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Renal cortical mitochondrial transport of calcium in chronic uremia

AVIVA CONFORTY, RUTH SHAINKIN-KESTENBAUM, VARDA SHOSHAN, RINA KOL, JAYSON RAPOPORT, and CIDIO CHAIMOVITZ

Departments of Nephrology, Pathology and Biology, Ben-Gurion University of the Negev and Soroka Medical Center, Beer Sheva, Israel

Renal cortical mitochondrial transport of calcium in chronic uremia. Calcium overload of tubular cells may occur in uremia, and may be the underlying functional abnormality in the continued deterioration of renal function in chronic renal failure. In order to study this question further, the effect of chronic uremia on the calcium transport properties and respiratory rates was examined in mitochondria (Mi) isolated from the cortex of the remnant kidneys of subtotally nephrectomized rats (SNX) and sham operated controls (C). Plasma calcium concentration was similar in both groups of rats, but a significant hyperphosphatemia was seen in SNX, 8.6 ± 0.6 mg%, as compared to 7.2 ± 0.2 mg% in C ($P < 0.001$). Mi calcium and phosphate concentrations (nmol/mg protein) were significantly elevated in SNX, 49.9 ± 7.9 and 35.1 ± 4.2, respectively, in SNX compared to C, 21.2 ± 4.2 and 21.4 ± 2.7, respectively ($P < 0.01$). Mi respiratory control ratio and ADP/O were similar in both experimental groups. Kinetic parameters for calcium uptake (Ca^{2+} concentrations in the medium of 1.25 to 16 μM) revealed initial velocities 1.5-fold higher in SNX Mi than in C. Mi retention of calcium in the presence of medium Ca^{2+} concentrations up to 500 μM was studied. Calcium retention was reduced in SNX: the Mi were unable to retain calcium at concentrations of 250 μM. The addition of ruthenium red to the medium substantially improved calcium retention by the uremic Mi. Chronic parathyroidectomy did not correct either the increased calcium uptake or the poor retention of uremic Mi. In conclusion: 1) Chronic uremia in rats is associated with a marked alteration in calcium transport of renal cortical Mi, which may result in Mi calcium overload. 2) The enhanced calcium uptake and poor calcium retention of uremic Mi is not PTH dependent. 3) Since Pi is known to cause a higher initial velocity of calcium uptake and to impair calcium retention of normal mitochondria, it seems that cellular accumulation of phosphate in chronic uremia may be involved in this abnormality.

Renal calcification in uremia (uremic nephrocalcinosis) has been well documented in humans and experimental animals with chronic renal failure [1–3]. The mechanisms underlying this widespread parenchymal calcification remain unclear. Nonetheless it has been postulated that renal calcification may play an important role in the deterioration of renal function in the remnant kidney model of chronic renal failure [4]. Thus prevention of uremic nephrocalcinosis by means of restriction of dietary phosphate or treatment with calcium channel blockers has been shown to slow functional deterioration and to reduce histologic damage in experimental chronic renal failure in rats [4, 5].

The pathogenic role of cellular overload of calcium as a mediator of cell injury in the kidney has long been recognized [6–8]. Therefore, it is important to determine if calcium overload of renal cells accompanies the phenomenon of uremic renal calcification. If, indeed, calcium accumulates in renal cells, it could be a contributory factor to the continuing loss of functioning nephrons in chronic renal failure.

Active sequestration of calcium by intracellular organelles, such as the endoplasmic reticulum and mitochondria, and the Ca^{2+} ATPases of the plasma membrane, are the mechanisms involved in the maintenance of cytosolic calcium at a very low concentration. The mitochondria in particular are capable of accumulating large amounts of calcium, thereby playing a most important role in buffering massive intracellular loads of calcium. Thus, study of mitochondrial calcium transport and efficiency of mitochondrial oxidative phosphorylation may have substantial relevance in unveiling major abnormalities in cellular metabolism of calcium.

