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COLORADO UNIV AT DENVER MEDICAL CENTER R W SCHRIER
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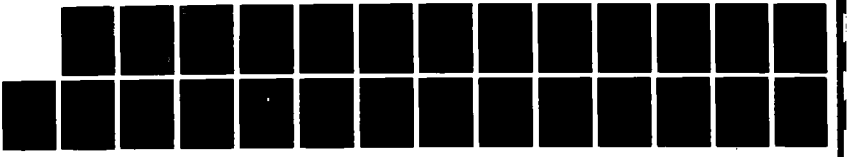

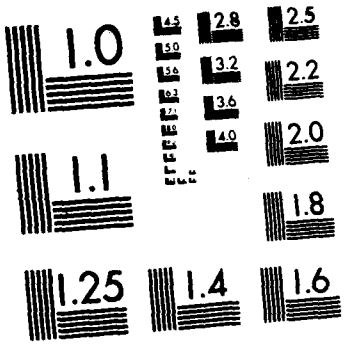

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PATHOGENESIS AND PREVENTION OF ACUTE RENAL FAILURE

Annual Report

Robert W. Schrier

October 1987

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Several in vitro studies on the isolated perfused kidney (IPK) and renal tubules freshly isolated from the rat were performed to evaluate the mechanism of oxygen deprivation injury and protection. Emopamil, a calcium channel blocker, prevented both warm and cold ischemia in the IPK. Cold perfusion alone is also of benefit and can be enhanced by verapamil. Atrial peptide III (the atrial natriuretic factor; ANF) exerts protection to the IPK from renal ischemia injury as well as in the whole animal. Using NMR, the protection appears to involve preservation of adenine nucleotides and prevention of intracellular acidosis. Furthermore, fructose diphosphate is also protective possibly by providing glycolytic intermediates. The tubule studies were characterized by technical improvements in preparative and incubation techniques and development of methods to ensure anoxic injury and its sequelae. Reynolds

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SUMMARY

Several studies in the IPK, in whole animals and in isolated tubules, confirm that 1) ATP depletion in anoxia is a critical component of cell injury and that improvement in ATP can be achieved by ANF, by fructose diphosphate and by verapamil or emopamil. The critical determinant of injury appears to be an imbalance between the demand of the tissue at risk for oxygen and the work of transport/cell volume regulation. By simple cold perfusion, cells can be metabolically depressed but this has little practical advantage for combat injury. Other effector maneuvers including certain drugs or hormones appear to also protect cells by interfering with the devastating effects of an imbalance between oxygen supply and demand.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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PROJECT 1

Introduction

The focus of the research outlined in this report continues to be the pathogenesis and prevention of ischemic renal cell injury. The hypothesis to be tested is that ischemia induces cellular and membrane damage which is perpetuated during the reflow period with cellular calcium influx as well as the mitochondrial, vascular and epithelial component of renal ischemic injury. The cellular mechanisms of renal cell injury in the setting of acute renal failure (ARF) are complex and multifactorial. Our approaches in the last year were related to the use of the isolated rat kidney perfusion coupled with P-31 NMR in vivo to test the specific effect of 1) calcium channel entry blocker, 2) exogenous atrial natriuretic factor (ANF), and 3) the effects of lowering intracellular phosphates, ATP or the addition of metabolic substrates on ischemic ARF in the rat.

Calcium Channel Entry Blocker

To understand the specific cellular effect of calcium channel entry blocker on the kidney, and to avoid the complications of hemodynamic effect of the drug, calcium channel entry blocker was studied in the isolated perfused kidney (IPK). ARF is a common complication in the early post-transplant period and the usual causes are related to the effect of warm and cold ischemia prior to the transplant operation. IPK offers a unique setting for studying the beneficial effect of calcium entry blocker and for testing the hypothesis related to the role of cytosolic calcium in the pathogenesis of ischemic injury. Therefore, we have extended the use of verapamil in the isolated perfused rat kidney (1) to different models of kidney preservation. The purpose is to investigate the potential benefit of adding verapamil to the organ flushing solution (Collins' solution) in ameliorating cellular and organ damage during warm and cold ischemia.

Effect of a new calcium channel entry blocker (emopamil)

We had previously shown that verapamil was protective against both warm and cold injury in the isolated perfused kidney (1). In this study, warm and cold ischemia were performed in different settings. We therefore designed a new protocol to include sequential warm and cold ischemia in the same model and used it to examine the utility of emopamil (Figure 1), a verapamil analogue with little cardiodepressant effect, in preventing warm and cold ischemia.

