Protective Effect of Intrarenal Calcium Membrane Blockers Before or After Renal Ischemia

Functional, Morphological, and Mitochondrial Studies

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Abstract. The present study examined whether a pre- or postischemic infusion of verapamil (V) or a postischemic infusion of nifedipine (N), drugs which block calcium (Ca^{++}) influx across plasma membranes, provides protection against ischemic acute renal failure (ARF) in dogs. Renal hemodynamics and excretory function were examined 1 h (initiation phase) and 24 h (maintenance phase) after a 40-min intrarenal infusion of norepinephrine (NE). In each case, the uninfused contralateral kidney served as control. Four groups were studied: (a) dogs receiving NE alone; (b) dogs receiving an intrarenal infusion of V for 30 min before NE (V + NE); (c) dogs in which intrarenal V was infused for 2 h, beginning immediately after completion of NE infusion (NE + V); and (d) dogs in which intrarenal N was infused for 2 h, beginning immediately after completion of NE infusion (NE + N). Glomerular filtration rate (GFR) in the NE kidneys, as assessed by inulin clearance, at 1 and 24 h averaged 2.4 ± 1.1 and 5.0 ± 2.0 ml/min, respectively, as compared with control kidney GFRs of 28.0 ± 3.5 and 43.8 ± 5.0 ml/min, respectively (both at least $P < 0.01$). In the V + NE group, GFR at 1 and 24 h averaged 15.0 ± 5.5 and 31.0 ± 4.5 ml/min, respectively, both at least $P < 0.05$ as compared with values from NE kidneys. GFRs in the NE + V group averaged 15.0 ± 2.4 and 16.3 ± 3.6 ml/min at 1 and 24 h, both at least $P < 0.02$ as compared with values from NE kidneys. GFR in the NE + N group averaged 18.6 ± 6.0 ml/min

at 24 h ($P < 0.05$ as compared with GFRs in the NE kidneys). In addition, function of cortical mitochondria (Mito) was examined at the end of the 40-min NE infusion and after 1 and 24 h of reperfusion in the NE alone and NE + V groups. Mito respiration, assessed by acceptor control ratios, was reduced at each period in the NE alone kidneys. After 24 h, these Mito had accumulated Ca^{++} and exhibited reduced Ca^{++} uptake and increased Ca^{++} release rates. Mito from NE + V kidneys respired normally, did not accumulate Ca^{++} , and exhibited no alterations in Ca^{++} uptake or release. Light and electron microscopy also demonstrated morphological protection of V against tubular necrosis and cell injury. Mito from the NE + N kidneys also respired normally and did not accumulate significant amounts of Ca^{++} . The results of the present studies therefore demonstrate that chemically dissimilar calcium entry blockers exert substantial functional, cellular, and morphological protection against experimental ischemic ARF. These findings are compatible with the hypothesis that increased cytosolic Ca^{++} is critically important in the maintenance of renal vasoconstriction and the development of cellular necrosis with subsequent tubular obstruction in NE-induced ischemic ARF. V or N may provide protection against renal injury by retarding any increase in cytosolic Ca^{++} in renal vasculature and epithelium.

Introduction

Norepinephrine (NE)¹-induced acute renal failure (ARF) in the dog is characterized by low glomerular filtration rate (GFR)

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1. Abbreviations used in this paper: ACR, acceptor control ratio; ARF, acute renal failure; CEB, Ca^{++} entry blocker; FE_{Na} , fractional sodium excretion; GFR, glomerular filtration rate; Mito, mitochondria, mito-

a secondary effect of renal vasoconstriction with reduced glomerular capillary pressure and by tubular obstruction (1). Certain drugs that cause renal vasodilation and increase solute excretion such as prostaglandin E₂, mannitol, and furosemide have been shown to reduce the severity of renal dysfunction in this model of ARF (1–5). Other vasodilators, however, such as secretin and acetylcholine, which increase renal blood flow (RBF) with or without enhanced solute excretion, are not protective in this model of ischemic ARF (4–6).

Since intracellular calcium (Ca⁺⁺) in vascular smooth muscle and renal tubular epithelial cells could be involved in renal vasoconstriction (7) and epithelial cell necrosis (8), respectively, it seemed important to examine whether a blocker of cellular Ca⁺⁺ influx would protect against the functional and cellular consequences of renal ischemia. This was a particularly intriguing prospect, since studies from our laboratory had shown that intrarenal verapamil (V) causes profound renal vasodilation and increases solute excretion (9). The present study was therefore undertaken to examine the intrarenal effect of the Ca⁺⁺ entry blocker (CEB), V, on the functional, morphological, and mitochondrial (Mito) consequences of acute renal ischemia. Early (initiation) and late (maintenance) studies were performed as was intervention with V before and after the ischemic renal insult. Nifedipine (N) was also infused after norepinephrine (NE) to test whether a dissimilar CEB would exert effects similar to those observed with V treatment.

Methods

Mongrel dogs of both sexes were used in this study. Anesthesia was induced with intravenous sodium pentobarbital (25 mg/kg). The method of creating unilateral NE-induced ARF has been described in detail previously (1, 3, 4). Contralateral uninfused kidneys served as controls.

