The Role of High-Energy Phosphate in Norepinephrine-Induced Acute Renal Failure in the Dog

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SUMMARY **Previous studies have demonstrated that pretreatment with mannitol, furosemide, or bradykinin can attenuate the severity of norepinephrine-induced renal functional impairment. The present studies were designed to evaluate the possibility that these agents are protective, in part, by preserving cellular metabolic integrity. The renal cortex was repetitively biopsied during the course of this study, and high-pressure liquid chromatography was used to analyze the tissue content of adenine nucleotides (expressed in nanomoles per gram of wet tissue). The adenine nucleotide charge ratio (CR) and total adenine nucleotide (TAN) content were calculated as indices of cellular metabolic integrity. In addition to the above-established protective agents, phenoxybenzamine was used to evaluate a direct toxic effect of norepinephrine on renal tissue. Inulin clearance at 3 hours post infusion (expressed as a percent of control) was 7% with norepinephrine alone and, in the protected groups, 36% with bradykinin, 61% with furosemide, 51% with mannitol, and 100% with phenoxybenzamine. There was no change in CR or TAN with phenoxybenzamine. In contrast, during norepinephrine administration CR fell significantly in all other groups. Three hours after stopping norepinephrine, CR had returned toward control values and the level of CR was significantly better in all protected groups when compared with norepinephrine alone. Similarly, the levels of TAN were significantly diminished in the norepinephrine-alone group when compared to all protected groups, and there was significantly more tubular necrosis as well. The maintenance of higher levels of TAN and the preserved ability to regenerate adenosine triphosphate in the protected groups, when compared to the norepinephrine-alone group, support the contention that these agents offer protection, at least in part, by preserving cellular metabolic integrity. (*Circulation Research* 1986;59:93–104)**

KEY WORDS • mannitol • furosemide • bradykinin • norepinephrine-induced renal impairment • cellular metabolic integrity • adenosine triphosphate • total adenine nucleotide content

THE experimental model of intrarenal-norepinephrine-induced acute renal failure is associated with severe renal hypoperfusion due to vasoconstriction. The glomerular filtration rate is reduced to less than 10% of control values in this model at 3 and 48 hours after norepinephrine administration.[1-3] Previously, studies from this laboratory and others have shown that pretreatment with prostaglandin E_2, mannitol, furosemide, and bradykinin can attenuate the severity of norepinephrine-induced renal functional impairment.[1,3,4] However, the mechanism(s) by which these agents offer protection is not clear.

The evaluation of hemodynamic parameters such as glomerular filtration rate or renal blood flow during the initial insult or the early phase of recovery in this model does not predict the potential for renal functional recovery. Other factors such as tubular obstruction, back-leak of glomerular filtrate, or decreases in K_f may be critical in this regard, although studies of back-leak of glomerular filtrate in the rat would suggest that its role is minimal.[5] Alternatively, it is possible that metabolic alterations may occur early in acute renal failure and play a critical role in determining the degree of functional recovery from acute renal failure.

High-energy phosphates play a key role in maintaining an appropriate metabolic milieu within cells and are thus necessary for the survival of living tissues.[6-8] Dead or dying cells are unable to regenerate the high-energy phosphate pool essential for functional recovery. Although the energy state of the cell during ischemia is not necessarily a reflection of cell viability,[6] the rate and degree of recovery of the cellular energy state during reflow may be a more reliable indicator of cellular functional viability.[9-13] This study evaluates the adenine nucleotide profile of kidney cortex in control conditions, during and up to 3 hours following norepinephrine-induced acute renal failure, with and without pretreatment with the protective agents, mannitol, bradykinin, and furosemide. The α-blocking effect of the agent, phenoxybenzamine, was also studied.

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If the critical effect of these protective agents was to enhance solute clearance and thus facilitate cast removal, one would not expect cellular metabolism to be altered by these drugs. On the other hand, if these agents offered their protective effect by preserving cellular metabolic integrity, one would expect the adenine nucleotide profile to be better preserved in kidneys pretreated with these protective agents when compared to kidneys exposed to norepinephrine (NE) alone. The results of the study support the latter contention.

Materials and Methods

Mongrel dogs (17–35 kg) were deprived of food 18 hours prior to study. The animals were anesthesized with IV sodium pentobarbital 30 mg/kg, and additional small doses were administered as needed during the experiment to maintain light general anesthesia. After endotracheal intubation, the animals were ventilated with a Harvard respirator (Harvard Apparatus Co. Inc., Millis, Mass.). The jugular vein and femoral artery were cannulated. Following an 800-mg IV bolus dose of inulin, an infusion of Ringer's solution containing 1.5% inulin was given at 0.5–1.0 ml/min through the jugular vein cannula. The femoral artery was used for collecting 5-ml blood samples. In addition, the femoral artery cannula was connected to a pressure transducer and recorder (Hewlett-Packard Co., Waltham, Mass.) for continuous monitoring of mean arterial blood pressure. The left and right kidneys were exposed through flank incisions; both ureters were cannulated with PE 160 polyethylene tubing; and both kidneys were carefully stripped of their capsular adhesions. An electromagnetic flow probe (3.0–4.0 mm i.d.; Zapeta Instruments, Seattle, Wash.) was fitted around the renal artery of the experimental kidney, taking care not to disrupt any visible renal nerves. This served for continuous monitoring of renal blood flow. These flow probes were calibrated *in vitro*, and zero flow was set *in vivo* by a brief occlusion of the experimental artery distal to the flow probe in each experiment. A curved 23-gauge needle was inserted into the renal artery near its origin, and patency was maintained with Ringer's solution at 0.8 ml/min. All the experimental agents were infused through this needle at 0.8 ml/min. At least 30 minutes were allowed following surgery before any experimental protocol was started.

Renal cortical biopsies were obtained using an 8-cm length of 3-mm-i.d. stainless steel hypodermic tubing sharpened at one end and connected by a short length of flexible tubing to a 13 × 100-mm glass tube partially filled with isopentane chilled to gelation (−160° C) in liquid nitrogen (Figure 1). A vacuum of −30 mm Hg was applied to the glass tube in order to draw the biopsy tissue from the kidney, through the tubing, and into the chilled isopentane. The biopsy was obtained by rapidly pressing the sharpened end of the 3-mm-i.d. stainless steel tubing through the outer cortex using a twisting motion. This ensured the specimen's being aspirated and frozen to −160° C in less than 1 second.

Preliminary studies were performed to evaluate the effect of this biopsy procedure on glomerular filtration rate (GFR). There was less than 5% fall in GFR when 1 to 8 biopsies were obtained from a single kidney. No more than 6 biopsies were obtained from a single kidney during the course of the study. Renal cortical bleeding was obviated by occluding the renal cortical biopsy hole with an appropriately sized cotton-tipped occluder.