The experiments described in this paper were designed to assess calcium transport and respiration parameters of isolated renal cortical mitochondria of the remnant kidneys of rats with chronic renal failure induced by partial renal ablation. Since parathyroid hormone administration causes nephrocalcinosis in a pattern similar to that described in chronic uremia [9], mitochondrial calcium transport was also determined in chronic uremic rats following parathyroidectomy.

Methods

The experiments were performed on male Charles River rats (Charles River Laboratories, Wilmington, Massachusetts, USA) weighing 250 to 300 g. Animals were fed regular rat laboratory chow and allowed water at libitum. Experimental animals were subjected to 5/6 nephrectomy (SNX) performed via flank incisions in two steps separated by one week. In a group of 5/6 nephrectomized rats, selective parathyroidectomy (PTX) was performed by cautery in the fifth week following the partial kidney ablation. Parathyroidectomy was considered successful if the plasma calcium level on the third day after surgery was below 7.5 mg%. Thereafter the rats were supplemented with calcium lactate in their drinking water. Another group of rats underwent sham operations consisting of flank incisions and kidney decapsulation. At the end of six weeks after partial

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nephrectomy or sham operation the rats were killed by cervical dislocation. In each experiment, four SNX and two control kidneys were immediately placed in ice-cold medium containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA (to avoid accumulation of calcium into mitochondria during separation procedure), 2 mM K-HEPES pH 7.4 and 0.5% bovine serum albumin. The cortices were dissected, coarsely minced and then homogenized by a Dounce homogenizer: three strokes using pestle A and two using pestle B. This homogenate was then centrifuged twice for 10 minutes at 4°C at 750 × g to remove cellular debris. The resultant supernatant was then centrifuged at 6,800 × g for five minutes to obtain the mitochondrial pellet which was washed twice in a similar buffer as above, omitting EGTA and BSA (wash buffer). The final pellet was suspended in a wash buffer containing 0.5% BSA to a concentration of approximately 10 mg mitochondrial protein/ml. For mitochondrial protein determination 50 μl aliquots of the final mitochondrial suspension were diluted in 1 ml of isotonic buffer, centrifuged at 12,000 × g for one minute. The supernatant was discarded and the pellet suspended in distilled water. The protein content was determined by the method of Lowry et al [10].

Mitochondrial characteristics

Mitochondrial calcium and phosphate content. Following isolation of mitochondria, aliquots of known protein content were removed, dried overnight at 130°C and digested for calcium determination with 250 ml of concentrated HNO_3. The test tubes were heated in boiling water for one hour, then 125 ml of $HClO_4$ were added. Lanthanum was added to the solution to give a final concentration of 1%. Calcium content was determined by atomic absorption spectrophotometry, using Varian atomic absorption spectrophotometer model No. 1200.

For phosphate determination, aliquots of mitochondria were precipitated with cold 4% $HClO_4$. After centrifugation, the supernatant was decanted and immediately titrated with 5 N KOH and 5 M Tris to neutral pH to avoid hydrolysis of phosphorylated nucleotides. Determination of phosphate was performed by the Malachite green method [11].

Mitochondrial ^{45}Ca uptake. Calcium uptake was initiated by adding mitochondria to the medium at a concentration of 1 mg protein/ml. The medium contained 120 mM KCl, 5 mM succinate, 2 mM K_2HPO_4, 0.4 μM rotenone, 20 mM HEPES, pH 7.0, 0.5 mCi ^{45}Ca/ml. Free calcium concentrations (1.25 to 16 μM) were obtained by buffering 100 μM calcium with various EGTA concentrations. The free calcium concentrations were calculated with a computer program using the EGTA/calcium association constant reported by Fabiato and Fabiato [12]. Experiments were performed at 27°C. The reactions were stopped at desired times in intervals up to five minutes by vacuum filtering through 0.45 μm pore-size filters. The radioactivity of the pellet was determined by liquid scintillation spectrophotometry (Packard Counter 3255, Hewlett Packard, Elkhart, Indiana, USA). For calculation of the initial velocity the values obtained at 10 seconds in the various Ca^{2+} concentrations were considered.