NE infused alone (NE) group

Initiation phase of ARF. For these experiments ($n = 9$), studies were performed before and for 1 h after NE-induced ARF. Both ureters were isolated through a midline abdominal incision and then cannulated. The left renal artery was isolated, instrumented with a flow probe, and curved 25-gauge needle was placed in the artery near its junction with the aorta. Systemic blood pressure was measured with a transducer (model 377; Harvard Apparatus Co., Inc., The Ealing Corp., South Natick, MA) attached to a femoral artery catheter. Infusions were administered through a femoral vein catheter. Inulin was infused intravenously to establish a plasma concentration of ~20 mg/dl. After an equilibration period of 1 h and two 15-min control clearance collections, NE (0.75 μ g/kg per min) was infused through the renal artery catheter for 40 min. Flowmeter readings indicated that RBF fell to zero within 10 s after the NE infusion was begun. Renal function was reassessed 1 h later. Inulin clearance was used as an estimate of GFR.

chondrial; N, nifedipine; NE, norepinephrine; NE + N, N infused after NE; NE + V, V infused after NE; RBF, renal blood flow; S3, state 3 respiration; S4, state 4 respiration; V, verapamil; V + NE, NE infused after V.

Maintenance phase of ARF. For these experiments ($n = 6$), studies were performed only 24 h after the NE infusion. To create ARF, sterile isolation of the left femoral artery was undertaken after anesthesia as described above; a radioopaque catheter was then threaded from the femoral artery into the aorta and guided under fluoroscopic control into the left renal artery. NE was infused for 40 min; then the catheter was withdrawn, the artery was ligated, and the incision was closed with 4-0 chromic gut. 24 h later, animals were reanesthetized and prepared for acute clearance studies as described above. Two 20-min urine collections were obtained to measure plasma clearances.

V infused before NE (V + NE) group

In these experiments, the pre-drug clearance collections were obtained as described above; then V (dissolved in water, 5 μ g/kg per min; Knoll Pharmaceuticals Co., Whippany, NJ) was infused at 0.5 ml/min into the left renal artery for 30 min ($n = 6$). During the final 10 min of this unilateral infusion, two 5-min urine collections were obtained. V infusion was then stopped and the 40-min infusion of NE was immediately initiated. RBF decreased to zero within 10 s. The remainder of these acute studies were similar to those described above for the initiation phase of ARF. In studies of the maintenance phase of ARF, V was also infused for 30 min before NE, and these animals were then studied 24 h later ($n = 6$).

V infused after NE (NE + V) group

In these experiments, intrarenal V infusion (also 5 μ g/kg per min at 0.5 ml/min) was begun immediately after the 40-min NE infusion ($n = 7$) was completed. Kidneys were removed for Mito studies 1 h after V infusion was started; in other experiments V was infused for 2 h after NE. In the latter, evaluations of renal functions were made 24 h after ischemia ($n = 6$).

N infused after NE (NE + N) group

N was dissolved in absolute ethanol (15 ml) and polyethylene glycol (15 ml, 300–400 mol wt) and brought up to a final volume of 120 ml with distilled water. In these experiments, intrarenal N infusion (2.0 μ g/kg per min at 1.0 ml/min) was begun immediately after completion of the 40-min NE infusion and continued for 2 h ($n = 4$). Functional studies were performed 24 h later and the kidneys were removed for Mito studies. N in the reservoir and perfusion line was protected from exposure to light throughout the preparatory and infusion period.

Mito respiration studies

In these experiments, at the end of the NE infusion (before reflow) or at either 1 h (initiation phase) or 24 h (maintenance phase), each renal artery was clamped and the control and infused kidneys were removed. Mito studies were not performed in the V + NE group since these data have been previously reported from our laboratory (10). Cortex was separated from medulla and immediately immersed in chilled media containing 210 mM mannitol, 70 mM sucrose, 0.1 mM EGTA,² 0.5% bovine serum albumin (BSA) and buffered to pH 7.4 with 2 mM

2. This concentration of EGTA (0.1 mM) may not totally prevent in vitro Ca⁺⁺ uptake by Mito during the separation phase performed at 4°C, but 1.0 mM EGTA may actually decrease Mito Ca⁺⁺ in vitro. Recent studies have shown results that are qualitatively similar to the present results when the Mito isolation in vitro was performed in the presence of ruthenium red which totally blocked in vitro Mito Ca⁺⁺ uptake (11, 12). This approach, however, does not allow for assessment of the uptake and release of Ca⁺⁺ by Mito.

K-Hepes. The tissue was scissor minced and homogenized (20% wt/vol) on ice for ~5–10 s by use of a homogenizer (Polytron; Brinkmann Instruments Inc., Westbury, NY). The homogenate was centrifuged at 4°C at 750 g twice to remove cellular debris. The resultant supernate was then centrifuged at 8,000 g to obtain the Mito fraction. The Mito pellet was washed twice with media from which the BSA and EGTA were omitted, and resuspended at a concentration of ~100 mg protein/ml. Mito protein content was determined by the method of Murphy and Kies (13) using a spectrophotometer (model 25; Beckman Instruments Inc., Fullerton, CA).

Mito O₂ consumption was measured polarographically in a closed reaction vessel fitted with a Clark O₂ electrode (Yellow Springs Instrument Co., Yellow Springs, OH) at 25°C in a volume of 1.7 ml. The incubation medium was composed of 120 mM KCl, 2 mM potassium-phosphate, 4 μ M rotenone, and 1 mM EGTA and buffered to pH 7.1 with 5 mM Tris HCl (14). The osmolality of this medium after buffering was 255 mOsm/kg H₂O.

Mito (2 mg protein) were energized with the addition of 5 mM K-succinate. After a short equilibration period (~30 s), state 3 respiration was induced by the addition of 300 nmol of ADP (Sigma Chemical Co., St. Louis, MO). After all of the added ADP had been phosphorylated to ATP, state 4 respiration was measured. The ratio of the O₂ consumption in the presence of ADP (state 3) to that in its absence (state 4), termed the acceptor control ratio (ACR), was used as an indicator of the integrity of the Mito preparation. In addition, a matrix enzyme (fumarase) and a membrane enzyme (succinic Iodonitrotetrazolium reductase) were assayed by conventional techniques (15, 16).