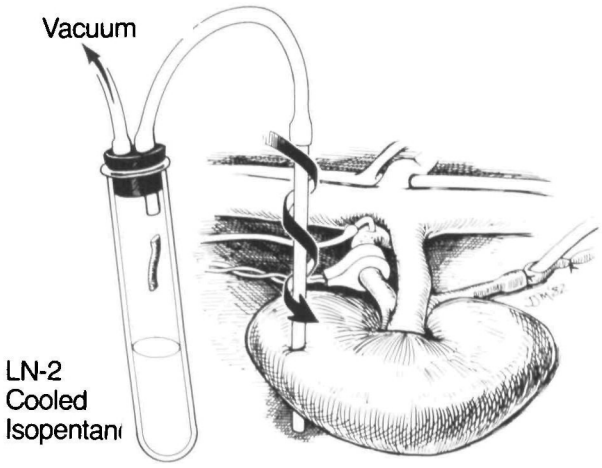

FIGURE 1. *The biopsy technique. This approach allows the aspiration and freezing of renal cortical tissue, of 185 mg average weight, to −160° C in less than 1 second. The sharpened steel tubing attached to vacuum is rapidly pressed through the renal cortex using a twisting downward motion. Having traversed the renal cortex, the core of cortical tissue is rapidly sucked into the test tube containing isopentane cooled to −160° C.*

Study Groups

Seven groups of studies were performed.

Group I: Norepinephrine Alone (n = *8*). The general protocol for these studies is shown in Figure 2. After two 15-minute control clearance collections were obtained, a control renal cortical biopsy was obtained from each kidney. Norepinephrine (1 μg/kg/min) was then infused into one renal artery for 40 minutes. Renal cortical biopsies were taken from the experimental kidney at 15 and 30 minutes after initiation of the NE infusion and at 20, 80, and 180 minutes after termination. Contralateral renal biopsies were taken at 30 minutes after initiation of the NE infusion and at 80 and 180 minutes after termination. Two 15-minute clearance collections were obtained at 165 and 185 minutes following the NE infusion. A final renal biopsy was obtained from each kidney for histology following the final clearance collection. Renal blood flow was monitored with the electromagnetic flow probe throughout the experiment.

Group II: Bradykinin (n = *8*). The general protocol for Groups II through V is shown in Figure 2. After two 15-minute control clearance collections and bilateral control renal cortical biopsies were obtained, bradykinin was infused at 0.75 μg/kg/min via the renal artery needle. A further 15-minute clearance collection and bilateral renal biopsies were obtained near the end of this collection. Norepinephrine (1 μg/kg/min) was

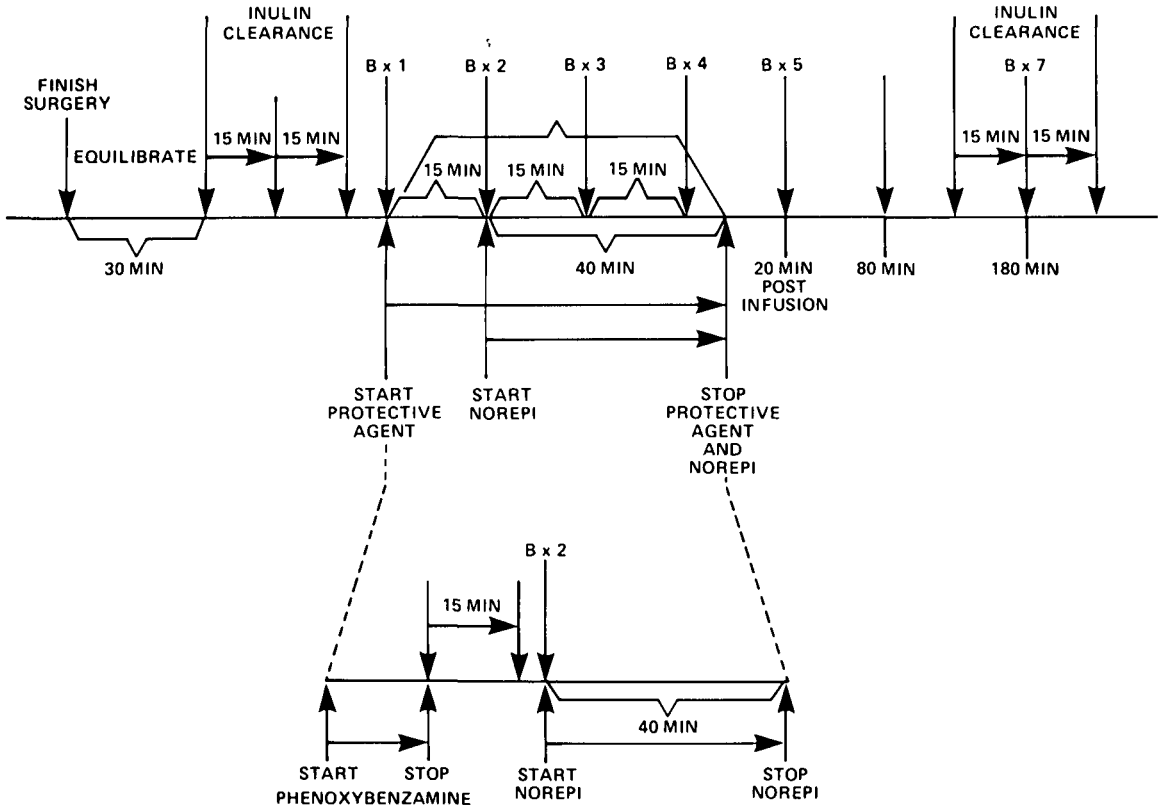

FIGURE 2. *Protocol for all of the experimental groups.*

added to the renal artery infusion after 15 minutes of bradykinin infusion. Both infusions were then maintained for 40 minutes. Renal cortical biopsies were obtained at the same time intervals as with Group I.

Group III: Furosemide (n = 7). To determine the effect of furosemide on the adenine nucleotide profile in NE-induced ARF, furosemide (4 mg/kg/hr) with a subsequent addition of NE (1 μg/kg/min) was infused using the same protocol as with Group II. Renal cortical biopsies were obtained at the same time intervals as in Group II.

Group IV: Mannitol (n = 7). To determine the effect of an osmotic agent on the adenine nucleotide profile in NE-induced acute renal failure, 10% mannitol with a subsequent addition of NE (1 μg/kg/min) was infused using the same protocol as in Group II. Renal cortical biopsies were again obtained at the same time intervals as in Group II.

Group V: Phenoxybenzamine (n = 5). To determine if the effect of NE was related to a direct cellular effect independent of α-receptor occupancy, phenoxybenzamine (0.3 mg/kg/min), a competitive inhibitor of NE, was infused via the renal artery for 15 minutes, starting 30 minutes prior to the beginning of the NE infusion (Figure 2). This was done to allow adequate binding of phenoxybenzamine to α receptors. Renal cortical biopsies were taken at similar intervals as in Group II, except that the second biopsy was obtained just prior to the beginning of the NE infusion, which was 15 minutes after the end of the phenoxybenzamine infusion. As in all groups of studies, volume replacement was performed with Ringer's solution in an amount that slightly exceeded urinary output.

