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Chemical and functional correlates of postischemic renal ATP levels

(NMR/nucleotides/nucleosides)

MICHAEL E. STROMSKI*, KERRY COOPER†, GUNILLA THULIN‡, KAREN M. GAUDIO‡, NORMAN J. SIEGEL‡, AND ROBERT G. SHULMAN*

Departments of *Molecular Biophysics and Biochemistry, †Medicine, and ‡Pediatrics, Yale University School of Medicine, New Haven, CT 06510

Contributed by Robert G. Shulman, April 30, 1986

ABSTRACT **Renal energy metabolism was investigated before, during, and after ischemic insults of varying durations with *in vivo* ^{31}P NMR spectroscopy. The postischemic recovery of renal ATP was found to be a biphasic process regardless of the length of the ischemia. This two-stage recovery consisted of a quick initial component immediately upon reflow followed by a slower, more gradual return toward preischemic levels. To characterize the source of each phase of the recovery, kidneys were extracted with perchloric acid at the end of the different periods of ischemia (before reflow). Concentrations of adenine nucleotides and breakdown products adenosine, inosine, and hypoxanthine were determined by ^{1}H NMR spectroscopy. Excellent correlation was found between the residual nucleotide pool and the magnitude of the initial phase of ATP recovery. Additionally, the renal ATP content after 120 min of reflow was shown to have a strong correlation with subsequent functional recovery. These experiments show that *in vivo* ^{31}P NMR can provide new and dynamic information concerning the biochemical recovery from ischemia. Furthermore, this data has the potential to predict the eventual functional recovery of the organ.**

It is well-known that during renal ischemia, ATP levels fall to less than 25% of preischemic values and remain low for the duration of the insult (1–9). After an ischemic episode, the recovery of ATP is incomplete (2–5, 10). Previous studies from our laboratory (8, 9) using *in vivo* ^{31}P NMR indicated that, after 45 min of bilateral renal artery occlusion, the recovery of cellular ATP is a biphasic process. There is a rapid initial recovery of ATP immediately upon reflow and a slower, more gradual recovery that continues as ATP returns toward its preischemic levels. In addition, these previous studies showed that postischemic augmentation of cellular ATP by the infusion of adenine nucleotides was associated with significant improvement in renal function 24 hr after the initial injury (8, 9, 11, 12).

The present study evaluates both of these previous observations by varying the duration of the insult in order to modulate the biochemical and functional severity of the ischemic injury. In this manner, we determined the components of the biphasic recovery of cellular ATP and also the relationship of functional recovery to the renal ATP concentration after 2 hr of reflow. The magnitude of the initial recovery of ATP was found to be a good index of the residual adenine nucleotide pool in the kidney at the end of the ischemic interval. In addition, there is a direct linear correlation between the tissue concentration of ATP following 120 min of recovery and renal function 24 hr later. Moreover, the pattern of recovery of renal ATP during this time period provides an assessment of the net rate of ATP resynthesis. Thus, delineation of the recovery of cellular ATP by *in vivo* ^{31}P NMR provides new and dynamic information concerning the biochemical and functional recovery from an ischemic renal injury.