Finally, maximal rates of O₂ consumption were measured with the addition of 0.2 nmol of FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazine (Sigma Chemical Co.), an uncoupler of oxidative phosphorylation.

Mito Ca⁺⁺ handling studies

Measurements of free Ca⁺⁺ movements were carried out in a water-jacketed reaction vessel by use of a Ca⁺⁺ sensitive electrode (Radiometer F2110) and a reference electrode (Radiometer K401) at 25°C. The signal from the Ca⁺⁺ electrode was amplified through a Radiometer PHM 82 pH meter (Radiometer America Inc., Westlake, OH). The signals from the O₂ electrode (monitored with a model 53 O₂ monitor [Yellow Springs Instrument Co.]) and the pH meter were both fed into a dual-channel strip-chart recorder (Linear Instruments Corp., Irvine, CA). The Ca⁺⁺ electrode was calibrated with CaCl₂ standards added to each reaction medium as required, and the logarithmic response was linearized graphically. The incubation medium for Ca⁺⁺ uptake and release experiments was the same as for the respiration studies with the omission of EGTA. Mito (5 mg protein) were suspended in a 3-ml incubation volume. The rate of Ca⁺⁺ uptake was measured in the presence of 66.7 μ M CaCl₂ and 5 mM K-succinate. Ca⁺⁺ release was measured in the absence of added CaCl₂ after the addition of 5 nmol ruthenium red, an inhibitor of Mito Ca⁺⁺ exchange. Tissue and Mito Ca⁺⁺ content were measured by atomic absorption spectroscopy (model 370; Perkin Elmer Corp., Coleman Instruments Div., Oak Brook, IL).

Biochemical analyses

Inulin in plasma and urine was measured by autoanalyzer techniques. Plasma and urine sodium were measured by flame photometry (model 343; Instrumentation Laboratory Inc., Lexington, MA). Plasma and urine osmolality were measured by freezing point depression (model 312; Advanced Instruments, Inc., Needham Heights, MA).

Calculations

Inulin clearance to estimate GFR, as well as solute excretion and fractional excretion of sodium, were calculated by conventional formulas.

Morphological studies

After an infusion of NE for 40 min ($n = 4$) or an infusion of NE + V for 2 h ($n = 5$), the kidneys were fixed 3 h after NE by intraaortic perfusion (17) of one-half strength Karnovsky's fixative buffered with potassium phosphate (pH 7.4), at a perfusion pressure maintained equal to or slightly greater than mean arterial pressure. A total of 300–400 ml of fixative was used in each animal. This methodology has been previously used in this model (3).

Pieces of kidney from each animal were embedded in paraffin according to routine histologic methods. Sections 5 μ m thick were cut and stained with hematoxylin and eosin for light microscopic analysis.

Tissue for transmission electron microscopy was minced and washed in 0.2 M phosphate buffer, pH 7.4, for 1 h. The tissue was then postfixed in 2% osmium tetroxide buffered in 0.1 M *s*-collidine (pH 7.2–7.4) for 1 h at room temperature, stained *en bloc* with uranyl acetate in veronal acetate buffer at pH 5.0, dehydrated in a graded series of alcohols, treated with propylene oxide, and embedded in epoxy resin. Ultrathin sections were cut on a Sorvall MT-2 ultramicrotome (E. I. Du Pont de Nemours & Co., Inc., Sorvall Instruments Div., Newtown, CT), stained sequentially in 7.5% uranyl magnesium acetate and 0.15% lead citrate, and examined with a Siemens 102 transmission electron microscope (Siemens Mfg. Co. Inc., Freeberg, IL). Semithin epoxy sections (0.5–1.0 μ m thick) also were cut, stained with toluidine blue, and viewed under a light microscope.

Quantitation of structural alterations

Paraffin sections were used for the quantitative analysis of injury. In each kidney, five areas each from the outer cortex, the midcortex, and the inner cortex were analyzed. The data from each region were then combined to give a single value for the outer cortical, midcortical, and inner cortical areas. Tubular injury was evaluated in the proximal tubule since it represented the major site of injury along the nephron. By means of a Leitz-Wetzlar overhead projector microscope (E. Leitz, Inc., Rockleigh, NJ), each area was first centered at low power magnification, and then the high power objective was positioned for viewing the field at random, without moving of the specimen stage to avoid bias of the chosen area. The image was projected upon a screen on to which 144 points had been inscribed. The cell or structure lying under or nearest each point was classified by one observer without the observer's having prior knowledge of the treatment given to the specimen being examined. Each of the counted proximal tubular cells was assigned to one of the following categories: (a) normal when indistinguishable from controls; (b) injured when the cell revealed no evidence of necrosis but the cell shape was obviously altered to a low cuboidal or squamous type or revealed extensive apical vesiculation or vacuolization in addition to the loss of brush border; or (c) necrosis when the cell showed irreversible damage such as loss of membrane integrity or loss of nuclear staining.

Statistical analyses

All morphological results are expressed as mean $\pm$ SEM. The *t* test (two tailed) was used to evaluate the significance of differences between the different groups studied.

Where appropriate, linear regression analysis, analysis of variance and the *t* test were used to determine significant differences among

Figure 1. (A) V administered for 30 min before NE (cross-hatched bars, top) significantly attenuated the fall in inulin clearance (C_{in}) induced by NE alone (open bars) both 1 and 24 h after NE. (B) V administered for 2 h after NE (stippled bars, bottom) also significantly attenuated the fall in C_{in} both 1 and 24 h after NE.

treatments and/or between animals. Significance was accepted at $P < 0.05$. All values reported represent mean $\pm$ SEM.