Group VI: Capsular Stripping (n = 9). Because of the possibility that some variability in the degree of freeing the renal capsule of all vascular attachments might have led to some bias in potential collateral flow through the capsule to the renal cortex, 4 dogs were studied according to the Group I protocol (NE alone) and 5 dogs were studied according to the Group II protocol (bradykinin). The only differences were that protocols were consciously alternated between the NE and bradykinin protocols and a single investigator consciously checked each kidney twice prior to study to confirm that all capsular adhesions and vessels had been severed; thus there was no way for blood to reach the renal cortex from the capsule. Kidney biopsies were not performed in Group VI.

Group VII: Total Collection of Renal Blood Flow. This group of studies was performed to evaluate the possibility that a protective agent (mannitol) might in some manner maintain renal blood flow (RBF) at a higher level during NE infusion than that found in studies with NE alone. Because RBF was quite low during NE infusion, the electromagnetic flow probe was not accurate. To measure renal blood flow in Group VII, a system for the total collection of renal venous outflow was devised.

The dogs were prepared as described previously. All capsular adhesions were carefully stripped from the kidneys. The animal was then systematically heparinized with an IV bolus of 250 U heparin. The control

kidney renal artery was briefly clamped, and a 4-mm-i.d. flexible rubber catheter was inserted into the renal vein of the control kidney. At the same time a similar catheter was advanced across the inferior vena cava into the contralateral (experimental) renal vein. Both catheters were then secured in their respective renal veins with purse string sutures. Both catheter ends were attached to an appropriately sized three-way connector that was attached to a larger flexible rubber catheter sutured (as a common return) into the inferior vena cava. Thus, both kidneys drained into the inferior vena cava via a loop of tubing. The loop was kept as short as possible (10–20 cm) to avoid any blood flow resistance in the loop. Clamping of the control kidney renal artery for this surgical procedure was kept to less than 5 minutes. This procedure thus avoided any clamping of the experimental kidney. Electromagnetic flow probes were then placed on both renal arteries as described previously. A curved 23-gauge needle for infusion was then placed in the experimental renal artery as described previously. Renal blood flow was measured by direct collection of renal venous outflow, via the three-way connector, from each kidney. The collections were compared to the calibrated flow probe readings. Collections were made at zero time, at 15 minutes of the protective agent mannitol, at 5, 10, 20, 30, and 40 minutes of NE and NE + mannitol, and then at 10, 20, and 80 minutes following the termination of the infusions. The control and 15-minute mannitol collections were taken as the average of two 15-second collections. The collections during and following the NE infusion varied from 1 to 40 minutes. Any blood collected was immediately reinfused via the jugular vein catheter. These collections did not affect blood pressure.

Assays

Inulin was measured using an autoanalyser (Technicon Corp., Ardsley, N.Y.). Plasma and urine osmolality were determined by freezing-point depression with an osmometer (Advanced Instruments, Inc., Newton Highland, Mass.). Plasma and urine sodium concentrations were determined by flame photometry (Instrumentation Laboratory, Lexington, Mass.).

The general procedure for adenine nucleotide analysis was adapted from Khym.[14] Renal biopsies were removed from cooled (−40° C) isopentane, quickly weighed in the frozen state, and then returned to cooled isopentane to await homogenization. Renal biopsies weighing, on average, 185 mg were homogenized in the frozen state in cooled 5% trichloracetate (0–4° C) using a Brinkman homogenizer. The resulting mixture was spun for 5 minutes at 3300 rpm at 10° C to precipitate denatured proteins, and the supernatant was decanted into an equal volume (2.5 ml) of 0.5 N tri-N-octylamine (Sigma chemicals) in trichloro-trifluroethane (Matheson Gas Products: freon-TF) and agitated in a capped 13 × 100-mm glass tube on a vortex agitator for 3 minutes. Further centrifugation for 2 minutes at 2,000 rpm facilitated aspiration of the top aqueous phase containing the extracted adenine nucleotides at a pH of 3.5–5.0. The samples were then stored at −80° C prior to analysis on a Waters high-performance liquid chromatograph (HPLC).

Routinely, a 200-μl sample size of standard or sample extract was injected onto a Whatman PXS 10/25 SAX cation exchange column. The mobile phase consisted of deionized, double-distilled, millipore-filtered water [solvent A (pump A, Waters model 6000A)] and 3.4% wt/vol potassium monohydrogen phosphate and 3.7% KCl, pH 5 [solvent B (pump B, Waters model 45)]. A solvent programmer (Waters model 660) was set at a flow rate of 2 ml/min. Initial conditions and an equilibration period of 30 minutes were set with solvent A 95%, solvent B 5%. Samples were eluted with a 35-minute concave gradient (Curve #7) from 5 to 100% solvent B with a total elution time of 45 minutes, including a final 10 minutes of 100% solvent B. This gave excellent separation of all adenine nucleotides with a stable baseline (Figure 3). Standardization was accomplished by preparing 10 nmol of each adenine nucleotide (Sigma) in 200 μl of water, and this standard mixture was injected every 10th sample. Peak identification was performed by injection of known standards individually, in combination, and by addition to tissue sample extracts. The Waters Wisp automatic injector (Wisp 710B) was used to automate injection of samples. Absorbance was measured at 254 nm with a Waters fixed wavelength detector (Model 440), and peaks were identified, integrated, and calculated in nanomoles per gram of wet tissue and displayed by the Waters Data module (Model 730). This method had an intraassay variation of ±3% and an interassay variation of ± 10%. If an individual column was used for more than 50 samples, this degree of variability increased; thus, the cation exchange column was regularly replaced after 40–50 samples had been analyzed. Each tissue sample was regularly analyzed once unless mechanical errors of injection or computer analysis required repeat analysis. All results of nucleotide analysis were thus expressed per gram of wet tissue. Preliminary experiments revealed that the water content of normal vs. norepinephrine-treated kidney biopsies varies by less than 6%. Thus, the possible change in water content of the tissue should not contribute in an important way to the results of nucleotide analysis since these changes were regularly greater than 50% of control.

The stability of the extracted adenine nucleotides was evaluated by repeating the HPLC analysis on several samples and standards up to 9 months apart. Little to no change was observed when samples were stored at −80° C. The stability of nucleotide content over time was confirmed in several preliminary studies in which serial biopsies were obtained from the same kidney without other pharmacological intervention.