Mitochondrial ^{45}Ca retention. The retention experiments were carried out at 27°C at calcium concentrations of 100, 200, and 500 μM. One ml of the medium contained 120 mM KCl, 5 mM succinate, 20 mM HEPES, pH:7.15, 2 mM K_2HPO_4, 0.4 mM rotenone, 0.5 μCi ^{45}Ca and the above mentioned concentrations of $CaCl_2$, respectively. Sampling, protein analysis and the amount of radioactivity were performed and determined as in section **Mitochondrial ^{45}Ca uptake**.

Measurement of mitochondrial respiration

Oxygen consumption was measured polarographically at 30°C within a temperature controlled, closed vessel fitted with a stirrer and O_2 electrode. The incubation medium contained 120 mM KCl, 2 mM K_2HPO_4, 0.4 μM rotenone, 1 mM EGTA, 5 mM HEPES pH 7.1. Mitochondria (0.5 to 1 mg protein) were introduced anaerobically to 2 ml buffer in the incubation cell followed by 5 mM succinate as substrate. The addition of 450 nmoles of ADP initiated state 3 of respiration. State 4 of respiration was measured at the slower respiratory rate following depletion of added ADP. Respiratory rates were expressed as nanoatom equivalents of oxygen consumed per milligram of mitochondrial protein per minute. The respiratory control ratio (RCR) was expressed as the quotient of state 3/state 4. The ADP/oxygen ratio (ADP/O) was evaluated using the amount of oxygen consumed during state 3.

Transmission electron microscopy

The mitochondrial pellet was fixed in 2% glutaraldehyde in sodium cacodylate buffer 0.1 M pH 7.2 for one hour at room temperature. Post-fixation was done with 1% osmium tetroxide in cacodylate buffer for one hour at 4°C. The pellet was then dehydrated in ethanol and embedded in araldite 502. Thin sections were stained with uranyl acetate and lead citrate and examined with Philips 201C Transmission Electron microscope.

Plasma levels of creatinine and phosphate were determined by spectrophotometric methods using a KDA Autoanalyzer and calcium by atomic absortion spectrophotometry.

Statistical analyses were performed using Student's unpaired *t*-test. Values are shown as mean ± SE.

Results

Renal function

By the sixth week after subtotal nephrectomy plasma creatinine levels determined in 20 rats were significantly higher in the SNX rats, 1.8 ± 0.1 mg% in comparison to 0.6 ± 0.1 mg% in the control rats ($P < 0.001$). A similar rise in plasma creatinine was seen in 20 SNX parathyroidectomized rats (1.9 ± 0.1 mg%). Plasma calcium concentration remained normal in the SNX rats. The parathyroidectomized rats remained mildly hypocalcemic despite calcium supplementation. Plasma phosphate concentration in the SNX rats was significantly higher (8.6 ± 0.2 mg%) than control rats (7.2 ± 0.1, $P < 0.001$). Following parathyroidectomy, plasma phosphate concentration was 8.8 ± 0.1 mg% in the SNX-PTX rats (P = NS compared to SNX rats).

Mitochondrial calcium and phosphate content, electron microscopy and respiratory parameters

Tables 1 and 2 summarize calcium and phosphate levels and respiratory function of renal cortical mitochondria isolated from the three experimental groups of rats.

It can be seen that both the mitochondrial calcium and phosphate content were significantly higher in the SNX rats. Similarly, in SNX-PTX rats, calcium and phosphate content of

Table 1. Calcium and phosphate content of mitochondria

	Control	SNX	SNX-PTX
Calcium	21.2 ± 4.2	49.9 ± 7.9[a]	36.8 ± 3.2[a]
	$N = 8$	$N = 8$	$N = 4$
Phosphate	21.4 ± 2.7	35.1 ± 4.2[a]	45.2 ± 3[a]
	$N = 8$	$N = 8$	$N = 4$

Mitochondrial calcium and phosphate are expressed as nanomols/mg mitochondrial protein. N, number of experiments. Values are means ± SE. The P values compare SNX vs. control, and SNX-PTX vs. control.
[a] $P < 0.05$.