Results

Effect of unilateral V + NE or NE + V on GFR (Fig. 1). Previously we reported that GFR is reduced 1, 3, and 24 h after NE in this model (1). The present results confirm those of the previous study; GFR at 1 and 24 h averaged 2.4 ± 1.1

and 5.0 ± 2.0 ml/min, respectively, in the NE alone group. Furthermore, it is clear that administration of unilateral, intrarenal V for 30 min before NE, which did not increase GFR of either kidney during this period, protects GFR at both 1 and 24 h (15.0 ± 5.5 vs. 2.4 ± 1.1 ml/min, $P < 0.05$, and 31.0 ± 4.5 vs. 5.0 ± 2.0 ml/min, $P < 0.001$, respectively). It can also be seen that V infused after NE also exerts a substantial protective effect on GFR at 1 and 24 h (15.0 ± 2.4 vs. 2.4 ± 1.1 ml/min, $P < 0.001$, and 16.3 ± 3.6 vs. 5.0 ± 2.0 ml/min, $P < 0.02$, respectively). At 24 h, V administered for 30 min before NE was somewhat more effective in protecting GFR than when V was administered for 2 h after NE (31.0 ± 4.5 vs. 16.3 ± 3.6 ml/min, $P < 0.05$).

Effect of unilateral V + NE or NE + V on RBF (Table I). In the NE alone group, RBF was 220 ± 25 ml/min before NE and was reduced to zero during NE; RBF then recovered slowly after NE to 56 ml/min at 30 min, and 72 ml/min at 45 min, and it averaged 82 ± 38 ml/min 1 h after NE ($P < 0.05$ as compared with control). V + NE kidneys had an RBF at 1 h, after NE which was somewhat higher (162 ± 32 ml/min) and not different from control (P , NS). RBF was significantly higher at 1 h in the NE + V group than in the NE alone group (207 ± 18 vs. 82 ± 38 ml/min, $P < 0.025$).

Effect of unilateral V + NE or NE + V on solute excretion, urine flow, and fraction sodium excretion (Table I). V infused before NE increased solute excretion from 251 ± 55 to 546 ± 63 mOsm/min ($P < 0.005$). 1 h after NE in V + NE animals, solute excretion averaged 111 ± 30 mOsm/min, a value not significantly different from that seen at 1 h in the NE alone group (101 ± 5 mOsm/min). As described above, however, GFR was very low in the NE alone group and substantially improved in the V + NE group. When V was infused after NE there was an immediate solute diuresis and solute excretion averaged 398 ± 88 mOsm/min at 1 h, a value significantly higher than that observed in both the NE alone group (101 ± 5 mOsm/min) and in the V + NE group (111 ± 30 mOsm/min; both $P < 0.005$).

V infused before NE increased urine flow from 0.19 ± 0.06 to 1.23 ± 0.24 ml/min ($P < 0.001$) and fractional sodium excretion

Table I. Effect of V on RBF, Solute Excretion, Urine Flow, and FE_{Na} Before and After NE-induced Ischemia*

	Cont	V alone	1 h after NE			24 h after NE		
			NE alone	V before NE	V after NE	NE alone	V before NE	V after NE
RBF (ml/min)	220 $\pm$ 25	319 $\pm$ 38‡	82 $\pm$ 38‡	162 $\pm$ 32	207 $\pm$ 18§	ND	ND	ND
Solute excretion (mOsm/min)	251 $\pm$ 55	546 $\pm$ 63‡	101 $\pm$ 5‡	111 $\pm$ 30‡	398 $\pm$ 88§	96 $\pm$ 25‡	ND	198 $\pm$ 29§
Urine flow (ml/min)	0.19 $\pm$ 0.06	1.23 $\pm$ 0.24‡	0.05 $\pm$ 0.02‡	0.17 $\pm$ 0.05§	0.96 $\pm$ 0.42‡§	0.18 $\pm$ 0.05	0.55 $\pm$ 0.19	0.34 $\pm$ 0.08
Fractional sodium excretion (%)	1.20 $\pm$ 0.46	4.30 $\pm$ 0.86‡	2.86 $\pm$ 1.10	0.93 $\pm$ 0.25	8.0 $\pm$ 2.0‡	4.05 $\pm$ 1.40	ND	2.9 $\pm$ 1.4

Cont, control; ND, not determined. * Mean $\pm$ SE. ‡ At least $P < 0.05$ as compared with control. § At least $P < 0.05$ as compared with NE alone at the same time or at least $P < 0.05$ as compared with V before NE at the same time.

Figure 2. S3 respiration during and after NE infusion (hatched bars) decreased progressively. V infused after NE (solid bars) prevented the decrease in S3 at both 1 and 24 h. Contralateral (Cont, control) kidneys are represented by open bars.

tion (FE_{Na}) from 1.2 ± 0.46 to $4.30 \pm 0.86\%$ ($P < 0.01$). In dogs infused with NE alone, urine flow was low at 1 h (0.05 ± 0.02 ml/min) and FE_{Na} averaged $2.86 \pm 1.10\%$. The V + NE group had a slightly higher urine flow at 1 h as compared with the NE alone group (0.17 ± 0.05 vs. 0.05 ± 0.02 ml/min, $P < 0.05$); FE_{Na} was not significantly different between the V + NE and NE alone groups (0.93 ± 0.25 vs. $2.86 \pm 1.10\%$, P , NS).