For light microscopy renal cortical biopsies were fixed in 10% formalin and processed by standard techniques. Coded sections were stained with hematoxylin and eosin and evaluated by an observer without knowledge of specimen identity. Tubular degenerative changes, including swelling, formation of blebs within

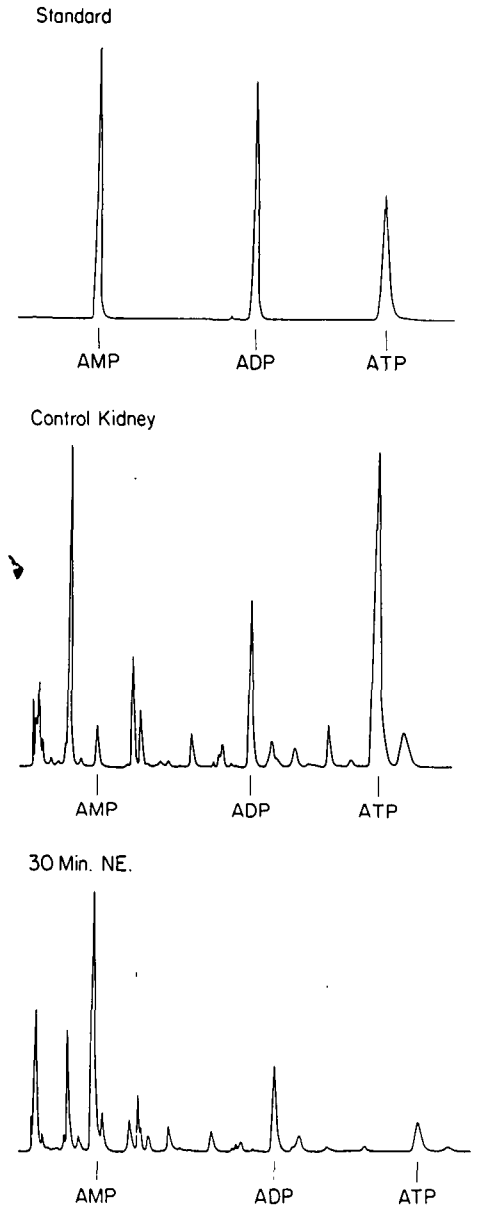

FIGURE 3. *The similar elution times and peak identification of prepared standards (top) of adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP); results from a control renal cortical biopsy (middle); and results from a renal cortical biopsy obtained at 30 minutes of norepinephrine infusion (bottom). Note the fall in ATP and rise in AMP concentration when the results at the bottom are compared with the results in the middle.*

proximal tubule lumina, nuclear pyknosis, desquamation, and necrosis, were graded on a 0 to 4+ scale.

Results in the figures and in Tables 1–4 are expressed as the mean ± 1 SEM, and paired and unpaired comparisons were made using Dunnett multiple group comparison procedure.[15] Histology was evaluated using the Spearman rank correlation test.[16]

Calculations. Renal functional parameters were calculated in the usual manner. Adenylate CR[17] was determined by using the following formula: (ATP + ½ ADP)/(ATP + ADP + AMP). Total adenine nucleotide (TAN) content was determined by summing the ATP, ADP, and AMP content of individual samples (and expressing the results as nanomoles per gram of wet tissue).

Results

Group I: Norepinephrine (n = 8). The results of these studies are summarized in Tables 1 and 2 and Figure 4. During the 40-minute NE infusion, renal blood flow (RBF) in the experimental kidney (EK) fell to essentially zero as determined by the flow probe. There was no measurable urine output during the infusion. Adenylate CR fell to 20% of control at 30 minutes of the NE infusion (Figures 4 and 5) and recovered to 80% of control at 180 minutes post infusion respectively. Adenosine triphosphate content fell to 7% of control and recovered to 28% of control values at 180 minutes post NE (Figures 4 and 6). Total adenine nucleotide fell to 62% of control by 30 minutes of NE and continued to fall to its lowest level of 27% at 80 minutes and recovered only slightly to 35% of control at 180 minutes (Figures 4 and 7). There were no significant changes in any of these parameters at any time in the control kidney (CK). Inulin clearance averaged 7% of control at 180 minutes, and RBF averaged 78% of control at this same time (Table 1).

Group II: Bradykinin (n = 8). During the administration of BR prior to NE infusion, there was a significant 53% increase in RBF (Table 1). Adenylate charge ratio (Figure 5 and Table 2), ATP content (Figure 6), and TAN content (Figure 7), did not change significantly with BR alone. On starting the NE infusion RBF fell to unrecordable levels as measured by flow probe. Adenylate CR fell to 27% of control at 30 minutes of NE + BR infusion in the EK and recovered to 97% at 180 minutes post infusion. Adenosine triphosphate content fell to 19% of control at 30 minutes of NE infusion and recovered to 74%. Total adenine nucleotide content fell to 75% of control at 20 minutes post infusion and changed little thereafter, remaining at 75% at 180 minutes. The recovery of adenylate CR, ATP content, and TAN content was significantly greater than in the NE-alone (Group I) studies (Figures 5–7). Inulin clearance was reduced to 36% of control values at 180 minutes, a value that was significantly higher than that found in the NE-alone group (Table 1). Renal blood flow recovered to 88% of control. There were no significant changes in any of these parameters in the CK.

Group III: Furosemide (n = 7). With furosemide alone RBF did not change significantly. There was a significant rise in urine volume, fractional sodium, and osmolar excretion from both kidneys (Table 1). Adenylate CR did not change with furosemide alone. Adenosine triphosphate and TAN contents increased slightly, but not significantly. During the NE infusion RBF fell to unrecordable levels. Adenylate CR fell to 43% of control at 30 minutes of NE infusion and recovered to 94% at 180 minutes post infusion (Figure 5 and Table 2). Adenosine triphosphate content fell to 28% of control at 30 minutes of NE infusion and recovered

TABLE 1. *Renal Functional Parameters*

Group	Time	GFR (ml/min/kg b.wt.)	RBF (ml/min/kg b.wt.)	V (ml/min)	FeNa (%)	UosmV (osm/min)
I	Control	1.3±0.6	7.3±1.3	0.13±0.02	0.5±0.1	225±47
Norepinephrine	180 min	0.1±0.02*	5.7±1.1	0.05±0.02*	3.5±1.9*	38±28*
II	Control	1.8±0.2	8.2±0.5	0.31±0.14	1.0±0.5	279±67
Bradykinin and	15 min BR	1.7±0.3	12.7±0.9*	1.20±0.40*	3.4±0.9*	312±95*
norepinephrine	180 min	0.7±0.2†	7.2±0.6	0.43±0.14	2.7±0.9	190±58
III	Control	1.6±0.1	6.9±0.3	0.53±0.19	2.1±0.4	325±70
Furosemide and	15 min FUR	1.7±0.4	7.9±0.9	3.65±1.37*	11.0±2.2*	1331±330*
norepinephrine	180 min	0.9±0.1†	5.3±0.4	2.48±0.12*	12.9±6.5*	679±193*
IV	Control	1.6±0.1	8.0±0.6	0.29±.07	1.3±0.3	300±47
Mannitol and	15 min MAN	1.4±0.1	8.1±0.6	1.07±0.28*	3.0±1.0*	612±127*
norepinephrine	180 min	0.8±0.1†	6.5±0.6	0.17±0.04	0.4±0.1	134±21
V	Control	1.6±0.2	9.5±0.6	0.20±0.04	1.0±0.2	283±72
Phenoxybenzamine	15 min PHE	1.4±0.2	10.3±0.9	0.40±0.14	1.2±0.4	331±41
and	15 min PHE+NE	1.3±0.3	11.3±1.4	0.35±0.09	0.9±0.1	227±18
norepinephrine	180 min	1.6±0.1†	10.2±0.6	0.30±0.07	1.1±0.4	303±69

NE = norepinephrine, BR = bradykinin, FUR = furosemide, MAN = mannitol, PHE = phenoxybenzamine. Results for only the experimental kidney are shown.