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MATERIALS AND METHODS

***In Vivo* NMR Experiments.** Male Sprague–Dawley rats (200–250 g) were anesthetized with sodium thiobutabarbital (Inactin, 80 mg/kg of body weight, i.p.) and tracheotomized. A catheter (P.E. 50) was inserted into the right jugular vein. The renal arteries were exposed via a midline abdominal incision and blunt dissection. A silastic sling was looped around the aorta distal to the origin of the celiac artery but proximal to the origin of both renal arteries. Animals were given heparin (500 units per kg), and fluid losses were replaced by an infusion of saline (1.2 ml/hr) throughout the experiment. The left kidney was exposed by a flank incision and placed in a micropuncture cup containing a NMR radiofrequency coil that fit snugly about the organ. Fourier transform ^{31}P NMR spectra were obtained with an Oxford Research Systems TMR-32/200 spectrometer operating at 32.5 MHz. Spectra were 7 min long and consisted of 2048 acquisitions, using 70° pulses and 0.2-sec recycle times. Control spectra contain well-resolved peaks for the α, β, and γ phosphates of ATP as well as inorganic phosphate, sugar phosphates, and methylphosphonate (an external standard attached to the micropuncture cup) as described (8, 9). Three control spectra were taken to ensure stability of the preparation, and then both kidneys were made ischemic for 15, 30, 45, or 60 min by placing tension on the silastic sling. After the ischemic interval, the tension was released and the animal was allowed to recover. Spectra were collected prior to, during, and for 120 min after the insult. Thus, each animal served as its own control. Tissue ATP levels were assessed by comparing changes in the intensity of the β-phosphate peak of ATP (ATP-β peak). In each spectrum the intensity of the ATP-β peak was scaled to that of the methylphosphonate peak, and changes in ATP are expressed as a percentage of the preischemic control value. In all groups of animals, ATP_{120} is the tissue concentration of ATP measured after 120 min of reflow, expressed as a percentage of the preischemic value.

To provide time-course data for ATP recovery, a linear regression line was calculated from all the data points collected during the first 120 min of reflow for each experiment. The initial rapid recovery of ATP (ATP_{init}) was determined from the y-intercept of the linear regression line calculated from all the data for a particular duration of ischemia. The rate of net resynthesis was evaluated from the slope of the linear regression line for each group of animals.

Abbreviations: ATP-β peak, peak for β phosphate of ATP; C_{In}, inulin clearance.

It should be noted that, after 60 min of ischemia, the tissue concentration of ATP was too low to be detected *in vivo* during the first 20 min of reflow. In this group of animals, calculations of linear regression lines were based on data obtained after reappearance of the ATP-β peak.

Extract Studies. Animals were prepared as described for *in vivo* studies, and the kidneys were made ischemic for periods of 15, 30, 45, or 60 min. At the end of the ischemic interval, the kidneys were quickly frozen *in situ* with aluminum tongs cooled in liquid nitrogen and were extracted with perchloric acid by well-established methods (13). The protein-free extracts were then triply lyophilized in ^2H_2O. ^1H NMR spectra of these extracts were obtained on a Bruker WM 500 spectrometer operating at 500 MHz. Spectra consisted of 200 acquisitions using 90° pulses and 10-sec recycle times. Peak assignments were made by comparison with standard spectra and by sequential addition of the compounds to extracts. Concentrations of the adenine nucleotides and adenine-related nucleosides and breakdown products were calculated by using glycerophosphocholine as an internal concentration standard.

Functional Studies. Animals were anesthetized with sodium pentobarbital (50 mg/kg) and subjected to the various ischemic intervals as previously described. At the end of the ischemic insult, the catheters were removed and the animal was allowed free access to food and water. Kidney function was determined by inulin clearance (C_{In}) 24 hr after the injury (14). The animals were anesthetized with Inactin (80 mg/kg, i.p.), tracheotomized, and catheters were inserted into the right jugular vein and bladder. After replacement of surgical losses with saline, a priming dose of 10 μCi (1 Ci = 37 GBq) of [*methoxy*-^{3}H]methoxyinulin (New England Nuclear) was given, followed by a sustaining infusion of 10 μCi/hr in 1.2 ml of saline. After a 45-min equilibration period, C_{In} was determined by the average of three 10-min urine collections. Blood samples were obtained from the tail at the midpoint of each urine collection. The concentration of [^{3}H]methoxyinulin was determined with a liquid scintillation counter. C_{In} was calculated by a standard formula (14).

Statistics. All values are expressed as means ± SEM. Linear regression lines were calculated by using the method of least squares. Comparison between groups was made by using analysis of variance and Student's *t* test, where applicable.