Table 2. Mitochondrial respiratory parameters in sham operated, SNX and SNX-PTX rats

	Control	SNX	SNX-PTX
RCR	4.9 ± 0.2	4.8 ± 0.2	4.4 ± 0.4
ADP/O	1.8 ± 0.1	1.7 ± 0.02	1.6 ± 0.1
S3	364 ± 42	302 ± 41	318 ± 54
S4	76 ± 6	63 ± 8	73 ± 9
N	8	8	8

Respiratory rates are expressed as nanoequivalents of oxygen consumed per milligram of mitochondrial protein. Respiratory parameters were not statistically different in SNX and SNX-PTX mitochondria compared to controls.

mitochondria were also significantly elevated. The respiratory control ratio (RCR) and ADP oxygen ratio (ADP/O) of the isolated cortical mitochondria were not significantly different in the two SNX groups of rats as compared to the values of the control animals (Table 2). Samples of mitochondria from sham operated and SNX rats were analyzed by electron microscope (Fig. 1). Mitochondrial morphology was similar in both groups. Thus damaged mitochondria, which have been described in tubules of rats with chronic renal failure, were probably removed during isolation procedure.

Calcium fluxes in mitochondria

Calcium uptake. The reaction was carried out in the presence of 2.6 μM free calcium. A maximum accumulation of calcium was reached in all groups after approximately five minutes of incubation. Figure 2 summarizes calcium accumulation in mitochondria of SNX and control animals after incubation of one and two minutes. Calcium accumulation was about 1.5-fold higher in the SNX mitochondria.

The possibility that a PTH-dependent mechanism may be involved in the increased mitochondrial calcium accumulation in SNX rats was investigated in the parathyroidectomized rats. Figure 2 shows that the increased mitochondrial calcium accumulation was not reversed by parathyroidectomy.

The initial velocity of calcium uptake was obtained from the uptake at 10 seconds at various free calcium concentrations (1.25 to 16 μM), and in all experiments an enhanced uptake was found in SNX in the presence of the Ca^{2+} concentrations tested. Figure 3 shows a representative experiment. A double reciprocal plot indicated no difference in the Ca^{2+} affinity between control and SNX mitochondria (insert).

Retention of calcium. Retention of calcium by the cortical mitochondria was measured at three high concentrations of calcium in medium: 100, 250 and 500 μM. At a Ca^{2+} concentration of 250 μM, the retention of Ca^{2+} in the SNX and SNX-PTX mitochondria were significantly lower than controls (Fig. 4). This calcium was released by a ruthenium red-sensitive pathway, since the addition of 8 μM ruthenium red (Sigma Chemical Co., St. Louis, Missouri, USA, dye purity 45%) to the retention experiments substantially improved calcium retention by the SNX mitochondria (Fig. 5). The possible role of secondary hyperparathyroidism in the impaired calcium retention of the SNX mitochondria was investigated in the parathyroidectomized rats. Figure 4 shows that mitochondrial calcium retention in SNX-PTX remained poorer than in the control rats. However, at 500 μM Ca^{2+} retention was markedly suppressed in all three experimental groups.