*Significantly different from the control values for this group of studies.

†Significantly different from the values for Group I at the same time intervals.

to 81% at 180 minutes (Figure 6). Total adenine nucleotides fell to 68% of control at 20 minutes post NE and recovered to 92% at 180 minutes (Figure 7). The recovery of adenylate CR, ATP content, and TAN content was significantly greater than in the NE-alone group (Figures 5–7). Renal blood flow recovered to 76% of control. Inulin clearance at 180 minutes averaged 61% of control, a value significantly higher than that found in the NE-alone group (Table 1). Fractional sodium and osmolar excretion were significantly greater than the other groups at 180 minutes. There were no significant changes in GFR or the adenine nucleotide profile in the CK.

Group IV: Mannitol (n = 7). During mannitol (MAN) administration prior to NE infusion there was no significant change in RBF. The adenine nucleotide profile did not change significantly. During the NE infusion RBF fell to unrecordable levels. Adenylate CR fell to 28% of control at 30 minutes of NE + MAN infusion and recovered to 96% at 180 minutes post NE

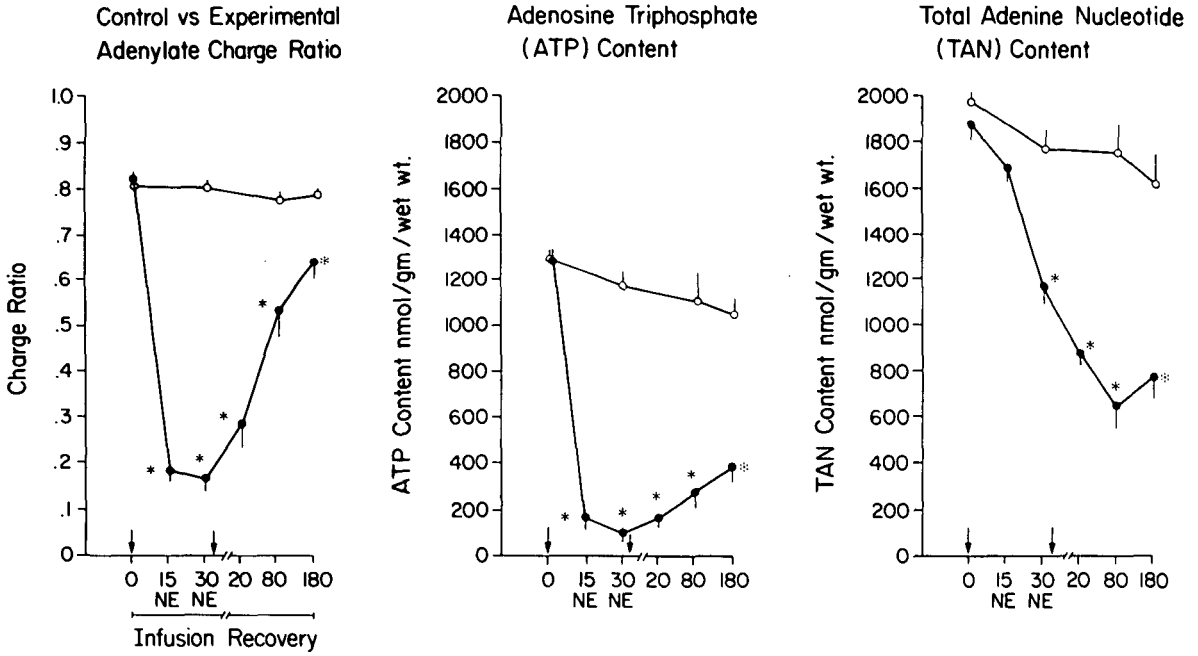

FIGURE 4. *Adenylate charge ratio (CR), adenosine triphosphate content (ATP), and total adenine nucleotide content (TAN) in the norepinephrine group (NE) of the experimental kidney (●) and the contralateral control kidney (○) at various times of infusion and recovery. Note the limited recovery of ATP content, the progressive and continuous decline, well into the recovery period, of the TAN content in the experimental kidney. Also note the relatively good recovery of the adenylate CR.* *p < .01 *control kidney vs. experimental kidney.*

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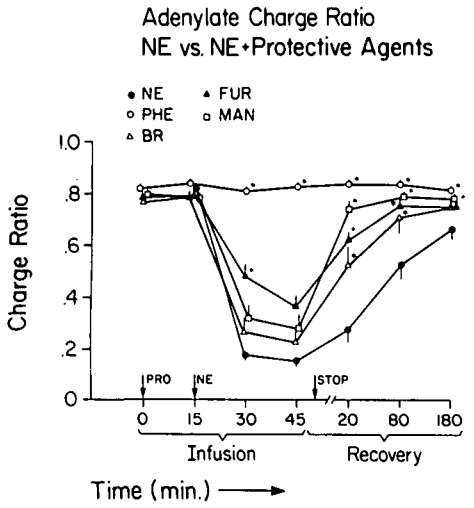

FIGURE 5. *Adenylate charge ratio (CR) from all of the experimental kidneys. There was no significant change in the adenylate CR with infusion of the protective agents alone. Once norepinephrine (NE) was started, there was a rapid fall in adenylate CR that is observed in all groups. At 20 and 80 minutes of recovery there was a significantly greater degree of recovery of the adenylate CR with the protective agents than with NE alone. At 180 minutes of recovery, only mannitol and phenoxybenzamine were significantly different from NE alone. * = p < 0.01 protective agents and NE vs. NE alone.*

infusion (Figure 5 and Table 2). Adenosine triphosphate content fell to 22% of control at 30 minutes of NE + MAN infusion in the EK and recovered to 67% at 180 minutes (Figure 6). Total adenine nucleotide content fell to 72% of control at 20 minutes post NE and changed little thereafter, staying at 72% at 180 minutes (Figure 7). The recovery of adenylate CR, ATP content, and TAN content was significantly greater than in the NE-alone group (Figures 5–7). Renal blood flow recovered to 81% of control. Inulin clearance at 180 minutes was 51% of control, a value significantly higher than that found in the NE-alone group (Table 1). There were no significant changes in any parameters in the CK.

Group V: Phenoxybenzamine (n = 5). There was slight (8%) increase in RBF with intrarenal phenoxybenzamine (PHE) administration. With the addition of NE, RBF increased by 11% associated with a rise in renal perfusion pressure, presumably due to NE spillover into the renal venous and then systemic circulation. Renal blood flow remained slightly higher than control throughout the NE infusion and during recovery despite mean blood pressure returning to control values. The GFR fell slightly during the NE infusion (1.62 to 1.4 ml/min/kg); however, it had returned to control values by 180 minutes post NE infusion (Table 1). The adenine nucleotide profile did not change throughout the experiment in either kidney (Table 2, Figures 5–7).