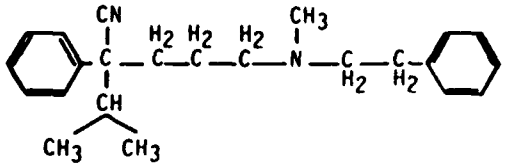


Figure 1. (-) emopamil (Knoll^{R}) Structure formula of (-) emopamil.

Following warm ischemia (15 min) and cold anoxia (stored at 0°C in 25 ml Euro-Collins solution for 4 hr), emopamil (5 nM/L) treated kidneys had significantly higher renal perfusate flow, inulin clearance, and tubular sodium transport during reperfusion (Table 1). The data show that emopamil has a salutory effect on kidney function following warm and cold ischemia (2).

The low effective dose and the unique property of emopamil, as demonstrated in this study and in parallel study in cell culture, make this new compound an exciting new calcium channel blocker to be used in the prevention of ischemic injury.

Table 1. Effects of emopamil on renal function following warm and cold ischemia[a]

Group	(N)	RPF (ml/min/g)	C_{In} (µl/min/g)	UV (µl/min/g)	FR_{Na}* (%)
Normal (no ischemia)	(7)	39±2	492±63	14±3	99±1
Control	(11)	33±1	58±7	30±4	48±4
Emopamil	(7)	39±1[b]	146±23[b]	34±5	76±4[b]

[a] Results, expressed as mean ± SEM for each kidney, are the average values of three 15-min collection periods during the last 45 min of reperfusion following a 15-min warm ischemic renal artery clamp and 4 hr of cold ischemia.

[b] $P<.01$ compared with control. Normal values (kidneys perfused without any warm or cold ischemia) are shown for comparison.

Effect of continuous hypothermic perfusion

In clinical practice, continuous cold perfusion has been used as a method of preservation in addition to the simple ice storage. Therefore, we have extended the rat model to include the use of verapamil in long-term continuous cold perfusion in the isolated rat kidney as the method of organ preservation. In this study, the effect of verapamil on renal function after 24 hr of hypothermic (4-7°C) perfusion was examined and compared with simple cold storage with Euro-Collins solution (4 hr), 4 or 24 hr cold perfusion without the addition of verapamil. The cold perfusion media consisted of 3% albumin in Krebs-Henseleit saline supplemented with 5 mM glucose. There was no increase in renal perfusate flow with verapamil but inulin clearance was significantly higher (Table 2). Preservation of tubular function by verapamil was demonstrated by an increase in fractional reabsorption of sodium and renal ATP concentration (Table 2 and Figure 2).

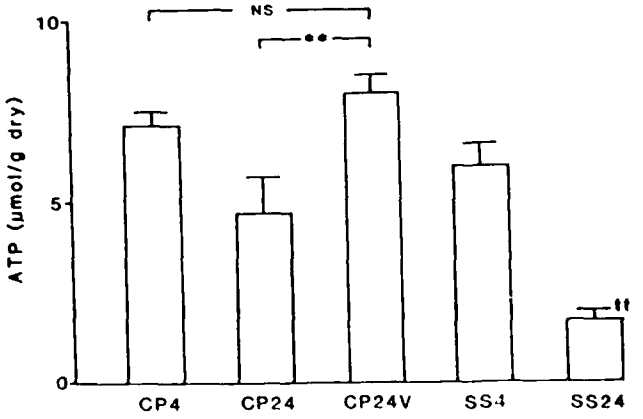


Figure 2. Tissue concentration of ATP following 60 min of warm reperfusion. CP4: 4 hr cold perfusion (n=6); CP24: 24 hr cold perfusion (n=9); CP24V: 24 hr cold perfusion with verapamil (n=5); SS4: 4 hr simple storage (n=6); SS24: 24 hr simple storage (n=4); Results expressed in mean±SE; **p<.01, ++p<.01, VS all other groups

Table 2. Renal function during 24 hr cold perfusion

	First 4 hr (n=9)	Last 4 hr (n=9)	First 4 hr (n=5)	Last 4 hr (n=5)
RPF (ml/min/g)	9±1	17±1	10±1	16±2
C_{In} (µl/min/g)	34±7	35±8	114±18	88±14
UV (µl/min/g)	15±9	20±5	28±10	54±11
T_{Na} (µmol/min/g)	4±1	2±1	13±2	6±1

Results expressed as mean±SE

Thus, verapamil appears not only to enhance kidney preservation with warm and cold ischemia but also improves renal function as assessed by GFR, tubular function and tissue ATP concentration with 24 hr cold perfusion (6). These data suggest the pivotal role of calcium in the pathogenesis of ischemic cell

injury and the specific cellular effect of calcium entry blocker in the prevention of warm as well as cold ischemic injury.