RESULTS

In all *in vivo* experiments, at the initiation of ischemia the tissue concentration of ATP quickly decreased to levels that were undetectable with *in vivo* ^{31}P NMR and remained low for the duration of the insult. Table 1 gives the initial and subsequent (after 120 min of reflow) recovery of ATP seen with 15, 30, 45, and 60 min of ischemia. Note the decline in both the initial recovery of ATP (ATP_{init}) and the ATP concentration after 120 min of reflow (ATP_{120}) with increasing durations of ischemia. Both ATP_{init} and ATP_{120} have significant inverse linear correlations with the duration of the insult: $ATP_{init} = -0.43T + 74.0$ ($r = 0.973$, $P < 0.05$), and $ATP_{120} = -0.79T + 100.4$ ($r = 0.966$, $P < 0.05$), where T is the duration of ischemia in minutes and ATP is expressed as a percentage of control values. This table also presents the difference (denoted by Δ) between ATP_{init} and ATP_{120}. The decline in Δ as a function of increasing ischemic interval indicates that ATP returns more slowly toward control values.

A measure of the rate of net resynthesis of ATP is provided by the slope of the linear regression line calculated from all data points collected during the first 120 min of reflow for each ischemic interval. Inspection of these linear regression lines (Fig. 1) and their slopes (Table 1) indicates that there was no difference between the rate of net resynthesis of ATP after 15 or 30 min of ischemia nor was there a difference between 45 and 60 min of ischemia. However, the rate of net resynthesis after 45 or 60 min of ischemia was significantly lower than that after 15 min ($P < 0.05$).

The chemical contents of kidneys freeze-clamped and extracted at the end of the different ischemic intervals (before reflow) are presented in Table 2. The concentrations of ATP, ADP, and total adenine nucleotides decreased as the ischemic period increased. Although AMP concentration was significantly increased at the end of each ischemic insult, the highest values were seen after only 15 min of ischemia and decreased significantly with each increase in ischemic intervals. The decline in total adenine nucleotide concentration after short periods of ischemia is due in great part to the decline in the tissue concentration of AMP, since the decreases in ATP and ADP were small after their initial decrease. Although tissue concentrations of the nucleosides adenosine and inosine and their breakdown product hypoxanthine were not detected in nonischemic control kidneys, all were visible after the ischemic insult. The sum of the concentrations of adenosine, inosine, and hypoxanthine remained constant at $\approx$1 μmol/g of wet weight regardless of the duration of the ischemia.

There is a significant linear correlation between the total adenine nucleotide concentration in the kidney at the end of the ischemic insult and the magnitude of the recovery of tissue ATP immediately upon reflow (Fig. 2). The initial recovery of renal ATP decreased from 69.3% of preischemic levels after 15 min of ischemia to 49.7% after 60 min of ischemia, whereas the total adenine nucleotide concentration in the kidney at the end of the ischemic interval decreased from 82.7% to 31.7% over the same period. The data indicate that the initial recovery of renal ATP following ischemia is a good index of the residual nucleotide pool in the kidney at the end of the insult.

Kidney function 24 hr after an ischemic insult decreased with increasing durations of the insult. In control nonischemic animals, C_{In} was 1016 ± 42, whereas 15, 30, 45, and 60 min of ischemia led to values of 856 ± 40, 387 ± 30, 313 ± 28, and 170 ± 36, respectively. There is a significant linear correlation between kidney function 24 hr after ischemia and renal ATP content after 120 min of reflow (Fig. 3).