V infused after NE resulted in a sustained increase in urine flow (0.96 ± 0.42 ml/min) which was significantly higher than urine flow (0.17 ± 0.05 ml/min) in the V + NE group ($P < 0.05$) and in the NE alone group (0.05 ± 0.02 ml/min, $P < 0.025$). FE_{Na} was also acutely increased at 1 h in the NE + V group ($8.0 \pm 2.0\%$).

Mito respiration. There was a significant and steady decrease in state 3 respiration (S3) as reflow ensued in the NE alone kidneys (Fig. 2). As compared with control values for S3 of 116 ± 7 ng atoms O/min per mg, S3 at the end of the NE infusion, but before reflow, was slightly reduced, to 96 ± 4 ng atoms O/min per mg, $P < 0.01$; after 1 and 24 h of reflow S3 had fallen to 67 ± 7 and 17 ± 5 ng atoms O min/mg, respectively ($P < 0.02$ and $P < 0.001$ as compared with control). V infused after NE prevented the decrease in S3 as compared with control at 24 h (86 ± 9 vs. 84 ± 9 ng atoms O/min per mg; P , NS) as well as at 1 h (105 ± 6 vs. 102 ± 9 ng atoms O/min per mg; P , NS). Similar patterns of protection were observed in measurements of state 4 respiration (S4), uncoupled respiration, and ACR (ACR = S3/S4) (Table II).

Mito and tissue Ca^{++} . Mito Ca^{++} was not increased during the 40-min NE infusion and before reflow (control 23.3 ± 0.9 nmol/mg Mito protein vs. NE 24.4 ± 1.4 nmol/mg Mito protein) (Fig. 3). There was also no difference after 1 h of reflow. After 24 h of reflow, however, Mito Ca^{++} increased (control 26.4 ± 4.0 nmol/mg Mito protein vs. NE 97.7 ± 12.0 nmol/mg Mito protein, $P < 0.01$) in the NE alone group. In contrast, at 24 h, Mito Ca^{++} was no different from control in the NE + V group (52.5 ± 17.8 vs. 51.1 ± 12.1 nmol/mg Mito protein).

It is important to recognize that the comparisons made were between Mito Ca^{++} content of contralateral (control) and ischemic kidneys. This seems to be the most appropriate comparison since renal cortical tissue Ca^{++} content in mongrel dogs may vary substantially even in control kidneys (Table III). The variability probably reflects the nonhomogeneous nature of mongrel dogs and differs appreciably from our observations of renal cortical tissue Ca^{++} levels in normal laboratory rats (unpublished observations). V and N also abolished the renal cortical tissue Ca^{++} accumulation at 24 h after NE (Table III).

Table II. Effect of V on S4, Uncoupled Respiration, and ACR During and After NE Infusion*

	Cont	During NE	1 h after NE				24 h after NE			
			Cont	NE alone	Cont	NE + V	Cont	NE alone	Cont	NE + V
S4 (ng atoms O/min per mg mito protein)	23.0 ± 1.0	23.0 ± 0.9	18.0 ± 1.0	17.0 ± 1.3	19.0 ± 1.7	20.0 ± 1.4	16.0 ± 1.2	7.0 ± 1.1	17.0 ± 0.8	17.0 ± 0.4
P value		NS		NS		NS		<0.001		NS
Uncoupled (ng atoms O/min per mg mito protein)	182.0 ± 8.0	184.0 ± 10.0	137.0 ± 17.0	118.0 ± 20.0	169.0 ± 18.0	173.0 ± 18.0	121.0 ± 16.0	27.0 ± 10.0	153.0 ± 20.0	151.0 ± 15.0
P value		NS		<0.05		NS		<0.001		NS
ACR (S3/S4)	5.10 ± 0.24	4.12 ± 0.17	4.87 ± 0.28	3.86 ± 0.21	5.24 ± 0.35	5.15 ± 0.32	4.75 ± 0.39	2.09 ± 0.36	4.63 ± 0.46	5.36 ± 0.44
P value		<0.005		<0.005		NS		<0.001		<0.05

Uncoupled, during FCCP. Cont, contralateral control kidney. * Mean ± SE. Variations among dogs accounts for some differences in control kidney values at different times.

Figure 3. Mito Ca^{++} content (hatched bars) was substantially increased at 24 h. V infused after NE (solid bars) prevented the increase in Mito Ca^{++} content at 24 h.

Mito Ca^{++} uptake rate (Fig. 4) during NE before reflow was no different from control (357 ± 26 vs. 371 ± 17 nmol/min per mg; P , NS). No decrease in Ca^{++} uptake was observed after 1 h of reflow. However, a significant decrease ($P < 0.005$) in Ca^{++} uptake was observed at 24 h (34 ± 17 vs. 167 ± 33 nmol/min per mg) in the NE alone group. V infused after NE prevented this decrease in Ca^{++} uptake rate at 24 h (335 ± 21 vs. 349 ± 13 nmol/min per mg; NS).

Mito Ca^{++} release rate (Fig. 5) was not affected by NE infusion as compared with control until 24 h of reflow, when Ca^{++} release had increased dramatically to 23.0 ± 7.2 nmol/min per mg ($P < 0.05$). The Ca^{++} release rate in the NE + V group did not increase as compared with control. The increase in Mito Ca^{++} release 24 h after the ischemic insult may be due to endogenous Mito Ca^{++} content, diminished electromotive force across the inner Mito membrane, or the presence of increased permeability through ruthenium red insensitive channels.

