dependent $^{32}$Pi uptake

Previous studies have shown that thyroid hormone [28,29] and LPD [30,31] increase Na-Pi uptake in BBMVs from normal rat kidney by increasing the expression of Na-dependent phosphate cotransporters. To determine whether pre-treatment with T3 or LPD could prevent the inhibition of Na-Pi cotransport due to ischemia, rats were pre-treated with T3 and/or fed with LPD for seven days prior to ischemia (Fig. 1). As shown in Fig. 6, BBM uptake of Na-Pi maximally decreased after 30 min ischemia 15 min reperfusion in normal phosphate diet (NPD) fed rats. Pretreatment with T3 or LPD prior to ischemia caused an initial decrease in Pi uptake after 30 min ischemia and 15 min reperfusion as compared to their respective sham-operated controls, however, to a less extent than in the NPD group. The decrease in Pi uptake due to 30 min ischemia recovered completely after 60 min reperfusion in T3 and/or LPD pretreated groups.

## 4. Discussion

The results of the present study demonstrated that ischemia caused marked reductions in the transport of $^{32}$Pi and L-$^3$H-proline, which was dependent on the duration of
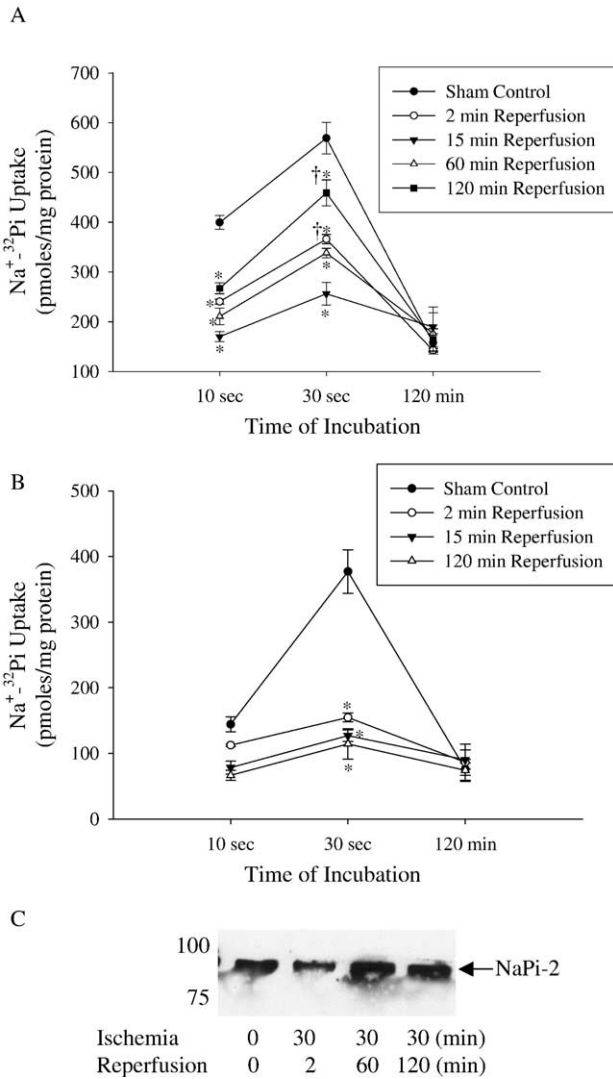
A



B



C



Fig. 4. Time course of net sodium-dependent phosphate after ischemia and reperfusion: Ischemia was produced by occlusion of the left renal artery for 30 (Panel A) or 60 min (Panel B) followed by blood reperfusion for the indicated time. BBMs from kidney cortex were prepared as described in Methods. Sodium-dependent and -independent phosphate uptake was measured at 10 s, 30 s, and 120 min as described in Methods. Values are expressed as net sodium-dependent uptake as mean ± S.E. from four different experiments. *Indicates significant difference from normal BBM, [†]indicates significantly different from 15 min reperfusion group ($P < 0.05$) by ANOVA followed by Bonferroni's analysis. Panel C: Representative Western blot for NaPi-2 in BBM from 30 min ischemia and reperfused kidneys.