Table 1. ATP recovery after ischemia as a function of the duration of the insult

Ischemic interval, min	No. of animals	Initial recovery, % of control	120-min recovery, % of control	Δ*	Rate of net resynthesis, % of control per min
15	5	69.3 ± 3.5	92.1 ± 4.5	22.8 ± 5.7	0.20 ± 0.05
30	5	59.1 ± 3.9	72.8 ± 6.8	13.7 ± 7.8	0.17 ± 0.05
45	6	53.0 ± 2.7	61.4 ± 3.0	8.4 ± 4.0	0.10 ± 0.03†
60	5	49.7 ± 4.4	56.2 ± 3.2	6.5 ± 5.4	0.08 ± 0.06†

*Δ = 120-min recovery − initial recovery.
†$P < 0.05$ compared to 15-min ischemia.

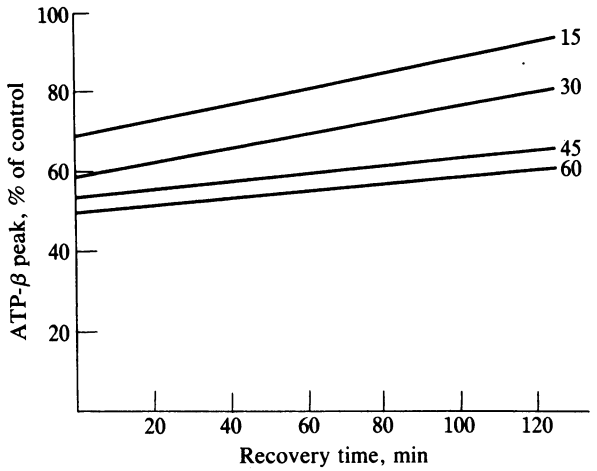

FIG. 1. Linear regression lines for ATP recovery after ischemic insults of various durations shown in minutes. The slopes of these lines represent net rate of resynthesis of ATP (Table 1). The derivation of the linear regression lines is described in the text.

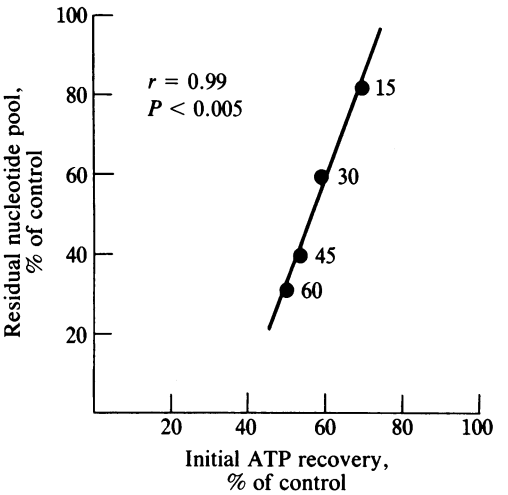

FIG. 2. The correlation between the total adenine nucleotide pool at the end of the ischemic episode (shown in minutes) before reflow and the rapid initial recovery of ATP, both given as a percentage of the preischemic value.

DISCUSSION

Previous investigations from our laboratory (8, 9) were concerned with accelerating the recovery of renal ATP after 45 min of renal ischemia by infusing adenine nucleotides combined with magnesium chloride. In the course of these experiments, we discovered that the postischemic restoration of ATP was a biphasic process whether or not exogenous nucleotides were infused. There was a rapid initial recovery of cellular ATP immediately upon reflow. The second component of ATP recovery was slower with a gradual increase throughout the first 120 min of reflow, and this phase was augmented by the postischemic infusion of adenine nucleotides (8, 9). These previous observations led us to examine the pattern of recovery of cellular ATP after ischemic insults of 15, 30, 45, or 60 min. The data presented in this study indicate that the restoration of cellular ATP after renal artery occlusion follows a biphasic pattern, and an analysis of each component provides information concerning (*i*) the residual nucleotide pool at the end of ischemia, (*ii*) the rate of net resynthesis of ATP, and (*iii*) the severity of functional impairment.