Discussion

The results of the present study demonstrate that mitochondria isolated from the renal cortex of SNX rats are overloaded with calcium, and contain higher phosphate levels. We were also able to demonstrate an increased accumulation of ^{45}Ca by SNX mitochondria. This could be explained by either an increased rate of uptake, or a decreased rate of efflux of calcium. This question was evaluated by measuring initial rates of uptake of calcium at different free Ca^{2+} concentrations. We found that calcium uptake by SNX mitochondria, as early as 10 seconds from the start of the experiment, was markedly increased as compared to controls. These data show that at least part of the augmented calcium accumulation in the SNX mitochondria can be accounted for by an increased rate of calcium uptake. We also examined the capacity of mitochondria to retain calcium in the presence of high concentrations of this cation in the medium. A marked reduction in calcium retention by the SNX mitochondria was detected: the SNX mitochondria did not retain the calcium at a calcium concentration of 200 μM, compared to the control mitochondria, where a similar impairment of retention was seen only at a calcium concentration of 500 μM. This rapid loss of calcium at high calcium concentrations by SNX mitochondria was partially blocked by ruthenium red [13], suggesting that mitochondrial calcium release at these high calcium concentrations was occurring via a pathway sensitive to ruthenium red. It is possible that this impaired calcium retention is due to the calcium and phosphate preloads in the SNX mitochondria. It is worth noting that the isolated mitochondria represent a population of mitochondria that possess normal respiratory control values, and seem to have normal morphology in EM. It seems probable that the damaged mitochondria which were previously observed by us in EM preparations of entire SNX cortices were removed during the isolation procedure [14].

The present data are consistent with prior investigations from our laboratory which have demonstrated that kidney slices from SNX rats accumulate larger amounts of ^{45}Ca than those from normal rats [15]. Treatment with verapamil corrected the exaggerated ^{45}Ca accumulation and considerably reduced renal tissue calcification in the rats with chronic renal failure [15]. These observations, together with the alterations in mitochondrial calcium metabolism described here, are strong, although indirect, evidence that in chronic uremia renal tubular cells are overloaded with calcium. One should, of course, be cautious in extrapolating in vitro data on calcium metabolism in isolated

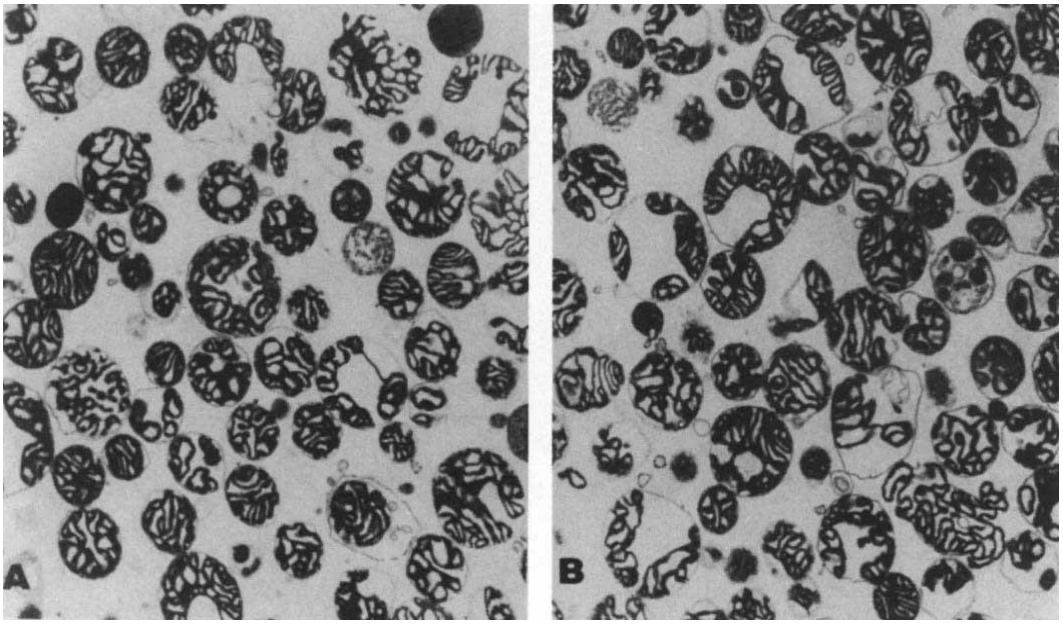

Fig. 1. *Electron microscopic examination of mitochondria isolated from sham operated* (A) *and SNX* (B) *rats.* The isolated mitochondria from SNX show normal morphology.