ischemia. Xiao et al. [20] in a recent study reported an increase in Pi uptake after an ischemia of 30 min. In our study we have observed an increase in the uptake of Pi at 30 and 60 min ischemia as compared to 15 min ischemic animals. To understand the mechanism of recovery in Pi transport, we measured the uptake of Pi in the absence of sodium and calculated Na-dependent versus Na-independent Pi uptake. Our results show that although total Pi transport increased, Na-dependent transport actually decreased. These results suggest that ischemic damage to the kidneys produced epithelial leak, as has been suggested by Molitoris et al. [12,14,15]. Ischemia also caused a compensatory

Table 2
Effect of 30 min ischemia and reperfusion for the indicated time on the kinetic parameters of Na-Pi uptake in BBMV

| Groups | $V_{max}$ (pmol/mg protein/10 s) | $K_m$ ($\times 10^{-4}$) |
| --- | --- | --- |
| Control | 769.23 ± 10.00 | 0.085 ± 0.019 |
| 2 min reperfusion | 571.90 ± 11.55* | 0.078 ± 0.034 |
| 15 min reperfusion | 333.71 ± 7.87* | 0.074 ± 0.01 |
| 60 min reperfusion | 540.93 ± 10.34*,[¶] | 0.090 ± 0.004 |
| 120 min reperfusion | 666.67 ± 8.94*,[¶] | 0.076 ± 0.001 |

BBMVs were prepared from rat kidneys subjected to 30 min ischemia and reperfusion for 2–120 min. [32]Pi uptake was measured in the BBMVs at different phosphate concentrations. The $V_{max}$ (pmol/mg protein/10 s) and $K_m$ ($10^{-4}$M) values were calculated from Lineweaver–Burk plots from three independent preparations. * Indicates significantly different from control (sham operated), and [¶] indicates significantly different from 15 min reperfusion group ($P < 0.05$) by ANOVA followed by Bonferroni's analysis.

increase in Pi uptake in BBM isolated from contralateral kidneys without any significant changes in the expression of NaPi-2 protein. The data reported here do not allow us to speculate on the molecular mechanisms by which ischemia caused the compensatory increase in the contralateral kidneys. Further studies are required to understand the mechanism of this increase in Pi uptake in the contralateral kidneys. Under similar conditions, Na-gradient-dependent transport of L-^{3}H-proline also decreased by ischemia, and the decrease was proportional to the duration of ischemia.

Reperfusion to ischemic kidneys initiate, the regeneration process and restores partially or completely, some of the kidney functions [3,5,8–10,32]. Regeneration is dependent both on the duration of ischemia and reperfusion. We have previously shown that the activities of BBM marker enzymes [9] and carbohydrate metabolism enzymes [10] are decreased by ischemia and restored by reperfusion in animals subjected to 30 min ischemia. However, after 60 min of ischemia, the activities of BBM marker enzymes were not restored by reperfusion [9]. Kwon et al. [21,22] have shown that the expression of the Na-coupled transporters in kidneys subjected to 30 min ischemia returns to normal levels after reperfusion for 5 days. This recovery was enhanced by treatment with α-Melanocyte stimulating hormone. However, the expression of Na-coupled transporters did not improve after 5 days of reperfusion in 60 min

Table 3
Effect of extravesicular sodium concentration on Na-Pi uptake in BBMV

| Groups | $Na_o > Na_i$ | $Na_o = Na_i$ |
| --- | --- | --- |
| Control | 259.23 ± 12.82 | 34.29 ± 9.84 |
| 2 min reperfusion | 98.12 ± 3.46* | 31.29 ± 1.37 |
| 15 min reperfusion | 65.37 ± 8.58* | 33.13 ± 5.62 |
| 60 min reperfusion | 159.16 ± 16.81*,[¶] | 33.58 ± 12.25 |
| 120 min reperfusion | 207.55 ± 10.43[¶] | 28.87 ± 8.48 |

