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Disassociation of Postischemic Recovery of Renal Adenosine Triphosphate and Cellular Integrity[1]

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ABSTRACT. **Previous studies from our laboratory have demonstrated that postischemic infusion of thyroxin (T_4) will augment the restoration of cellular ATP and enhance the recovery of renal function. It has not been clear, however, whether T_4 has a direct effect on mitochondrial ATP synthesis or an indirect effect by stabilization of the plasma membrane. To differentiate these putative effects, rats were subjected to 45 min of renal ischemia and given either normal saline (0.5 mL) or T_4 (20 μg/100 g body weight) during the first 15 min of reflow. Cellular ATP levels were assessed by ^{31}P-nuclear magnetic resonance spectroscopy, and release of lactate dehydrogenase (LDH) was used as an index of plasma membrane integrity at 30 and 120 min of reflow. In rats given normal saline, renal ATP had returned to only 57.9 ± 1.4% of preischemic values at 30 min of reflow and 66.1 ± 1.4% by 120 min. LDH release was 13 ± 0.89% at 30 min and 14.6 ± 1.6% at 120 min. In contrast, T_4-treated animals had ATP levels of 70.2 ± 2.0% at 30 min and 84.0 ± 1.9% at 120 min, whereas LDH release was elevated to values similar to those in normal saline–treated rats, 14.9 ± 1.5% and 14.4 ± 0.5% at 30 min and 120 min, respectively (nonischemic LDH 8.8 ± 0.8%). These data suggest that T_4 stimulates the recovery of renal ATP by a direct effect on synthesis rather than an indirect effect related to global improvement in cellular integrity. (*Pediatr Res* 33: 595–597, 1993)**

Abbreviations

T_4, thyroxin
NS, normal saline
NMR, nuclear magnetic resonance
LDH, lactate dehydrogenase
BW, body weight

It has been well established that treatment with T_4 has a beneficial effect in accelerating recovery after toxic (1, 2) and ischemic acute renal failure (3) in animals. The enhanced recovery of renal function has been demonstrated to parallel the augmented restoration of cellular ATP in animals treated with T_4 after the renal injury. Whether T_4 affects mitochondrial ATP synthesis directly or works by an indirect mechanism, such as restitution of cellular integrity, has not been established. The purpose of the present study was to determine whether the beneficial effect of postischemic treatment with T_4 involves stabilization of the injured plasma membrane of renal proximal tubule cells.

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MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 200 to 290 g were anesthetized with thiobutabarbital (Inactin, Byk Gulden, Konstanz, Germany) 80 mg/kg by intraperitoneal injection and placed on a heated animal board to maintain body temperature between 36 and 37°C. A tracheostomy was performed, and an external jugular vein catheter (PE-50) was placed. Isotonic saline was administered to replace surgical fluid losses (2% BW) and provide maintenance fluid at 1.2 mL/h. The abdominal aorta and right renal artery were isolated through a midline abdominal incision. Ten min before ischemia, 500 U/kg heparin were infused. A vascular clamp was placed proximal to the origin of the left renal artery, and a Silastic sling was placed around the right renal artery to induce 45 min of bilateral renal ischemia. After the ischemic period, the kidneys were allowed either 30 min or 120 min of reperfusion. Rats received either NS (0.5 mL) or T_4 (20 μg/100 g BW) during the immediate reflow period. Nonischemic control animals were anesthetized, sham-operated, and studied in the same manner as ischemic animals.

Tubule harvest. At the end of the reperfusion interval, an aortic catheter (PE-90) was placed distal to the origin of the renal arteries to perfuse the kidneys *in situ* with 90 mg of collagenase (Boehringer Mannheim, Indianapolis, IN) dissolved in 60 mL buffered solution over 20 min. After flushing with ice-cold buffer solution, the kidneys were rapidly excised and placed on ice, and capsules were removed. The cortex was gently scraped and suspended in a balanced buffered solution containing metabolic substrates (4), then stirred on ice. The suspension was filtered through a fiber mesh (Tetko, Depew, NY) to produce a suspension enriched in proximal tubules, which was then washed and sedimented by gentle centrifugation at 4°C. Microscopic inspection of the preparation was performed to ensure minimal contamination with glomeruli. Throughout the harvest procedure, the tubules were equilibrated with 95% O_2/5% CO_2 and kept on ice. Previous studies (5) have extensively validated this method of harvesting injured proximal tubule segments for *in vitro* evaluation after an *in vivo* ischemic insult. Such tubule segments recapitulate the metabolic and histomorphologic characteristics of ischemia.