mitochondria to the in vivo situation. However, the consistency of our results, both in isolated mitochondria and kidney slices, supports the supposition that cellular calcium overload in the kidneys of SNX rats indeed occurs. It should be noted that in other experimental models of nephrocalcinosis, such as vitamin D overdose and administration of pharmacological doses of PTH, mitochondrial damage was found in association with widespread tissue calcification [16, 9]. It is thus possible that the abnormalities of cellular calcium metabolism described in this study may be implicated in the development of uremic nephrocalcinosis. The potential of this cellular overload of calcium to mediate tubule cell injury and participate in the deterioration of renal function in experimental chronic uremia has been emphasized by the report of Harris et al [5]. These authors have demonstrated that chronic treatment with verapamil has a protective effect on progression of renal failure and improves the survival time of rats after subtotal nephrectomy.

Another goal of this study was to throw some light on factors involved in abnormal calcium transport by SNX mitochondria.

Parathyroid gland hyperplasia resulting in elevated parathyroid hormone levels may be detected in chronically uremic rats as early as 7 to 14 days after subtotal nephrectomy [17]. Borle and Uchikawa [18] have demonstrated that in cultured kidney cells, PTH stimulates calcium influx. The resulting increase in the total cellular calcium was accompanied by a marked trapping of this ion inside the mitochondria. It has been proposed, too, that some features of the uremic syndrome are related to a PTH-induced increase in intracellular calcium flux. Thus, PTH has been implicated in disturbed myocardial and CNS function, and decreased red blood cell survival of uremia [19–21]. These abnormalities seem to be dependent on the calcium ionophoric action of PTH, since they are partially corrected by verapamil.

Important evidence for the role of PTH in the pathogenesis of nephrocalcinosis is the renal calcification and severe cortical

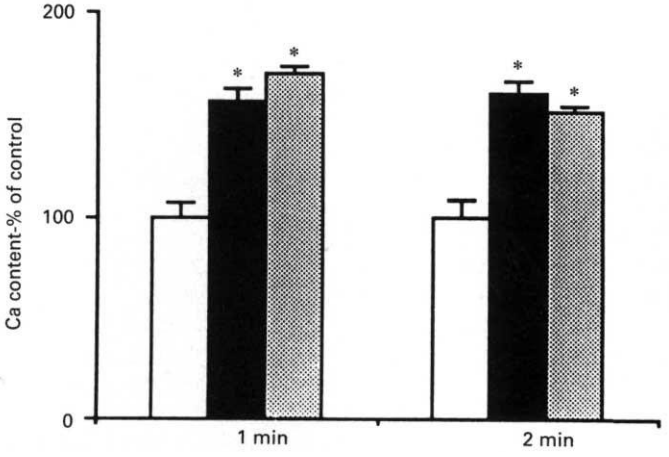

Fig. 2. *Ca^{2+} uptake by mitochondria after 1 and 2 min incubation with 2.6 μM Ca^{2+} in the medium.* Data are presented as percent changes in mitochondrial calcium content of SNX (■) and SNX-PTX (▨) vs. control (□). Values represent mean ± SE from 4 experiments in each group. $^*P < 0.05$ compared to control.

mitochondrial injury which follows administration of repeated doses of this hormone to mice [9]. Therefore, it was reasonable to assume that secondary hyperparathyroidism might be implicated in the pathogenesis of the calcium overload of renal tubular cells and cortical mitochondria in rats with chronic uremia.