The total adenine nucleotide concentration (the sum of ATP, ADP, and AMP) has been shown to decrease during an ischemic episode (1, 2, 4, 5, 7). These nucleotides are degraded to the purine nucleosides adenosine and inosine and to the purine base hypoxanthine, which have been shown to increase during ischemia (15–17). The data presented here (Table 2) indicate that the loss of adenine nucleotides with increasing durations of ischemia is mainly due to a loss of AMP. However, the loss of AMP cannot be accounted for by an increase in the tissue concentration of degradation products, since the sum of the adenosine, inosine, and hypoxanthine concentrations remains constant. Our data show a correlation between the size of the total adenine nucleotide pool at the end of the ischemic insult and the initial component of ATP recovery.

We considered the possibility that the second phase of postischemic ATP recovery is due to salvage of adenosine, inosine, and hypoxanthine in the kidney at the end of ischemia. This is not a very likely explanation. Although tissue concentrations of these degradation products increased with short durations of ischemia, the sum of their concentrations remained constant and did not change significantly with increasing lengths of ischemia, and these purine nucleosides and base are membrane-permeable and wash out of the kidney upon reflow (15, 17). It seems more probable that this slower component of recovery of ATP reflects the net resynthesis of cellular ATP. As blood flow is returned to the kidney, nutrients and endogenous precursor compounds that would support resynthesis of the adenine nucleotides become available, and the rate of ATP resynthesis would be dependent on the severity or duration of the ischemic insult.

In the present study, we have shown that increasing the duration of ischemia from 15 to 60 min results in more severe functional impairment and that the magnitude of recovery of ATP after 120 min of reflow is inversely related to the ischemic interval. Moreover, our previous studies have shown that postischemic infusion of ATP/$MgCl_2$ (8) or AMP/$MgCl_2$ (9) will significantly augment this second component of ATP recovery. Consequently, the slower recovery of ATP may be affected by many factors that influence the resynthesis of ATP, including availability of precursor compounds, degree of cellular or membrane damage, and the residual nucleotide pool. The slope of the linear regression line derived from all data points during the first 120 min of reflow provides an assessment of the rate of net resynthesis

Table 2. Concentrations of purine breakdown products in the ischemic rat kidney as a function of the duration of ischemia

Ischemic interval, min	No. of animals	Adenine nucleotides, μmol/g wet weight				Nucleosides and product, μmol/g wet weight			
		ATP	ADP	AMP	Total	Ado	Ino	Hyp	Total
0	7	2.06 ± 0.24	1.25 ± 0.30	0.51 ± 0.11	3.82 ± 0.40	ND	ND	ND	ND
15	6	0.62 ± 0.11	0.60 ± 0.12	1.94 ± 0.20	3.16 ± 0.26	0.15 ± 0.03	0.28 ± 0.07	0.49 ± 0.10	0.92 ± 0.12
30	6	0.57 ± 0.09	0.31 ± 0.06	1.40 ± 0.15	2.27 ± 0.18	0.07 ± 0.01	0.28 ± 0.07	0.68 ± 0.06	1.03 ± 0.09
45	5	0.33 ± 0.04	0.31 ± 0.07	0.89 ± 0.15	1.53 ± 0.17	0.09 ± 0.02	0.19 ± 0.04	0.67 ± 0.15	0.95 ± 0.16
60	7	0.30 ± 0.05	0.18 ± 0.03	0.73 ± 0.19	1.21 ± 0.20	0.08 ± 0.02	0.25 ± 0.06	0.70 ± 0.06	1.03 ± 0.09

ND, below detectable levels; Hyp, hypoxanthine.