Effect of ANF

Mammalian atrial cells have long been known to contain granules that resemble those in endocrine cells; however, it was only recently that extracts from these granules were shown to induce natriuresis when injected in vivo into rats. Since then substances from these granules have been purified and amino acid sequences have been determined in the rat as well as in human. Using a complementary DNA technique, the precursor forms of these peptides were also clarified.

ATRIAL PEPTIDE

1
Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-

Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr
24

AP-I (1-21 Amino acid)

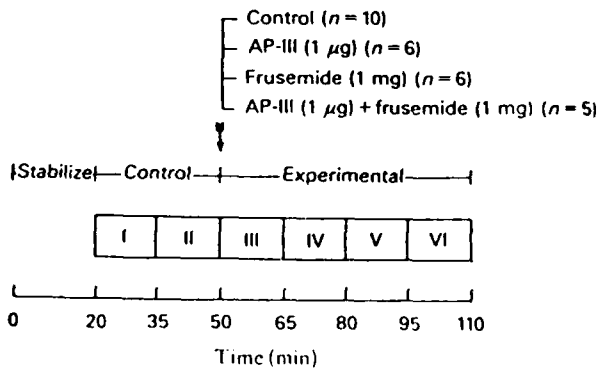
AP-II (1-22 Amino acid)

AP-III (1-24 Amino acid)

The exact mechanism of natriuresis of these peptides, however, is unclear. To avoid extrarenal hemodynamic, neural and hormonal changes in the in vivo animal, isolated rat kidney perfusion was used to study and compare the natriuretic response of the different atriopeptides. Atriopeptin III (AP-III) was found to be most effective among the three. Since the hallmark of ischemic ARF is a profound diminution in GFR and AP-III has shown to increase GFR independent of glomerular plasma flow, we reasoned that AP-III might provide unique protection against ischemic ARF. We therefore examined the utility of ANF in the preservation of renal function.

Use of isolated perfused rat kidney to study the mechanisms of action of ANF

To avoid systemic effect of AP-III, isolated rat kidney perfusion was used to examine the dose-response of different synthetic ANF and to compare the natriuretic response of AP-III with furosemide. Protocol for the experiments is described as follows. AP-III was shown to increase GFR (Figure 3) and enhance distal delivery of sodium. In addition, the effect of AP-III and furosemide are additive (Figure 4), thus supporting potential therapeutic implications for edematous state and other renal disorders (3).



Protocol for the experiments. The start of the perfusion is denoted as 0 min. After 20 min of stabilization the samples were collected every 15 min (periods I-VI). Drugs were added into the reservoir after two control collection periods, shown by the arrow.

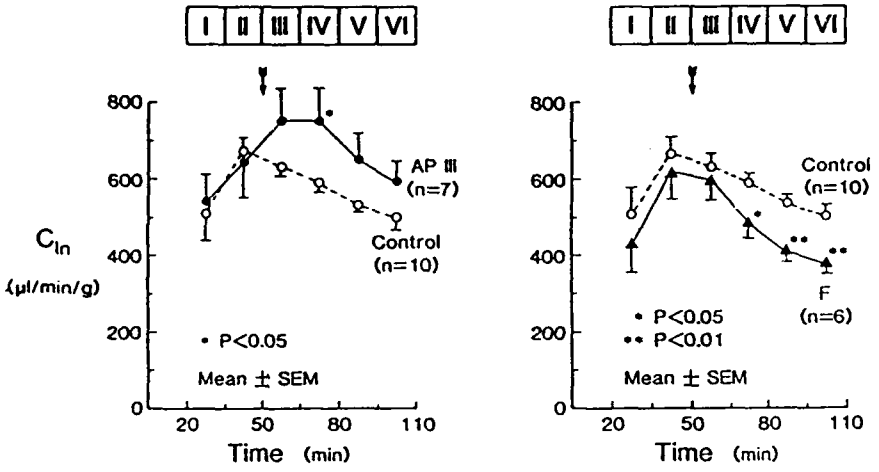


Figure 3. Effects of AP-III on inulin clearance.