BBMVs were prepared from rat kidneys subjected to 30 min ischemia and reperfusion for 2–120 min. [32]Pi uptake was measured in the BBMVs under high extravesicular sodium concentration ($Na_o > Na_i$) or under equal ($Na_o = Na_i$) intra- and extravesicular concentration of Na as described in Methods. The data are expressed as mean [32]Pi uptake (pmol/mg protein/20 s) ± S.E. from three independent preparations. *Indicates significantly different from control (sham operated), and [¶]indicates significantly different from 15 min reperfusion group ($P < 0.05$) by ANOVA followed by Bonferroni's analysis.
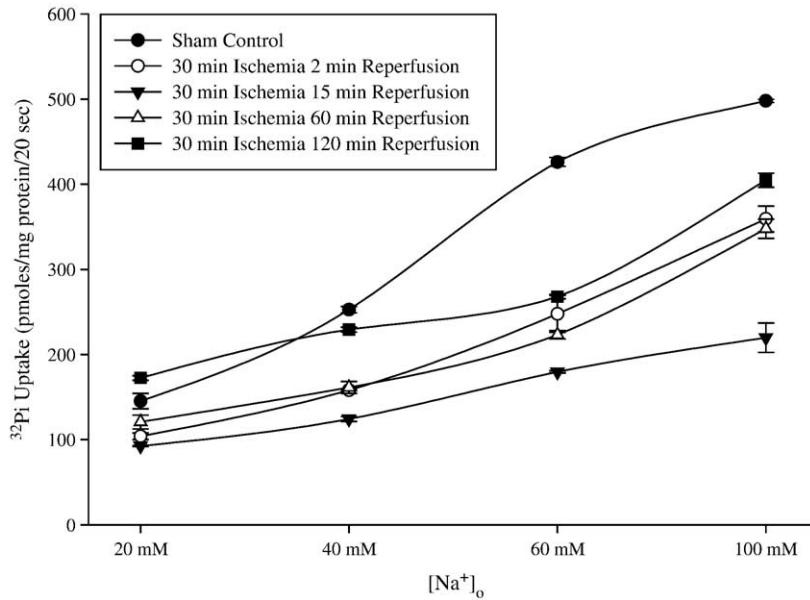
Fig. 5. Effect of extra-cellular sodium concentration [Na$_o$] on Sodium-dependent phosphate uptake in BBM from 30 min ischemic and reperfused rat kidneys: Ischemia was produced by occlusion of the left renal artery for 30 min followed by blood reperfusion as described in Methods. BBMs were prepared from rat kidneys as described in Methods. Phosphate uptake at different extracellular sodium concentration [Na$_o$] was measured at 10 s as described in Methods. Values are plotted as v (phosphate uptake/mg protein/10 s, vertical axis) to extra-cellular sodium concentration ([Na$_o$], horizontal axis. Values are expressed as mean±S.E. from three independent experiments.

ischemic rat kidneys. Recently, Rubinger et al. [33] demonstrated that NaPi-2 mRNA levels were decreased maximally after a bilateral ischemia for 60 min followed by reperfusion for 48 h. The results of the present study also indicate that 30 min ischemia caused a reversible decrease in the uptake of Na-coupled Pi and L-proline uptake. However, 60 min ischemia caused an irreversible reduction in the uptakes of Pi and L-proline. The present data also suggest that the decrease in Pi uptake produced by ischemia is due to a decrease in the $V_{max}$. These data correlate well with the earlier observations by Kwon et al. [21,22] and Xiao et al. [20] who showed decreased expression of NaPi-2 and other Na-coupled transporters in the BBM of the ischemic kidneys. We also confirmed the decrease in the expression of NaPi-2 cotransporters in the BBM of ischemic kidneys following ischemia and increase in the BBM expression of rat type II Na-Pi cotransporter, NaPi-2.