Effects of NE + N at 24 h. In Table IV are listed the effects of NE + N on renal and Mito function at 24 h as compared

with the contralateral, control kidneys. N infused intrarenally for 2 h after NE also protects renal and Mito function to a degree similar to that seen with V.

Mito enzymes. The concentration of both the matrix enzyme (fumarase) and the inner membrane enzyme (succinic INT reductase) were similar in Mito pellets from NE-treated kidneys, control kidneys, and V-treated kidneys 1 h after NE. Control, NE alone, and V-treated Mito fumarase levels averaged 2.72 ± 0.18 , 3.04 ± 0.28 , and 2.23 ± 0.03 $\Delta\text{OD}/\text{min}$ per mg Mito protein (all P , NS), respectively; succinate-Iodonitrotetrazolium reductase levels averaged 0.66 ± 0.04 , 0.67 ± 0.02 , and 0.58 ± 0.04 $\Delta\text{OD}/\text{min}$ per mg Mito protein (P , NS), respectively. These results suggest that comparable amounts of Mito were harvested from control, NE-treated, and V-treated kidneys. The differences in Mito respiration indicate that both normal and damaged Mito were harvested. The differences in Mito dysfunction would be even greater if normal Mito were preferentially harvested; we, however, have no evidence for this in the present study or in previous studies in the rat (18).

Morphological analysis of protection in NE + V-treated dogs. In Table V are listed the comparative effects of NE alone or NE + V on the percentage of normal, injured, and necrotic tubules in dog kidneys 3 h after NE. Similar degrees of injured and necrotic cells were seen in outer, mid, and inner cortex (~ 50 and 5% , respectively). V infused for 2 h after ischemia dramatically reduced these levels approximately 10-fold (~ 8 and 0.5% , respectively). Both light and transmission electron microscopy also demonstrate substantial protection in V-treated dogs (Fig. 6).

Discussion

The present results demonstrate that the CEBs, V and N, provide functional protection against NE-induced ischemic ARF in the dog. The protection was demonstrable when V was administered for 30 min before or for 2 h after the NE-induced ischemia. Protection was also observed when N was infused for 2 h after NE. Although RBF, as assessed by flow meter, the paleness of the kidney, and the collapsed peritubular

Table III. Comparison of Renal Cortical Tissue Ca^{++} Content (mmol/kg dry wt) in Dogs*

	NE alone			NE + V		NE + N
	During (n = 9)	1 h (n = 9)	24 h (n = 6)	1 h (n = 7)	24 h (n = 6)	24 h (n = 4)
Control kidney	8.2 ± 0.67	21.2 ± 4.0	11.9 ± 1.3	23.6 ± 9.7	23.6 ± 9.7	16.1 ± 6.4
Ischemic kidney	8.1 ± 1.1	25.4 ± 5.8	45.3 ± 11.0	25.6 ± 8.4	24.9 ± 11.5	30.1 ± 10.8
P value	NS	NS	<0.05	NS	NS	NS

* Mean $\pm$ SE.

Figure 4. Mito Ca^{++} uptake (hatched bars) was decreased 24 h after NE. V infused after NE (solid bars) prevented the decrease in Mito Ca^{++} uptake 24 h after NE.

capillaries which prevented micropuncture measurements of vascular pressures, appeared to be absent during the NE infusion in the animals pretreated for 30 min with V, an undetectably small preservation of RBF could not be totally excluded in those experiments. However, since significant protection was also demonstrable when V or N was administered after NE ischemia, the preservation of RBF during the ischemic insult can be excluded as a necessary factor accounting for the observed protection. This observation is important since, although at least three groups (19–21) have now confirmed our preliminary observation (22) that V administration before an ischemic insult affords protection against ischemic injury as assessed by preservation of GFR, two of these groups (19, 20) have failed to demonstrate protection when V is administered after the insult. In one study in which ischemia was produced by renal artery clamping for 70 min in the rat,

Figure 5. Mito Ca^{++} release (hatched bars) was increased 24 h after NE. V infused after NE (solid bars) prevented the increase in Mito Ca^{++} release 24 h after NE.

Table IV. Effect of N Infused for 2 h after NE on Renal and Mito Function at 24 h ($n = 4$)^{*}

	Contralateral (24 h)	NE + N (24 h)	P value
GFR (ml/min)	39.6±3.4	18.6±6.0‡	<0.05
U (ml/min)	0.17±0.05	0.13±0.02	NS
FE _{Na} (%)	0.07±0.06	0.85±0.72	NS
Solute excretion (mOsm/min)	186±32	96±10	NS
S4 (ng atoms O/min per mg)	23.9±1.3	22.7±2.9	NS
S3 (ng atoms O/min per mg)	113±7	90±19	NS
Uncoupled (ng atoms O/min per mg)	219±16	202±59	NS
ACR	4.74±0.33	3.84±0.46	NS
Mito Ca^{++} (nmol/mg)	34.6±9.5	80.3±21.0	NS

U, urine flow rate; uncoupled, during FCCP.

^{*} Mean±SEM.

‡ This value was significantly increased as compared with the inulin clearance 24 h after NE in the absence of a CEB (5.0±2.0 vs. 18.6±6.0 ml/min, $P < 0.02$). Thus, N afforded significant functional and Mito protection. V at this dose is, however, somewhat more protective to Mito function than is N.

the V was infused systemically for only 15 min after the insult, and no effect on GFR was observed after 24 h (19). In the other study, also in the rat, the 40-min intrarenal NE infusion was followed by a 2-h intrarenal infusion of V, and no protection was observed (20). It is difficult to reconcile these negative results with those of the present study. It is clear, however, that this latter model in the rat differs from previous dog or rat studies with respect to the recovery of RBF after NE. Specifically, the RBF recovered to almost normal levels within 20 min (20), whereas we (1, 3) and others (2, 5, 6) have reported that RBF recovery is usually quite slow after NE. Whereas most other investigators have used pentobarbital as the anesthetic while studying renal ischemia induced by NE (1–6, 23), the latter group (20) used inactin. Inactin, but not pentobarbital, is known to disrupt renal autoregulation in the rat (24).