LDH assay. The tubule suspension was warmed to 37°C and bubbled with 95% O_2/5% CO_2 in a shaker bath for 10 min, then centrifuged to produce a supernatant sample and a cellular-plus-supernatant sample. LDH was assayed immediately on each sample spectrophotometrically by following the disappearance of NADH at 340-nm wavelength as described by Bergmeyer *et al.* (6). LDH was expressed as a percentage of LDH released into

the supernatant compared with LDH in the sonicated cellular-plus-supernatant sample.

ATP determination. Animals were prepared as previously described in Materials and Methods. Each animal's body temperature was maintained at 36 to 37°C with a recirculating water bed. After exposure of the renal arteries through a midline abdominal incision, rats received heparin 500 mg/kg BW via the jugular catheter. A balloon cuff vascular occluder (In Vivo Metric, Healdsburg, CA) was looped around the aorta, distal to the origin of the celiac artery but proximal to the origin of both renal arteries. A femoral artery catheter was placed to continuously monitor arterial pressure distal to the site of the vascular cuff to assure complete occlusion of the aorta during the ischemic period.

The left kidney was exposed by a flank incision and placed in a saddle-shaped NMR coil that fit snugly around the organ. ^{31}P-NMR spectra were obtained with the Bruker Biospec I 4.7 T spectrometer (Bruker Instruments, Inc., Billerica, MA), operating at 81 MHz. Each spectrum consisted of 128 acquisitions, using a 90° pulse 2-s relaxation delay to ensure that the β-ATP resonance (T_1 = 400 ms) was fully relaxed. Improvements in the radiofrequency transceiver coil and spectrometer have led to enhanced ^{31}P sensitivity, allowing us to acquire ^{31}P spectra with 3.5-min time resolution. Four control spectra were taken to ensure stability of the preparation and establish the preischemic ATP level. Both kidneys were then made ischemic for 45 min by inflating the balloon cuff of the vascular occluder. During this time, the femoral artery pressure was 0 mm Hg and constant. After 45 min of ischemia, the cuff was deflated, and the animals received either 0.5 mL NS or 20 μg/100 g BW T_4 during the first 5 to 7 min of reflow. There were no changes in coil loading or position during any of these maneuvers; therefore, reshimming was not required. Spectra were collected before, during, and for 120 min after the insult. Thus, each animal served as its own control. In control animals, the β-ATP peak was stable and varied by 0.33% of the preischemic ATP value over the 120 min of the study.

Spectra were processed using a convolution difference to remove the broad phospholipid resonance and a mild Lorentzian filter (20 Hz). Renal ATP levels were assessed by comparing changes in the area of the β-phosphate peak at each time interval with the preischemic values. Areas were determined using standard NMR software (Bruker).

Statistical methods. Results are presented as mean values ± SEM. Comparisons between groups were done by t test, with differences considered significant when the p value was <0.05.

RESULTS

Cell membrane integrity (Fig. 1). The effect of postischemic T_4 treatment on cell membrane integrity, as assessed by the percentage of LDH released from injured cells, can be evaluated by comparing NS-treated animals with those receiving T_4. Nonischemic controls had a mean LDH release of 8.8 ± 0.8% (n = 4 animals). At 30 min of postischemic reflow, NS-treated subjects had an LDH release of 13 ± 0.8% (n = 5 animals), and T_4 treatment resulted in a mean of 14.9 ± 1.5% (n = 4 animals). By 120 min of reperfusion, similar results were obtained. LDH release was 14.6 ± 1.6% (n = 5 animals) in the NS-treated group and 14.4 ± 0.5% (n = 5 animals) in the T_4-treated group. Although ischemic injury caused a significant increase in LDH release compared with controls ($p < 0.05$), no statistically significant difference was detected in plasma membrane integrity between NS- and T_4-treated groups at either time interval.