Another study which is particularly relevant to the present experiments is the effect of parathyroidectomy on the renal calcification in rats with secondary hyperparathyroidism due to a high phosphate diet [22]. In this study, rats kept on a high phosphate and low calcium diet had a significant increase in kidney and mitochondrial calcium content associated with an

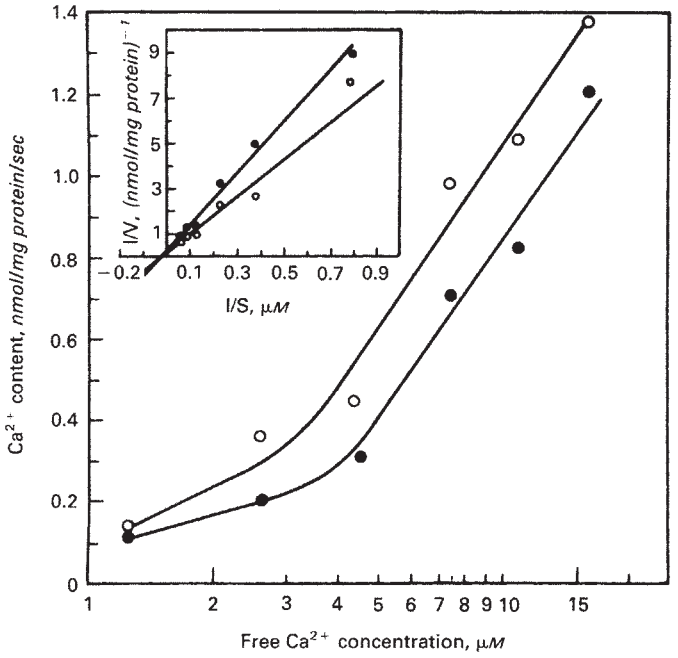

Fig. 3. *Initial velocity of Ca^{2+} uptake by mitochondria of control (●) and SNX (○) rats obtained from the uptake at 10 secs at various free Ca^{2+} concentrations in medium.* Insert shows double reciprocal plot.

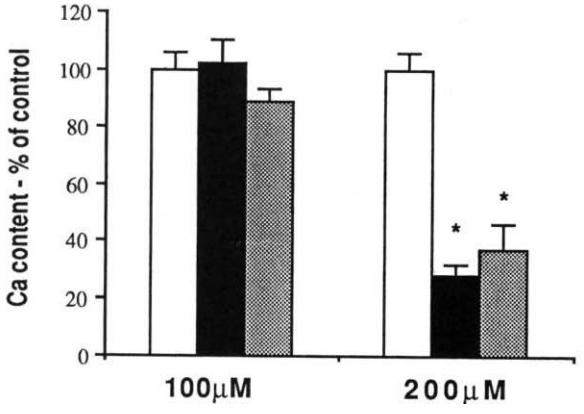

Fig. 4. *Mitochondrial accumulation of Ca^{2+} in SNX (■), SNX-PTX (▨) and control (□) rats after 90 secs of incubation at calcium concentrations of 100 and 200 μM in the incubation medium.* At 200 μM Ca^{2+}, calcium accumulation was significantly lower in SNX and SNX-PTX mitochondria compared to controls, $P < 0.05$. Data are presented as percentage of control, as for Fig. 2. Values represent data for 4 experiments in each group, mean ± SE.