Thus, differences among species, the chemical versus mechanical nature of the ischemic insult, the route of administration, and the amount and duration of the infusion postischemia may be very important factors in modulating the protective

Table V. Morphometric Analysis of Normal, Injured, and Necrotic Tubules in NE Alone and NE + V-treated Animals 3 h After NE*

	Outer cortex (%)			Mid cortex (%)			Inner cortex (%)		
	Normal	Injured	Necrotic	Normal	Injured	Necrotic	Normal	Injured	Necrotic
NE (n = 4)	41.5±7.7	53.1±8.4	5.3±2.3	34.3±5.3	59.7±6.8	6.0±2.7	42.8±5.7	55.2±5.6	2.1±0.9
NE + V (n = 5)	93.0±3.6	4.8±1.7	0.2±0.2	90.1±3.8	8.9±3.5	0.6±0.3	91.3±3.0	8.5±3.0	0.1±0.1
P value	<0.001	<0.001	<0.05	<0.001	<0.001	NS	<0.001	<0.001	NS

Necrosis is seldom seen at 1 h in this model and therefore studies were done 3 h postischemia. * Mean±SE.

effect of CEBs. Specifically, in the present study V and N were administered intrarenally to avoid any extrarenal peripheral vasodilation and reflex renal vasoconstriction that could occur and diminish renal perfusion during systemic administration of V. Second, the duration of the intrarenal V infusion postischemia also appears to be quite important. In this regard, preliminary experiments in our laboratory demonstrated that a 1-h infusion of V postischemia, although able to improve renal function transiently at 1 h, was insufficient to provide functional protection 24 h postischemia (unpublished results). In the present study a 2-h infusion of either V or N was clearly protective when renal function was evaluated 24 h after ischemia. Moreover, even the much longer (2 h) duration of the intrarenal CEB after the ischemia failed to provide the level of preservation of GFR observed with V pretreatment for 30 min. Thus, the protective effect of the CEB relates also, at least in part, to the relative proximity of the ischemic result. The protective effect of V against the ischemic insult is demonstrable not only in NE-induced ARF in the dog but also in the mechanical occlusion model as well. Wait et al. (25) have demonstrated significant protection of renal function in dogs given intrarenal V after a 1-h renal ischemic insult induced by mechanical occlusion of the renal artery.

Since micropuncture studies have demonstrated that NE-induced ARF involves an early (initiation) vasoconstrictor and a later (maintenance) tubular obstruction phase, without evidence of tubular back-leak (1, 6), the protective efficacy of the CEB must be considered on this background. There seems little doubt now that hormone-mediated vasoconstriction involves enhanced Ca^{++} movement into smooth muscle cells (26). Recent in vivo studies in the conscious rat have demonstrated that two chemically dissimilar CEBs, V and N, abolish the pressor and renal vasoconstrictor responses to NE, angiotensin, and vasopressin (7). It thus seems quite likely that a component of the protective effect of the CEBs, V and N, involves attenuation of the postischemia renal vasoconstriction. In this regard, during the initiation phase (1 h after NE) of the ARF both the pre- and postinsult V studies were associated with higher RBF as compared with the animals infused with NE alone. There is, however, considerable evidence that makes it unlikely that the vascular effect of V constitutes the only

component of the agent's protective effect. For example, V or isotonic saline infused intrarenally after renal ischemia increased RBF to nearly normal levels within 30 min; only V, however, exerts functional protection (25). We have also reported that RBF after NE recovered at a similar rate in mannitol-treated, polyethylene glycol-treated and hypertonic saline-treated dogs (23). Nevertheless, protection against ischemia-induced functional impairment was observed in the mannitol-treated and polyethylene glycol-treated dogs but not in the hypertonic saline-treated dogs (23). Finally, Williams et al. (27) reported that after mechanical occlusion of the renal artery for 60 min, RBF in the dog recovers to 164% of preischemic levels within 5 min after release of the clamp and remains at this level for at least 30 min. No protection of GFR was, however, observed 18 h later. Thus, recovery of renal function after ischemic injury does not depend upon whether RBF, and corresponding oxygen delivery, improves quickly (6, 23, 25, 27) or slowly (1–6, 23). Nevertheless, it is possible that an as yet undiscovered effect of V on intrarenal RBF redistribution or specific regional vascular sites might contribute to improved recovery from ischemic injury. Other agents such as furosemide (4) and mannitol (1, 3), which produce somewhat less or, at most, no greater renal vasodilation than does acetylcholine (4) or secretin (5), have been found to be very potent protective agents against this form of ischemic ARF. An assessment of several protective agents led to the proposal that the rate of solute excretion, as an index of intratubular pressure and flow rate, is also an important variable in the protection against ischemic ARF. Results of micropuncture studies (1) supported this proposal since early (1 h) after the ischemic insult, dogs pretreated with mannitol exhibited significantly higher intratubular pressures than did untreated animals (31 vs. 16 mmHg; $P < 0.001$). Moreover, tubular micropuncture studies (1) demonstrated the presence of tubular obstruction in the untreated but not mannitol treated animals. These results led to the conclusion that both renal vasodilation and prevention of secondary tubular obstruction are necessary components in the prevention of ischemic ARF. This conclusion provided an explanation for the efficacy of mannitol and furosemide in protecting against ischemic ARF as well as for the failure of secretin or acetylcholine to offer protection, since either no, or

a very modest, solute diuresis was observed with the latter two agents. Taken together, these results suggest that factors in addition to vascular events are involved in the protective effect of V. Moreover, since the induced solute diuresis by V and N is substantially less than observed in the mannitol (1, 2) and furosemide (4) studies, factors other than the rate of solute excretion must be invoked. The results in the present study suggest that the additional protective factor might be some event that occurs at the cellular level.