Group VI: Capsular Stripping (n = 9). This group of studies was conducted to determine whether the degree of capsular stripping would explain the differences between the Group I (norepinephrine) and Group II (bradykinin) studies. The results of these studies are shown in Table 3. There was consistently a higher level of GFR present in the kidneys exposed to bradykinin and norepinephrine than in those exposed only to NE. These differences were highly significant and quite similar to differences presented above for Group I and Group II studies. These observations would suggest that it was the bradykinin and not the capsular stripping that was responsible for these differences.

Group VII: Total Collections of Renal Blood Flow. This study was conducted to evaluate the possibility that the agent mannitol might offer protection by, at least in part, maintaining renal blood flow at a higher level during the norepinephrine infusion. The protocols were similar to the Group I (norepinephrine) and Group IV (mannitol) studies, with the exception that the animals were prepared for total collection of RBF at indicated time points. The results of these studies are shown in Table 4. The average RBF to the experimental kidney of both groups during control collections was 2.94 ml/min/g kidney. Following the initiation of the NE infusion total RBF diminished by at least 95% in both groups. At only one of the time points was there a significant difference between the kidney infused with NE and that infused with NE + mannitol. At 5, 10, 20, and 30 minutes during the NE infusion there was no significant difference between the values for total RBF of the kidneys infused with NE or NE + mannitol.

Pathology. Tubular degenerative changes and necrosis were observed consistently in kidneys subjected to norepinephrine alone. The degree of these changes

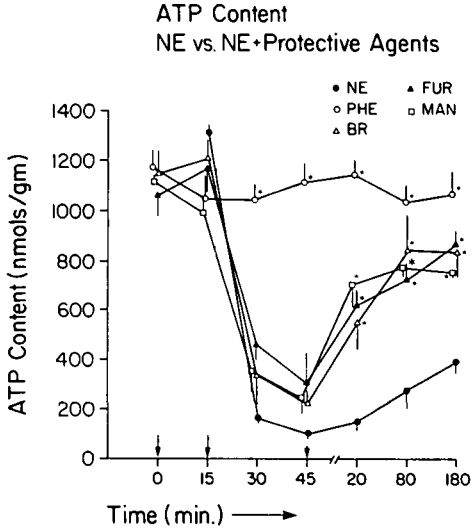

FIGURE 6. *The ATP content of all experimental kidneys. There was no significant change in ATP content during the administration of the protective agents. At the beginning of the norepinephrine (NE) infusion, there was a relatively steep fall in ATP content in all groups. At 20, 80, and 180 minutes of recovery, ATP content had recovered significantly more with the protective agents bradykinin, furosemide, and mannitol when compared to NE alone. * = p < 0.01 protective agents and NE vs. NE alone.*

TABLE 2. *Adenine Nucleotides (in Nanomoles per Gram of Wet Tissue)*

	Group I Norepinephrine, $n=8$			Group II Bradykinin, $n=7$		
	ATP	ADP	AMP	ATP	ADP	AMP
Control	1305±33	448±36	112±7	1144±104	544±55	171±23
15 P				1215±81	552±64	133±11
15 NE	158±26*	302±31	1214±63	337±121	489±100	1285±128
30 NE	97±19*	185±23	881±63	222±71	277±44	1172±86
20	146±35*	179±23	549±73	559±114†	373±49	462±85
80	270±71*	184±43	186±28	847±144†	378±58	182±13
180	389±38*	238±24	150±45	849±132	411±42	134±13

15 P = 15 minutes of protective agent; 15 NE = 15 minutes of norepinephrine with or without protective agent; 30 NE = 30 minutes of norepinephrine with or without protective agent; 20, 80, 180 = 20, 80, 180 minutes following discontinuation of infusion.
Results for only the experimental kidney are shown.
*Significantly different from control values.
†Significantly different from NE alone.

was significantly less in kidneys treated with the protective agents bradykinin, furosemide, mannitol, and phenoxybenzamine (Figure 8 and Table 5). The respective mean scores for NE, BR, furosemide, MAN, and PHE were: 2.0, 1.0, 0.5, 0.6, and 0.5. The corresponding score for the pooled "control" kidneys was 0.5. The differences between the protected groups and the NE group were each statistically significant by the Spearman rank test ($p < .05$) or less. Tubular casts were not a prominent feature of the norepinephrine-induced lesion in the period studied. However, they were more frequent in the NE group than in the other groups.

Discussion

These results confirm previous findings from this laboratory that pretreatment with bradykinin, mannitol, or furosemide attenuates the renal functional impairment noted after intrarenal NE infusion. In addition, the work with phenoxybenzamine implies that NE has a deleterious effect on the kidney as a result of interaction with α receptors, since blockade of α receptors with phenoxybenzamine totally protects against acute renal failure.

It should be recognized that the NE model of acute renal failure used in the present study may not lead to renal failure simply by reducing RBF. In a recent study using direct renal artery occlusion in the dog,[18] administration of mannitol, furosemide, or bradykinin did not attenuate the subsequent decrease in GFR. Since there is evidence that NE may have a direct toxic effect on renal tubular cells,[19] some consequence of α-receptor occupancy per se, possibly independent of hemodynamic consequences, may play an important role in the cellular damage induced in the NE model of acute renal failure.

In order to evaluate the possible role of metabolic factors in the protection afforded by certain agents against the NE-induced acute renal failure model, the present studies were performed to evaluate the adenine nucleotide profile before, during, and following NE administration. The adenine nucleotide profile is extremely labile, and ATP content can change within seconds. Thus the method for tissue sampling is critical. The method used in this study whereby renal cortical biopsies were frozen to −160° C within less than 1

TABLE 3. *Group VI: Studies of Capsular Stripping*

	GFR (ml/min/kg b.wt.)	
	Norepinephrine	Bradykinin
Control kidney	1.33±0.04	2.16±0.61
Experimental kidney	0.21±0.07	1.37±0.55*
% Protection in experimental kidney	16.2±5.3	57.8±6.9*

All values are those found at 180 minutes. Control kidney was untreated. Experimental kidney received norepinephrine or bradykinin and norepinephrine.
% Protection in experimental kidney = (GFR experimental/GFR control) × 100.
*$p < 0.01$ significantly different from norepinephrine alone.