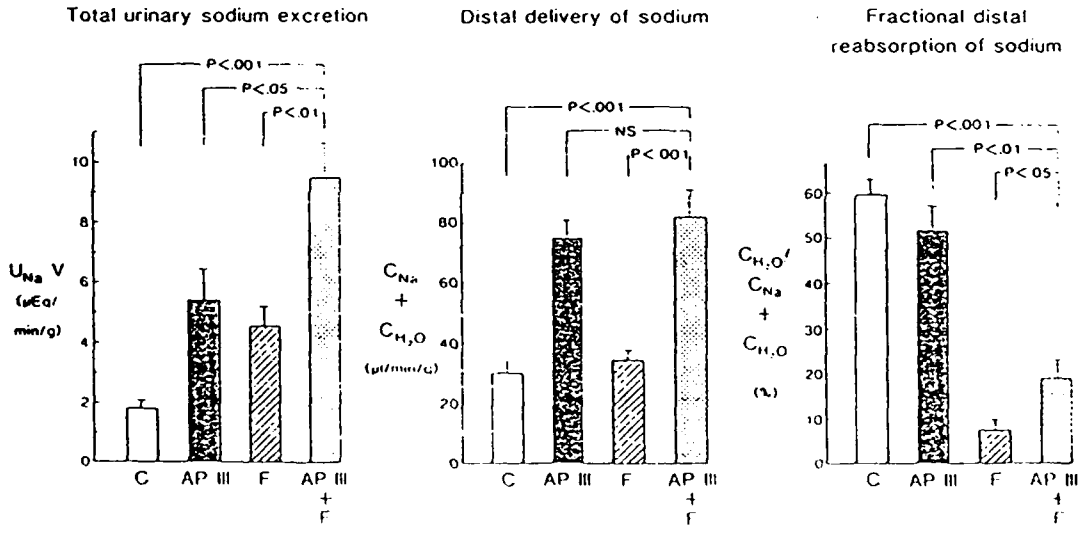


Figure 4. Combined effect of AP-III and furosemide (F).

In vitro protective effect

Encouraged by this result, we have extended the study of AP-III to include its utility in the setting of ischemic ARF. The effect of AP-III was first examined in the isolated perfused rat kidneys (clamped for 1 hr and reperfused for 30 min without therapy and perfused with either 0 (control) or 100 µg/dl AP-III. In this system, AP-III significantly improved plasma flow, inulin clearance, urine flow, and net tubular sodium reabsorption as compared with control (Table 3 and Figure 5).

Table 3. Effect of AP-III on renal function after ischemia in the isolated kidney

	Time	Control	AP-III	AP-III
	min	*0 μg/dl*[a]	*10 μg/dl*[b]	*100 μg/dl*[b]
Renal plasma flow (*ml/min/g*)	0–30	27.6±3.1	23.8±2.2	25.6±2.7
	30–60	33.5±1.4	36.5±1.4	39.1±1.8[d]
	60–90	32.2±2.1	34.7±2.0	39.6±2.4[d]
C_{In} (*μl/min/g*)	0–30	11.6±4.2	3.9±1.6	4.3±0.8
	30–60	17.0±4.7	13.6±2.8	123.4±44.0[d]
	60–90	24.6±6.2	28.4±8.4	182.6±49.2[d]
V (*μl/min/g*)	0–30	3.3±0.4	2.6±1.2	1.3±0.4
	30–60	4.8±0.8	6.3±1.6	31.4±8.0[d]
	60–90	7.1±0.8	10.1±2.1	52.9±12.1[d]
T_{Na} (*μmol/min/g*)	0–30	1.3±0.8	0.2±0.1	0.8±0.4
	30–60	2.3±0.8	1.4±0.3	14.8±6.0[d]
	60–90	2.9±0.9	3.0±1.2	21.2±6.6[d]
$U_{Na}V$ (*μmol/min/g*)	0–30	0.7±0.3	1.0±0.1	0.5±0.1
	30–60	0.7±0.1	0.9±0.2	3.6±0.8[d]
	60–90	0.8±0.2	1.2±0.2	5.6±1.3[d]

Results expressed as mean ± SEM. All data normalized per gram of left kidney weight.
[a] $n = 5$. [b] $n = 6$. [c] $P < 0.05$. [d] $P < 0.01$ vs. control.