Table 4
Effect ischemia and reperfusion on Pi uptake in BBMV at different extravesicular concentration of sodium

| Groups | $n$ | $[S]_{0.5}$ mM |
|---|---|---|
| Control | 2.00±0.08 | 31.62±1.46 |
| 2 min reperfusion | 1.67±0.06* | 31.62±2.58 |
| 15 min reperfusion | 1.58±0.03* | 25.12±2.46 |
| 60 min reperfusion | 1.25±0.05* | 39.81±1.59 |
| 120 min reperfusion | 1.33±0.03* | 31.62±4.67 |

BBMVs were prepared from rat kidneys subjected to 30 min ischemia and reperfusion for 2–120 min. $^{32}$Pi uptake was measured in the BBMVs under different extravesicular sodium concentration [Na$_o^+$] as described in Methods. The data are calculated from Hill's Plot from three independent preparations and is expressed as mean±S.E. *Indicates significantly different from control group ($P < 0.05$) by ANOVA followed by Bonferroni's analysis.

Previous studies suggest alterations in the intrinsic properties of transporter molecules due to ischemia [14]. Reduced phlorizin binding was demonstrated when Na-dependent glucose transport was lowered due to ischemia indicating reductions in the number of glucose transporting molecules in the membrane [15]. Our data also suggest that the properties of the Na-dependent transporter with respect to [Na]$_o$ changed due to ischemia/reperfusion injury. Sodium serves both as a driving force, in terms of gradient (Na$_o$>Na$_i$) for the translocation of Pi across BBM, and as a factor modulating the interaction of Pi with Na-Pi co-transporter in the BBM [31,34–37]. The allosteric modulatory component of Na$_o$ action [31,36,37] likely accounts for sigmoid dependence of Pi transport on [Na$_o$]. This sigmoidal dependence of Pi was more apparent in controls, but not in ischemia/reperfused animals. A transformed Hill plot of the data yielded a straight line and the calculated $[S]^{0.5}$ values were not significantly different between the groups suggesting that $V_{max}$ for sodium did not change due to ischemia and/or reperfusion. The calculated $n$ value, which indicates the number of Na ions required for the transport of one molecule of Pi, was 2 in the normal rat kidneys. This observation is in agreement with earlier reports that two sodium ions are required to transport one molecule of phosphate in BBM isolated from normal rat kidney [31,37,38]. Biophysical studies have also shown that although the Pi transport is electrogenic, but there is no change in net charge during the Na-dependent Pi uptake process indicating that two sodium ions are transported into the cell to drive transport of one molecule of phosphate [38]. The data presented here demonstrate that ischemia significantly decreased the number of sodium ions involved in the uptake of one molecule of Pi suggesting that the intrinsic properties of
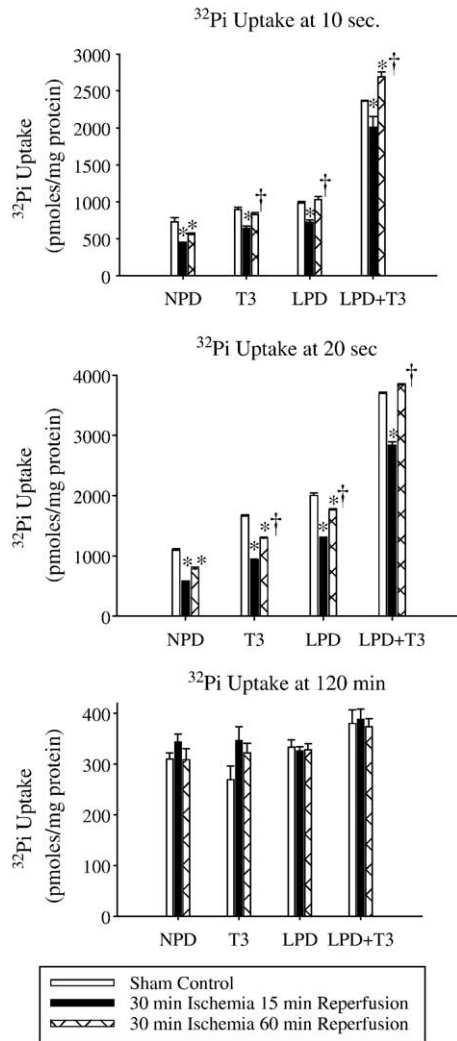
Fig. 6. Effect of T3 treatment and LPD on net sodium-dependent phosphate uptake after ischemia–reperfusion injury: Rats were treated with T3 and fed either normal or low phosphate diet for 7 days prior to ischemia–reperfusion injury as described in Methods and Fig. 1. Ischemia was produced for 30 min followed by reperfusion for 15 or 60 min, BBMs were prepared, and sodium-dependent and -independent phosphate uptake was measured as described in Methods. Values are expressed as net sodium-dependent uptake as mean ± S.E. from three different experiments. *Indicates significant difference from normal BBM, †indicates significantly different from 15 min reflow group ( $P < 0.05$ ) by ANOVA followed by Bonferroni's analysis.