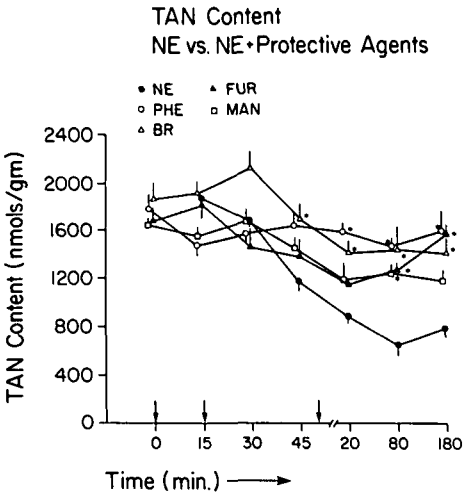

FIGURE 7. *Total adenine nucleotide (TAN) content for all experimental kidneys. In all groups the TAN content declined gradually during the infusion of norepinephrine (NE). Only the NE group, however, continued to decline during the recovery period. At 80 minutes of recovery the TAN content of the NE group was significantly less than that found with all of the protective agents. * = p < 0.01 protective agents and NE vs. NE alone.*

TABLE 2. *(continued)*

Group III Furosemide, $n=7$			Group IV Mannitol, $n=7$			Group V Phenoxybenzamine, $n=5$		
ATP	ADP	AMP	ATP	ADP	AMP	ATP	ADP	AMP
1060±90	524±53	95±15	1122±27	426±15	101±10	1242±74	406±52	101±11
1170±100	538±47	98±14	988±62	428±45	123±12	1074±102	391±34	94±12
448±63	441±53	534±115	362±78	371±74	906±70	1042±102†	397±23	104±14
299±113†	393±45	651±48	250±16	282±26	885±120	1111±79†	391±36	101±10
605±65	404±57	286±53	711±86†	330±54	141±25	1146±67†	321±16	98±8
727±63†	429±30	91±19	780±37†	335±34	91±12	1030±68†	32±25	83±8
861±48†	570±58	117±16	756±46†	329±45	95±12	1056±95†	407±53	122±24

second allowed for repeated measurements of adenylate CR and nucleotide content over time in this model. The finding of a CR in dog kidney cortex under control conditions of approximately 0.80 in this study is quite consistent with previous findings of others in which whole kidney cortex was frozen between two clamps previously cooled in liquid nitrogen.[20] The similar level of CR between the present and previous studies would suggest that the methodology used in the present study should be at least as good at stabilizing the ATP content as those used previously.

Adenosine tri-, di-, and monophosphates are in dynamic equilibrium, and there is considerable debate in the biochemical literature as to how best to evaluate

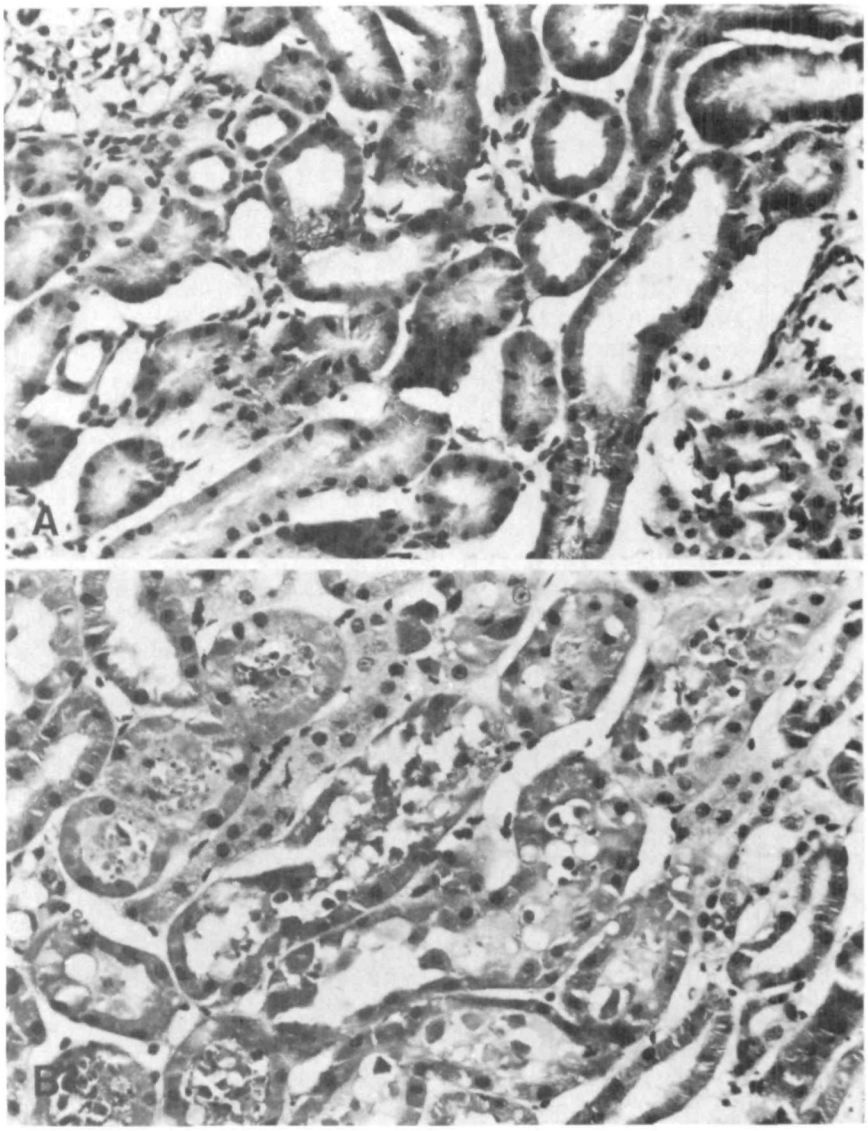

FIGURE 8. *Typical hematoxylin and eosin sections from a kidney treated with norepinephrine (NE) alone (bottom) and a kidney pretreated with mannitol followed by NE (top). Note the marked increase in necrosis in the bottom panel in the kidney treated with NE alone.*

TABLE 4. *Studies of Total Collection of Renal Blood Flow (ml/min/g Kidney)*

Time	Control	Protective Agent	5 NE	10 NE	20 NE	30 NE
Norepinephrine ($n=8$)	2.94±0.38		0.09±0.06	0.05±0.01	0.01±0.02	0.04±0.01
Mannitol ($n=6$)	2.94±0.63	2.63±0.63	0.13±0.03	0.11±0.03	0.07±0.02	0.07±0.08
Paired t test	NS		NS	NS	NS	NS

Data for only the experimental kidney are shown. 5 NE–40 NE indicate time of norepinephrine infusion. Other times indicate collection obtained after discontinuing NE.

cellular or tissue integrity in terms of energy availability.[6] The adenine nucleotide CR, the parameter used in this study, has been suggested as one of the better indexes of tissue energy availability.[17] As is obvious from the equation used to calculate this ratio, small decreases in ATP concentration will markedly lower the calculated tissue adenylate CR. On the other hand, the adenylate CR does not necessarily indicate integrity of the total organ. Since the CR only evaluates the ratio of the different adenine nucleotides, if a tissue has cells that contain no adenine nucleotides at all, these cells will not contribute to the adenylate CR. Thus, if there are a hundred cells, only 10 of which are biochemically intact, the adenylate CR of that tissue might be normal although 90% of the tissue might be dead. On the other hand, the evaluation of TAN content does indicate, to some extent, cell viability per unit weight of tissue. Thus, in the example given above, if only 10 of 100 cells were alive, the TAN content would still be reduced by 90%. Thus, in the interpretation of the adenine nucleotide profile in this study it seemed important to evaluate both adenylate CR and TAN content.