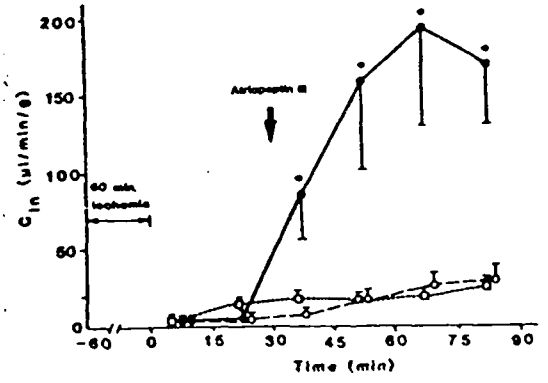


Figure 5. Effect of AP-III on inulin clearance (C_{In}) after ischemia in the isolated kidney. AP-III was infused at 100 μg/dl (solid circles) (n=6) or 10 μg/dl (open circles) (n=6). Control (open squares) (n=5) received no AP-III. Results of experiments are mean±SEM. *p<.05 compared with control.

In vivo protective effect: Acute studies

In the in vivo acute studies, when renal clamps were removed either 0, 0.02 or 0.2 μg/kg/min AP-III were infused for 1 hr in a randomized, blinded fashion. The AP-III was dissolved in 100 μl of 0.2 M acetic acid and then added to the saline given to each animal during the first hour after clamp removal. The result demonstrated that inulin clearance, urine flow and net tubular sodium reabsorption were significantly higher in AP-III treated rats than control during the hour of AP-III infusion (Table 4 and Figures 6 and 7).

Table 4. Effect of AP-III infusion on recovery of renal function over 2 hr after 60 min of ischemia in vivo

	Time	Control*	AP-III†	AP-III*
	min		*0.02 μg/kg/min*	*0.20 μg/kg/min*
C_{In} (*μl/min/100 g*)	0–60	15.8±8.2	21.4±3.6	244.4±25.1‡
	60–120	88.6±21.9	73.8±10.4	311.3±27.8‡
V (*μl/min/100 g*)	0–60	1.1±0.5	1.1±0.4	23.1±5.9‡
	60–120	2.6±0.4	4.3±0.6	12.8±3.8‡
FR_{Na} (%)	0–60	94.1±1.0	92.6±1.0	94.4±2.1
	60–120	97.2±0.6	94.8±0.8§	96.5±1.0
T_{Na} (*μmol/min/100 g*)	0–60	4.3±1.6	5.9±1.7	38.9±4.7‡
	60–120	15.4±4.4	10.3±1.4	49.1±3.6‡
$U_{Na}V$ (*μmol/min/100 g*)	0–60	0.2±0.1	0.2±0.1	3.3±0.8‡
	60–120	0.3±0.1	0.5±0.1	2.0±0.6§

Results expressed in mean±SEM. * $n = 6$. † $n = 4$. ‡ $P < 0.01$ vs. control. § $P < 0.05$.

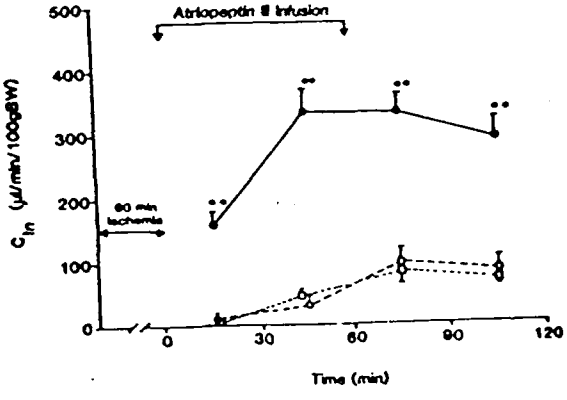


Figure 6. Effect of AP-III on inulin clearance (C_{In}) after ischemia in vivo. AP-III was infused at 0.2 μg/kg/min (solid circles) (n=6) or 0.02 μg/kg/min (pen squares) (n=4) for 60 min. Control (open circles) (n=6) received no AP-III. Results of experiments are mean±SEM. Results normalized per 100 g animal body weight. **p<.01 compared with control.