Cellular ATP recovery (Fig. 2). In all animals, renal ATP levels evaluated by ^{31}P-NMR spectroscopy fell to below 20% of control levels within 10 min of aortic occlusion and remained at that level during the remainder of the ischemic insult. ATP levels returned to only 57.9 ± 1.4% of nonischemic control values by 30 min of reflow in NS-treated animals (n = 8 animals). In contrast, administration of T_4 improved levels to 70.2 ± 2.0% at 30 min of reflow (n = 11 animals). By 120 min of reperfusion, the ATP levels increased to 66.1 ± 1.4% and 84.0 ± 1.9% in the NS and T_4 groups, respectively. At both time points, the difference between the experimental groups was statistically significant.

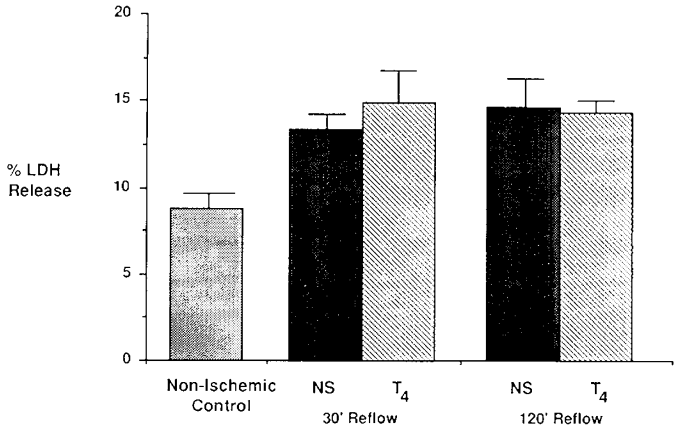

Fig. 1. Effect of T_4 on cell membrane integrity comparison of NS- and T_4-treated animals. Increase in LDH release was statistically significant ($p < 0.05$) for all groups with 45 min of renal ischemia compared with nonischemic controls. LDH released from injured cells was not significantly different between NS and T_4 groups at either 30 min or 120 min of reflow.

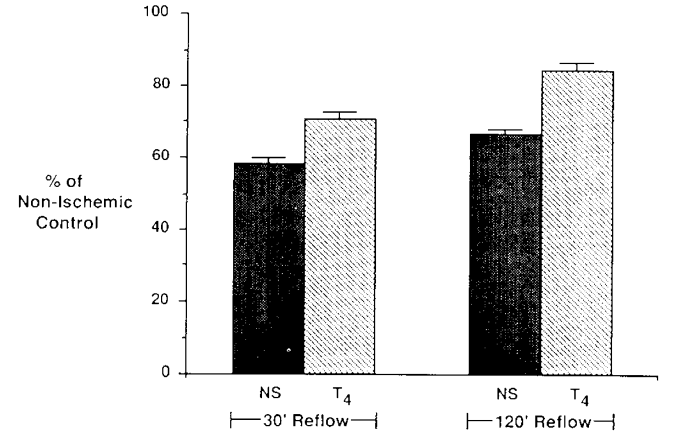

Fig. 2. ATP recovery by ^{31}P-NMR spectroscopy. NS- and T_4-treated ischemic animals compared at 30 and 120 min of reflow after 45 min of renal ischemia. A statistically significant ($p < 0.05$) improvement was seen in T_4-treated animals at both time intervals.

DISCUSSION

Although the beneficial effects of T_4 in enhancing recovery after acute renal injury have been well established (1–3), the mechanisms of T_4 action have not been elucidated. Recovery of injured tissue requires the reestablishment of cellular ATP levels to provide energy for repair of the cell, ongoing metabolic requirements, and support of the regenerative and proliferative process (1, 7–10). In fact, it has been shown that increases in glomerular filtration rate, filtered load, and tubular transport that are not accompanied by concomitant increases in cellular energy are associated with increased swelling and necrosis of injured renal tubule epithelium (8, 9). Moreover, cellular nucleotides have been shown to stimulate cell growth and repair (7, 10). Cellular energy is also required for regenerative processes such as heat-shock protein induction and function (11, 12). Therefore, the mechanism by which T_4 augments recovery of cellular ATP has been of interest.