the Na-Pi co-transporter might have changed due to ischemia. The changes in the intrinsic properties of the transporter are suggestive of adaptive changes to decrease in the number of transporters.

The kinetic analysis of the Pi transport showed that the decrease in the Pi uptake was due to decrease in the total number of apical Na-Pi transporter. LPD [30,31,39] and thyroid hormone [28,29] has been shown to increase the uptake of phosphate in normal kidneys by increasing the apical expression of Na-Pi cotransporters in the BBM. Changes in dietary phosphate results in adaptive changes in the renal reabsorption of phosphate. Low phosphorus diet has been known to increase Na-dependent Pi uptake in rats [39] and in opossum kidney cells, a proximal tubule cell line [40] by

increasing the total number of type II sodium coupled Pi transporters. Loghman-Adham [41] demonstrated that in chronic renal failure phosphate homeostasis is maintained primarily by a PTH-mediated adaptive increase in phosphate excretion by the remaining nephrons. Lopez-Hilker et al. [42] demonstrated that phosphate restriction corrected secondary hyperparathyroidism in dogs with advanced renal failure. Phosphorus restriction may result in an adaptive response to increased phosphate retention by renal tubular brush border membrane [43]. Humes et al. [44] and Rogers et al. [45] demonstrated that T3 enhances EGF receptor gene expression leading to increased EGF receptors in renal proximal tubules resulting in enhanced recovery following acute renal failure. Thyroid hormone has been shown to protect the heart from ischemic damage similar to ischemic preconditioning by decreasing the activation of p38 MAPK and activation of PKCδ [46,47]. However, recent clinical trials have shown that thyroxine treatment did not improve post-transplant acute tubular necrosis [48] and increased mortality in acute renal failure patients [49]. The data reported here suggest that LPD and/or treatment with T3 prior to renal ischemia may have produced an adaptive response resulting increased Pi uptake by the BBM. Although the basal reabsorption of Pi increased in BBM, ischemia caused a similar decrease in the Pi uptake as compared to rats fed with NPD. However, reperfusion caused early recovery of Pi uptake in BBM from rats fed with LPD and/or T3 treated animals than the rats fed with NPD.

In summary, the present study showed that ischemia caused decrease in the uptake of sodium-dependent Pi uptake. Reperfusion of blood to 30 min ischemic kidneys was reversible while 60 min ischemia caused an irreversible loss of Na-dependent Pi uptake. Further, this study suggests that the changes in the Pi uptake were due to changes in the intrinsic properties of the transporter. Further studies are necessary to determine the mechanisms by which LPD and T3 regulate Pi uptake in ischemia induced acute renal failure.

## Acknowledgements

## References

[1] B. Glaumann, H. Glaumann, I.K. Berezeky, B.F. Trump, Studies of cellular recovery from injury. II. Ultrastructural studies on the recovery of pars convoluta of the proximal tubules of rat kidney from temporary ischemia, Virchows Arch. 24 (1977) 1–18.