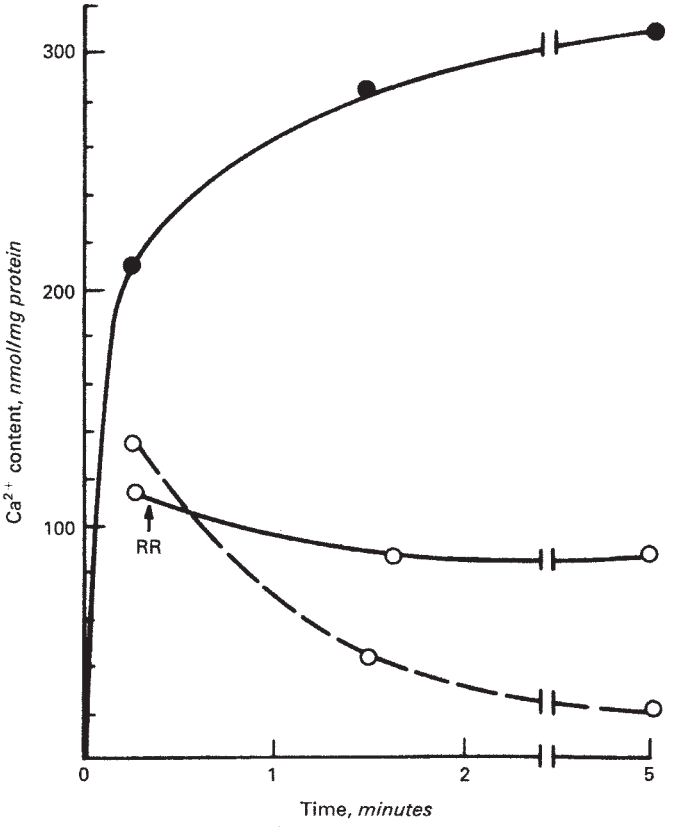

Fig. 5. *Time course of Ca^{2+} accumulation by mitochondria at a concentration of 200 μM Ca^{2+} in medium, expressed as nanomol/mg protein.* Controls (●——●); SNX (○- - -○); SNX after addition of ruthenium red to incubation medium (○——○). The initial time points are data after 16 secs of incubation with 200 μM Ca^{2+}

increase in circulating parathyroid hormone. Parathyroidectomy corrected this abnormality despite a persistent hyperphosphatemia. It was concluded that the nephrocalcinosis in this experimental model of secondary hyperparathyroidism is likely to be caused by a PTH-induced intracellular accumulation of calcium. Against this background, we studied the effect of parathyroidectomy on mitochondrial calcium content and transport of rats with chronic uremia. Our results show that isolated mitochondria from SNX-PTX rats continue to have a higher calcium and phosphate content than control mitochondria, with an increased rate of calcium uptake and impaired retention. No explanation is immediately apparent for the different effect of parathyroidectomy on renal mitochondrial calcium overload in these two experimental models of secondary hyperparathyroidism.

The present study disclosed that the SNX rats are hyperphosphatemic and there is a twofold rise in their cortical mitochondrial phosphate content. This phosphate retention in uremia may contribute to the abnormal calcium homeostasis in the kidney cells of SNX rats. There is evidence in cultured kidney cells that a rise in medium concentration of phosphate stimulates calcium influx into the cells and increases trapping of calcium inside mitochondria [23].

Phosphate is known to increase the initial rate of calcium uptake by isolated mitochondria of normal animals [24, 25]. Moreover, following massive calcium loading in the presence of phosphate, normal mitochondria will rapidly lose their calcium [13, 25, 26]. Thus, adding phosphate to the medium of normal mitochondria mimics the abnormal calcium uptake and retention seen in SNX mitochondria.

It has been shown that dietary phosphorous restriction has a protective effect on renal functional deterioration of rats with subtotal nephrectomy [4, 27]. This protective effect of phosphorous depletion has been attributed to a prevention of renal

calcification. Taken together, all these lines of evidence support the view that phosphate retention in chronic SNX rats may play a role in the abnormal calcium transport of renal cortical mitochondria. Further studies on mitochondrial calcium transport in SNX rats kept on a phosphate restricted diet are required to explore this subject further.

In summary, our data show marked abnormalities in calcium metabolism of renal mitochondria from rats with chronic uremia. Calcium content is elevated, initial rates of ^{45}Ca transport are increased, and calcium retention impaired. These mitochondrial abnormalities do not appear to be dependent on hyperparathyroidism, but may be related to the phosphate accumulation occurring in the uremic state. These findings are compatible with renal cellular calcium overload in chronic uremia, which may contribute to the continuing loss of functioning nephrons in chronic renal failure.

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Reprint requests to Aviva Conforty, M.D., Department of Nephrology, Soroka Medical Center, Beer Sheva 84101, Israel.

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