One proposed mechanism for the salutary effect of T_4 has been through an indirect stabilization of injured cells, thereby preventing the transcellular leakage of adenine nucleotides and precursors required for ATP resynthesis. Phospholipids play an important role in regulation and maintenance of cellular membranes and have been demonstrated to be significantly altered by renal ischemia (13). Ballard *et al.* (14) have shown that thyroid hormone stimulates the incorporation of phospholipids into developing rat and rabbit lung tissue. This study was designed to test the hypothesis that improvements in renal cell integrity would precede improvements in cellular energy levels.

LDH release by injured cells is a reliable indicator of plasma membrane integrity and has been used to evaluate many forms of tissue injury (4, 5, 15, 16). Takano *et al.* (17) found that release of LDH occurred progressively with increasing duration of anoxia in rabbit proximal tubules, reflecting an increase in the degree of cellular damage with prolonged anoxia. Doctor and Mandel (18) demonstrated a similar progressive increase in LDH release in rat tubules when anoxic intervals were extended to 60 min, suggesting that LDH release can be used as an index of the severity of the cellular injury. In the present study, a fixed period of *in vivo* renal ischemia was followed by reperfusion for 30 or 120 min. Postischemic treatment with T_4 did not significantly improve LDH release in proximal tubule cells at either reflow interval as compared with NS-treated control animals. These results indicate that postischemic administration of T_4 does not directly alter cellular integrity during the early reflow period.

To accurately determine cellular ATP levels *in vivo*, so that we could obtain the data necessary to test our hypothesis, we had to modify our previous techniques for the determination of ATP by ^{31}P-NMR spectroscopy. These technical improvements included a redesigned and rebuilt coil, use of a cuffed vascular occluder that did not change the load factor or position of the kidney during ischemia, and placement of a femoral artery line to be sure the vascular occlusion was complete and was effectively released. These improvements in NMR technique have now: *1*) allowed acquisition of spectra at intervals of 3.5 min instead of 7 min, as previously reported (19); *2*) obviated the need for reshimming, inasmuch as the kidney does not move during occlusion or reperfusion; and *3*) enhanced sensitivity with improved signal-to-noise ratio. Changes in cellular ATP during the early rapid phase of recovery, the first 30 min of reflow, can now be examined.

In the present study, ATP levels for T_4-treated animals were significantly higher at both 30 min and 120 min of reflow. Although we have previously reported the augmented recovery of cellular ATP at 120 min, the present study not only reconfirms these observations (19) but provides new data that postischemic ATP levels are increased very rapidly after the administration of T_4, *i.e.* within 30 min. The impact of this improvement in cellular ATP during early reflow and on the long-term recovery from ischemic acute renal failure has been delineated. Our laboratory has shown previously that improvement in cellular energetics is associated with enhanced renal function, amelioration of histomorphologic damage, and sustained recovery of glomerular and tubule function (3, 9, 19).

In conclusion, the present study has documented two new findings: *1*) postischemic recovery of cellular ATP is enhanced significantly by T_4 treatment as early as 30 min of reflow, but *2*) this augmentation of renal ATP levels is not preceded or accompanied by a concomitant improvement in cellular integrity. These results would suggest that ATP synthesis may be directly affected by T_4, by mechanisms such as stimulation of inorganic phosphate transport, or possibly by augmenting ADP translocator activity in mitochondria. In fact, mitochondrial nucleotide transporter activity has been shown to be reduced after ischemia (20, 21), and this transporter is known to be sensitive to thyroid hormones (22). Further investigation is required to explore these possibilities and to understand the complex interactions of the diverse cellular events that occur during recovery from ischemic acute renal failure.

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