[2] J.H. Stein, M.D. Lifschitz, L.D. Barnes, Current concepts on the pathophysiology of acute renal failure, Am. J. Physiol. 234 (1978) F171–F181.

[3] M.A. Venkatachalam, D.B. Jones, H.G. Renneke, D. Sandstorm, Y. Patel,

Mechanism of proximal tubule brush border loss and regeneration following mild renal ischemia, Lab. Invest. 45 (1981) 355–365.

[4] K.A. Reimer, C.E. Ganote, R.B. Jennings, Alterations in renal cortex following ischemic injury III. Ultrastructure of proximal tubules after ischemia or autolysis, Lab. Invest. 26 (1972) 347–363.

[5] M.A. Venkatachalam, D.B. Bernard, J.F. Donohoe, N.G. Levinsky, Ischemic repair and damage in the rat proximal tubule: differences among the S1, S2, and S3 segments, Kidney Int. 4 (1978) 31–43.

[6] J.F. Donohoe, M.A. Venkatachalam, D.B. Bernard, N.G. Lewinsky, Tubular leakage and obstruction in acute ischemic renal failure, Kidney Int. 13 (1978) 208–222.

[7] B. Glaumann, N. Glaumann, I.K. Berezeky, B.F. Trump, Studies on the pathogenesis of ischemic cell injury. Morphological changes in the pars convoluta (P1 and P2) of the proximal tubule of the rat kidney made ischemic in vivo, Virchows Arch. 19 (1975) 281–302.

[8] J.K. Paddock, W. Lada, L.H. Lowenstein, Regeneration of the renal brush border after renal ischemia in rats, Am. J. Physiol. 241 (1981) F28–F33.

[9] S.J. Khundmiri, M. Asghar, F. Khan, S. Salim, A.N.K. Yusufi, Effect of reversible and irreversible ischemia on marker enzymes of BBM from renal cortical PT subpopulations, Am. J. Physiol. 273 (1997) F849–F856.

[10] S.J. Khundmiri, M. Asghar, F. Khan, S. Salim, A.N.K. Yusufi, Effect of ischemia and reperfusion on enzymes of carbohydrate metabolism in rat kidney, J. Nephrol. 17 (2004) 377–383.

[11] B.A. Molitoris, Ischemia induced loss of epithelial cell polarity: potential role of the actin cytoskeleton, Am. J. Physiol. 260 (1991) F769–F778.

[12] B.A. Molitoris, W.J. Nelson, Alterations in the establishment and maintenance of epithelial cell polarity: role of tight junctions, J. Clin. Invest. 85 (1990) 3–9.

[13] M.J. Hanley, Isolated nephron segments in a rabbit model of ischemic acute renal failure, Am. J. Physiol. 239 (1980) F17–F23.

[14] B.A. Molitoris, L.K. Chan, J.I. Shapiro, J.D. Conger, S.A. Falk, Loss of epithelial polarity: a novel hypothesis for reduced proximal tubule Na+ transport following ischemic injury, J. Membr. Biol. 107 (1989) 119–127.

[15] B.A. Molitoris, R. Kinne, Ischemia induces surface membrane dysfunction. Mechanism of altered Na$^+$ dependent glucose transport, J. Clin. Invest. 80 (1987) 647–654.

[16] S. Maeda, M. Takano, T. Okano, K. Ohoka, K. Inui, R. Hori, Transport of organic cation in renal brush border membrane from rats with ischemic injury, Biochim. Biophys. Acta 1150 (1993) 103–110.

[17] T.P. Dousa, S.A. Kempson, Regulation of renal brush border membrane transport of phosphate, Miner. Electrolyte Metab. 7 (1982) 113–121.

[18] S.A. Kempson, T.P. Dousa, Current concepts of regulation of phosphate transport in renal proximal tubules, Biochem. Pharmacol. 35 (1986) 721–726.