Substantial evidence for an effect of V on epithelium has been provided by several other studies. In dogs in which an aortic clamp was used to lower renal perfusion pressure within the autoregulation range, the intrarenal infusion of V did not increase RBF but a solute diuresis was still observed, and recollection micropuncture studies demonstrated a depressed proximal reabsorption (9). These *in vivo* studies of course do not exclude small changes in GFR or intrarenal blood flow distribution as being what accounts for the solute diuresis. V had, however, also been shown *in vitro* to exert an effect on toad bladder epithelium (28) and on normal (29, 30) and anoxic (31) rabbit isolated proximal tubules.

In the present study the CEBs demonstrated substantial protection of Mito function as assessed by cellular respiration and calcium kinetics *in vitro*. It would appear that some cellular event is protected from ischemic injury by V and N administration. This conclusion is supported by the protective effect of these agents on the morphological appearance of the proximal tubules. The Mito studies provided important information about the subcellular events involved in the pathogenesis and prevention of this variety of ischemic ARF. In the untreated animals an increase in Mito Ca^{++} content, a decrease in active Ca^{++} uptake rate by Mito, and an increase in Mito Ca^{++} release rate were observed 24 h after the ischemic insult. These findings are compatible with death of tubular epithelial cells as demonstrated functionally by diminished Mito respiration in the present study and the morphological presence of tubular necrosis both 3 h (present study) and 24 h after NE (3). It seems reasonable to suggest that this cell death provided the debris necessary for the development of the observed tubular obstruction. If, as suggested by Farber (8) and Trump et al. (32), Mito Ca^{++} overload is not only the result of cell death but is also involved in the progressive deterioration of cell viability after ischemia, then a CEB such as V or N might be expected to attenuate these findings.

In the present studies it was clear that Mito Ca^{++} overload did not occur during the ischemic period. This is not unexpected

since the absence of active Mito proton extrusion during ischemia would abolish the electromotive driving force across the Mito inner membrane, which is necessary for active Ca^{++} uptake by Mito and retention of Ca^{++} within Mito (33). Furthermore, in animals treated with NE alone, Mito Ca^{++} loading was not observed in the first hour of reflow, when Mito respiration was diminished. This result in the dog clearly indicates that the early Mito dysfunction is probably a direct consequence of the ischemic insult rather than any consequence of increased Mito calcium concentration. Increases in Mito Ca^{++} have, however, been observed in the rat 1 h after ischemia induced by renal artery clamping (11, 18, 34, 35), and these increases might reflect the more rapid return of RBF in this model of ARF. In the present study, however, a correlation between decreased Mito respiration and Mito Ca^{++} overload at 1 h cannot be invoked. Since 1 h postischemia the RBF and GFR were enhanced by V, the Ca^{++} load to the tubular epithelium was even greater in these animals than in those receiving no treatment, yet the Mito Ca^{++} content was no higher than in contralateral (control) kidneys at 1 h.

The V- and N-treated animals demonstrated profound differences in Mito Ca^{++} and respiration as compared with the untreated animals. The progressive rise in Mito Ca^{++} and deterioration in Mito respiration observed in the kidneys with ischemic ARF were totally abolished by the administration of V and N, as were the abnormalities in Mito Ca^{++} uptake and release. Thus, CEBs provided protection not only against the functional, but also against the Mito, injury. This functional and Mito protection against the ischemic insult was also accompanied by prevention of the morphological changes of tubular necrosis. These findings are therefore compatible with the hypothesis (36–40) that Ca^{++} overload of tissue and Mito during reflow is an important factor in the progression of ischemic injury to cell death. CEBs therefore may exert a protective effect not only against renal vasoconstriction but also against the renal tubular necrosis after an ischemic insult. In the absence of tubular necrosis the cellular debris necessary for tubular obstruction and thus the maintenance phase of ischemic ARF may not be present. Moreover, attenuation of the renal vasoconstriction may prevent the lodging of any cellular debris that may result even in the presence of CEBs. Last, the fact that CEBs when administered after the ischemic insult were relatively more effective in protecting against the Mito dysfunction than in preserving GFR may suggest that factors in addition to cellular Ca^{++} are involved in ischemic ARF.

Figure 6. (Upper left) Light micrograph from a dog that had received NE for 40 min 3 h before sacrifice. Note the cell injury and necrosis present in the epithelium of the proximal tubule. $\times 380$. (Upper right) Light micrograph from a dog that had received V in addition to NE. The proximal tubular epithelial cells show normal structure. The normal proximal pars recta epithelium contains numerous lipid droplets. $\times 420$. (Lower left) Electron micrograph from a dog that

had received NE for 40 min 3 h before sacrifice. The proximal tubular cells show early injury with simplification of cell shape and rounding of the Mito. Necrotic debris is seen in the lumen of the tubule. $\times 6,700$. (Lower right) Electron micrograph from a dog that had received V in addition to NE. Note the normal appearance of the proximal tubular epithelium. $\times 6,700$.

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