The adenine nucleotide profile in this study showed that ATP content fell rapidly on initiation of the NE infusion (Table 2, Figures 4–7). Biochemical studies have shown that following the removal of oxygen from tissue such as kidney cortex, ATP production is maintained by a rapid change in cellular metabolism from aerobic to anaerobic glycolysis. Anaerobic ATP production produces only 1/18th the amount of ATP from an equal amount of glucose as aerobic glycolysis.[21] This being the case, glycogen stores are rapidly reduced under conditions in which renal blood flow is severely lowered. Several previous studies[9–13] of renal cortical metabolism have shown that cellular ATP depletion may be prolonged for some time and that restoration of ATP by endogenous ATP regeneration is regularly associated with cellular recovery. However, it would also seem that there is frequently a point of no return in most mammalian tissue after which normothermic ischemia is associated with major tissue damage.[22, 23] This period of time has regularly been found to be between 40 and 70 minutes. A recent preliminary study in the isolated perfused kidney has found that at certain levels of TAN reduction prior to ischemia there is no obvious relationship between TAN levels and GFR following 30 minutes of ischemia.[24]

TABLE 5. *Histological Scores (0–4+) of Necrosis*

	Control Kidney		Experimental Kidney	
	Mean	Range	Mean	Range
Norepinephrine	0.64	(0–1+)	2.00*	(1–3+)
NE+bradykinin	0.86	(0–2+)	1.00	(±–2+)
NE+furosemide	0	(0)	0.50	(±)
NE+mannitol	0.50	(0–1+)	0.60	(±–1+)
NE+phenoxybenzamine	0.50	(±)	0.50	(±)

Results were analyzed by the Spearman rank correlation test. There was significantly more necrosis found in the kidney exposed to norepinephrine alone than in all other groups.
* = $p < 0.05$.

Kidney cells apparently vary considerably in their ability to resist ischemic damage.[25] The P_3 segment of the proximal tubule appears to be one of the most vulnerable. This may be related in part to the blood supply available to this segment or to the fact that glycogen content is lower than in certain other nephron segments.[26, 27] However, the segment of the nephron that is most sensitive to ischemia would appear to be critical for changes in total nephron function, since significant damage anywhere along the nephron would result in that nephron failing as a functioning unit in terms of urine formation. Studies in the rat kidney have shown that following 25 minutes of ischemia, repair of the P_1 and P_2 segments is rapid, indicating that much of the cells' biochemical synthetic pathways are still intact.[28]

The findings in the present study demonstrate the extreme lability of the adenine nucleotide profile. The administration of NE into the renal artery was associated with considerable changes in this profile. At the first sampling point, 15 minutes following the initiation of NE, adenylate CR and ATP content had already fallen significantly in all groups, except phenoxybenzamine-treated animals. In the animals treated with NE alone, the CR never recovered to control values during or after the time NE was given. The finding of a depressed adenylate CR even 3 hours after discontinuation of NE would suggest that in these kidneys, marked changes in cellular metabolism had occurred. The return of adenylate CR to or towards normal in the animals pretreated with bradykinin, mannitol, and furosemide suggests that in some way, these agents are able to maintain tissue cellular integrity and that, thus, the cells from these "protected" kidneys are able to regenerate a normal adenylate CR. It is of interest that these kidneys also have higher GFRs than kidneys treated with NE alone. The changes in TAN content (Figure 7) give a slightly different impression. There is a progressive and virtually continu-

TABLE 4. *(continued)*

40 NE	10	20	80
0.04±0.01	0.27±0.06	0.52±0.12	0.77±0.13
0.26±0.10	0.50±0.17	0.95±0.26	1.16±0.31
<0.05	NS	NS	NS

ous fall in TAN content with time in the kidneys exposed to norepinephrine alone. In the kidneys treated with protective agents, there is a modest decrease in TAN content, but at the end of 3 hours following the administration of NE, all of the protected groups have significantly higher levels of TAN content than does the group treated with NE alone. Thus, in the kidneys pretreated with agents that protect against NE-induced acute renal failure, TAN content 3 hours after NE is higher and adenylate CR has returned virtually to control levels. These findings, in general, agree with the lesser degree of pathological changes found on histological study in these protected groups when compared with NE alone. Obviously these findings also qualitatively agree with the inulin clearance data.

Progressive decline in TAN in the kidney treated with NE alone may be the result of AMP being broken down to adenosine and inosine at a greater rate and for a greater length of time. Regeneration of adenine nucleotides via the salvage pathway and *de novo* synthesis requires guanosine triphosphate. Therefore, at a time when energy requirements for reparative processes are high, additional energy demand for adenine nucleotide synthesis puts the kidney treated with norepinephrine alone at a distinct disadvantage when compared to the protected groups. The protected groups have maintained their TAN levels reasonably well and are thus able to phosphorylate available AMP and ADP rapidly, restoring their CR almost to control values.

The most direct interpretation of the large fall in TAN content in the NE-alone group when compared with the other groups implies that cells from this set of kidneys are more severely injured and thus less able to maintain intercellular adenine nucleotides. This is also consistent with the lower level of ATP content and a lower level of adenylate CR found in this group 3 hours after NE is discontinued. On the other hand, the fact that ATP measurements agree with the histological changes and the differences in GFRs does not imply that the loss of adenine nucleotides is the primary event in this model of acute renal failure. It is just as likely that these changes are secondary to either a greater degree of ischemia or some other undetermined factor. Likewise, the mechanism whereby the protective agents, bradykinin, mannitol, and furosemide allow for the kidney to maintain a greater TAN content and restore adenylate CR following discontinuation of NE are not clear. The fact that adenylate CR can return back to control levels implies that many of the cells in these protected kidneys are still biochemically intact. This is consistent with a lesser change histologically and a greater level of GFR.

One explanation for the greater degree of protection with bradykinin in this model is that the fall in RBF may not be as marked as it is with NE alone. Preliminary data from this laboratory are consistent with this suggestion in that when RBF was quantitated by total renal venous collection during the administration of NE and bradykinin there was a significantly higher, although still quite small, value observed for RBF.[29] This explanation would not apply to the mannitol protected group (Group VII), however, since RBF in this group fell to levels similar to that found with NE alone. Since the degree of protection with all three agents is qualitatively similar, a unified explanation such as the metabolic one offered in this paper seems more attractive. An effect of mannitol to protect mitochondrial function has previously been demonstrated in a similar model of acute renal failure.[30]

In summary: The present findings confirm previous studies in that pretreatment with bradykinin, mannitol, or furosemide can attenuate the severity of norepinephrine-induced acute renal failure. Phenoxybenzamine, an α-receptor blocking agent, completely protects, suggesting that norepinephrine is nephrotoxic by binding to α receptors. Monitoring adenine nucleotides throughout the generation of and for 3 hours following NE-induced acute renal failure shows rapid decreases in adenylate CR during NE administration which returns to control levels in the protected groups, but not in the group given NE alone. Total adenine nucleotide content is also lower in the group treated with NE alone. The maintenance of a higher level of TANs and the preserved ability to regenerate ATP in the protected groups, when compared to the group treated with NE alone, support the contention that these agents offer protection, at least in part, by preserving cellular metabolic integrity.

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