[19] M. Silverman, H. Rose, J.B. Puschett, Modifications in proximal tubular function induced by nitrendipine in a rat model of acute ischemic renal failure, J. Cardiovasc. Pharmacol. 14 (1989) 799–802.

[20] Y. Xiao, R.R. Desrosiers, R. Beliveau, Effect of ischemia–reperfusion on the renal brush border membrane sodium-dependent phosphate cotransporter NaPi-2, Can. J. Physiol. Pharm. 79 (2001) 206–212.

[21] T. Kwon, J. Frokiaer, P. Fernandez-Llama, M.A. Knepper, S. Nielsen, Reduced abundance of aquaporin in rat with bilateral ischemia-induced acute renal failure: prevention by α-MSH, Am. J. Physiol. 277 (1999) F413–F427.

[22] T. Kwon, J. Frokiaer, J.S. Han, M.A. Knepper, S. Nielsen, Decreased abundance of major Na$^+$ transporters in kidneys of rats with ischemia-induced acute renal failure, Am. J. Physiol. 278 (2000) F925–F939.

[23] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[24] A.N.K. Yusufi, N. Murayama, S.M. Gapstur, M. Szczepanska-Konkel, T.P. Dousa, Differential properties of brush border membrane vesicles from early and late proximal tubules of rat kidney, Biochim. Biophys. Acta 1191 (1994) 117–132.

[25] S.A. Kempson, S.V. Shah, P.G. Werness, T. Brendt, P.H. Lee, L.H. Smith, F.G. Knox, T.P. Dousa, Renal brush border membrane adaptation to phosphorus deprivation: effects of fasting versus low phosphorus diet, Kidney Int. 18 (1980) 30–47.

[26] M. Szczepanska-Konkel, A.N.K. Yusufi, M. Vanscoy, S.K. Webster, T.P.

Dousa, Phosphonocarboxylic acids as specific inhibitors of Na$^+$ dependent transport of phosphate across renal brush border membrane, J. Biol. Chem. 261 (1986) 6375–6383.

[27] M. Szczepanska-Konkel, A.N.K. Yusufi, T.P. Dousa, Interactions of [$^{14}$C] phosphonoformic acid with renal cortical brush border membranes: relationship to the Na$^+$-phosphate co-transporter, J. Biol. Chem. 262 (1987) 8000–8010.

[28] A.N. Yusufi, N. Murayama, M.J. Keller, T.P. Dousa, Modulatory effect of thyroid hormones on uptake of phosphate and other solutes across luminal brush border membrane of kidney cortex, Endocrinology 116 (1985) 2438–2449.

[29] A.I. Alcalde, M. Sarasa, D. Raldua, J. Aramayona, R. Morales, J. Biber, H. Murer, M. Levi, V. Sorribas, Role of thyroid hormone in regulation of renal phosphate transport in young and aged rats, Endocrinology 140 (1999) 1544–1551.

[30] A.N. Yusufi, M. Szczepanska-Konkel, A. Hoppe, T.P. Dousa, Different mechanisms of adaptive increase in Na-Pi cotransport across renal brush-border membrane, Am. J. Physiol. 256 (1989) F852–F861.

[31] H. Murer, J. Biber, Molecular mechanisms of renal apical Na/phosphate cotransport, Ann. Rev. Physiol. 58 (1996) 607–618.

[32] J.K. Paddock, A.R. Taddeo, J.P. Paddock, L.M. Lowenstein, Glycoprotein reutilization in regenerating microvilli after renal ischemia in rats, Kidney Int. 28 (1985) 498–503.

[33] D. Rubinger, H. Wald, D. Gimelreich, N. Halihel, T. Rogers, M. Levi, M.M. Popovtzer, Regulation of renal sodium-dependent phosphate cotransporter NaPi2 (Npt2) in acute renal failure due to ischemia and reperfusion, Nephron, Physiol. 100 (2005) 1–12.