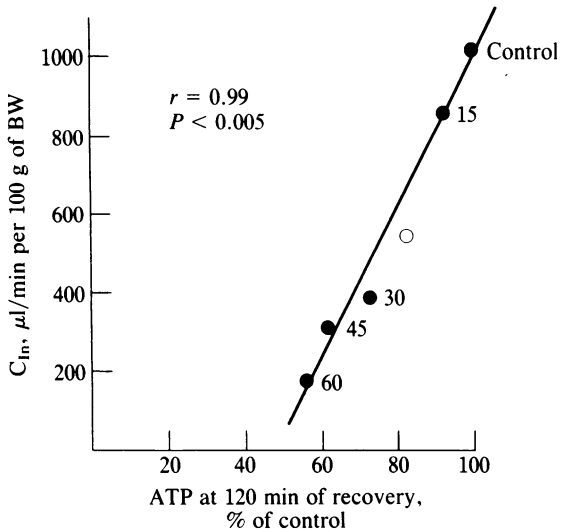

FIG. 3. The correlation between postischemic kidney function and the renal ATP content at 120 min of reflow. The closed circles are derived from the data in this study. The open circle represents data obtained after 45 min of ischemia, followed by the infusion of 25 μmol of ATP/$MgCl_2$ (9, 11). Duration of ischemia is shown in minutes. BW, body weight.

of ATP after a given ischemic insult. In the present study, the rate of net resynthesis of ATP was greater in animals subjected to either 15 or 30 min of ischemia as compared with animals with 45 or 60 min of injury (Table 1 and Fig. 1). In fact, the regression lines for ATP recovery are parallel in animals with the milder injury (15 or 30 min) and also in animals with the more severe injury (45 or 60 min), while the two groups are significantly different from each other. When these groups are compared, it is evident that the rate of recovery is significantly slower when ischemia exceeds 30 min. If one compares the magnitude of recovery at 120 min for animals with 15 min of injury to those with 30 min of ischemia, it becomes apparent that ATP_{120} is determined more by ATP_{init} than by differences in the rate of net ATP resynthesis. Similar observations occur if one compares animals with 45 min of ischemia to those with 60 min of injury. Thus, a more severe cellular and metabolic injury occurs when the interval of ischemia is extended beyond 30 min, and this is reflected, at least in part, by a diminished rate of net resynthesis of ATP.

The relationship between the concentration of ATP after 2 hr of recovery and subsequent renal function 24 hr after the ischemia was examined by varying the duration of the ischemic injury and comparing ATP_{120} with C_{In} 24 hr later. As expected, the severity of functional impairment was greater with longer duration of ischemic injury. In addition, there was a strong correlation found between ATP_{120} and postischemic renal function (Fig. 3). While the data do not require that the increased recovery of renal function is directly due to the improved recovery of ATP, the strong correlation between these two quantities implies that the renal ATP concentration after 120 min of reflow is a good indicator of the severity of the ischemic insult. The present observations are consistent with previous investigations from our laboratory that showed the beneficial effects of the postischemic infusion of adenine nucleotides (8, 9, 11, 12). These exogenous compounds have been shown to increase the return of ATP toward control levels by accelerating the second phase of the recovery process. In addition, infusion of these metabolites also resulted in increased C_{In} 24 hr after ischemia. As shown in Fig. 3, the relationship between ATP_{120} and C_{In} 24 hr later is maintained whether ATP_{120} occurs by an endogenous mechanism (varying the ischemic interval) or by pharmacologic manipulation (infusion of ATP/$MgCl_2$).

The data obtained in this study indicate that the postischemic recovery of renal ATP is a biphasic process regardless of the severity of the insult. The magnitude of the initial ATP recovery increases with the size of the residual adenine nucleotide pool in the kidney at the end of the ischemic period. The slower component is an index of the rate of net ATP resynthesis and can be influenced by both endogenous and exogenous factors such as the severity of the insult and the availability of precursors. The level of ATP achieved after 120 min of reflow is a good indicator of subsequent functional recovery. Further knowledge of the cellular and molecular mechanisms that influence each phase of ATP recovery should help in better understanding the adaptive mechanisms that occur in response to ischemia. These data also indicate that the continuous assessment *in vivo* of cellular recovery of ATP by ^{31}P NMR provides new and dynamic information about the mechanisms of renal injury.

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