[34] L. Cheng, B. Sacktor, Sodium gradient dependent phosphate transport in renal brush border membrane vesicles, J. Biol. Chem. 256 (1981) 1556–1564.

[35] P. Gmaj, H. Murer, Cellular mechanisms of inorganic phosphate transport in kidney, Physiol. Rev. 66 (1986) 36–70.

[36] N. Hoffmann, M. Thees, R. Kinne, Phosphate transport by isolated brush border vesicles, Pfluegers Arch. Eur. J. Physiol. 362 (1976) 147–156.

[37] H. Murer, A. Werner, S. Reshkin, F. Waurin, J. Biber, Cellular mechanisms in proximal tubular reabsorption of inorganic phosphate, Am. J. Physiol. 260 (1991) C885–C899.

[38] I.C. Forster, J. Biber, H. Murer, Proton sensitive transitions of renal type II Na-coupled cotransporter kinetics, Biophys. J. 79 (2000) 215–230.

[39] M. Levi, M. Lotscher, V. Sorribas, M. Custer, M. Arar, B. Kaissling, H. Murer, J. Biber, Cellular mechanisms of acute and chronic adaptation of rat renal Pi transporter to alterations in dietary Pi, Am. J. Physiol. 267 (1974) F900–F908.

[40] E.D. Lederer, S.S. Sohi, J.M. Mathiesen, J.B. Klein, Regulation of type II sodium phosphate cotransporter by protein kinase A and C, Am. J. Physiol. 275 (1998) F270–F277.

[41] M. Loghman-Adham, Adaptations to changes in dietary phosphorus intake in health and in renal failure, J. Lab. Clin. Med. 129 (1997) 176–188.

[42] S. Lopez-Hilker, A.S. Dusso, N.S. Rapp, K.J. Martin, E. Slatopolsky, Phosphorus restriction reverses hyperparathyroidism in uremia independent of changes in calcium and calcitriol, Am. J. Physiol. 259 (1990) F432–F437.

[43] M. Loghman-Adham, Renal and intestinal Pi transport adaptation to low phosphorus diet in uremic rats, J. Am. Soc. Nephrol. 3 (1993) 1930–1937.

[44] H.D. Humes, D.A. Cieslinski, L.B. Johnson, I.O. Sanchez, Triiodothyronine enhances renal tubule cell replication by stimulating EGF receptor gene expression, Am. J. Physiol. 262 (1992) F540–F545.

[45] S.A. Rogers, S.B. Miller, M.R. Hammerman, Altered EGF expression and thyroxine metabolism in kidneys following acute ischemic injury in rat, Am. J. Physiol. 270 (1996) F21–F30.

[46] C.I. Pantos, V.A. Malliopoulo, I.S. Mourouzis, E.P. Karamanoli, S.M. Tzeis, H.C. Carageorgiou, D.D. Varonos, D.V. Cokkinos, Long-term thyroxine administration increases heat stress protein 70 mRNA expression and attenuates p38 MAP kinase activity in response to ischemia, J. Endocrinol. 170 (2001) 207–215.

[47] C.I. Pantos, V.A. Malliopoulo, I.S. Mourouzis, E.P. Karamanoli, I.A.

Paizis, N. Steimberg, D.D. Varonos, D.V. Cokkinos, Long-term thyroxine administration protects the heart in a pattern similar to ischemic preconditioning, Thyroid 12 (2002) 325–329.

[48] C.G. Acker, R. Flick, R. Shapiro, V.P. Scantlebury, M.L. Jordan, C. Vivas, A. Greenburg, J.P. Johnson, Thyroid hormone in the treatment of post-transplant acute tubular necrosis (ATN), Am. J. Transp. 2 (2002) 57–61.

[49] C.G. Acker, A.R. Singh, R.P. Flick, J. Bernardini, A. Greenburg, J.P. Johnson, A trial of thyroxine in acute renal failure, Kidney Int. 57 (2000) 293–298.