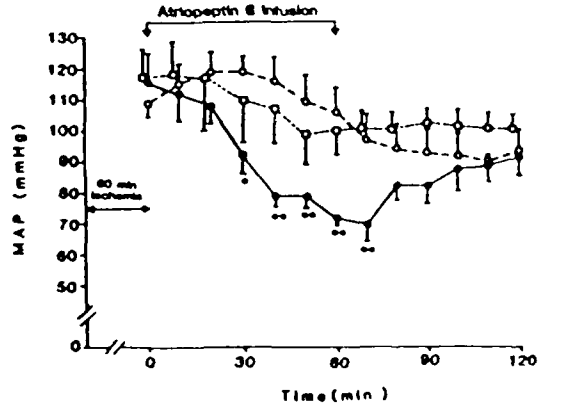


Figure 7. Effect of AP-III on mean arterial pressure (MAP) after ischemia in vivo. AP-III was infused at 0.2 μg/kg/min (solid circles) (n=6) or 0.02 μg/kg/min (open squares) (n=4) for 60 min. Control (open circles (n=6) received no AP-III. Results expressed as mean±SEM. *p<.01 compared with control.

P-31 NMR study

Three control and three experimental rats were studied with P-31 NMR.

These studies involved the identical procedure in the in vivo protocol except that after renal arterial clamp removal, a left flank incision was made with the exposed kidney and two turn 1.5 cm diameter surface coil was placed over the kidney. P-31 NMR examination was performed for 2 hr in a 1.89 Tesla 30 cm horizontal cryomagnet with a Biospec spectrometer (Bruker Instruments and Oxford Research Systems). At the end of the NMR experiment, kidney was freeze clamped and subsequently extracted with perchloric acid for determination of phosphate metabolites including ATP by enzymatic assay. P-31 NMR studies demonstrated that rats treated with the high dose of AP-III had a rapid recovery of renal ATP concentration than controls from 40 through 120 min post-ischemia (Table 5). Representative spectra are shown in Figure 8.

Table 5. Effect of AP-III infusion on intracellular ATP, inorganic phosphorus, and pH

Treatment	Time	ATP	Pi	pH
	min	*μmol/g dw*	*μmol/g dw*	
Control *(n = 3)*	20–40	1.15±0.11	4.60±0.21	6.96±0.06
	40–60	1.45±0.40	3.47±0.40	7.02±0.02
	60–80	1.68±0.08	3.52±0.39	7.04±0.03
	80–100	1.81±0.29	2.61±0.20	7.08±0.05
	100–120	1.80±0.05	2.44±0.28	7.08±0.05
AP-III *(0.20 μg/kg/min × 60 min; n = 3)*	20–40	1.40±0.16	4.28±0.54	6.97±0.04
	40–60	3.03±0.30*	3.52±0.36	6.96±0.04
	60–80	3.22±0.36‡	3.27±0.07	7.09±0.07
	80–100	3.92±0.53‡	2.26±0.30	7.13±0.04
	100–120	3.98±0.46‡	1.87±0.24	7.15±0.03

dw, dry weight. Results expressed as mean±SEM. * $P < 0.05$; ‡ $P < 0.01$ vs. control.

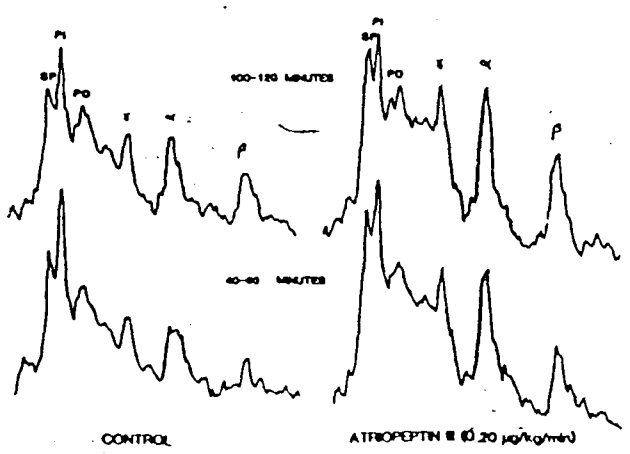


Figure 8. Effect of AP-III on representative P-31 NMR kidney spectra after ischemia in vivo. SP, sugar phosphate; Pi, inorganic phosphate; PD, phosphodiester; γ, gamma phosphate of ATP; α, alpha phosphate of ATP; β, beta phosphate of ATP.

Effect of AP-III on serum creatinine after ischemia in vivo

In this study, animals were acclimatized to metabolic cages, after which a baseline 24 hr urine collection was performed. The rat were then anesthetized and a baseline plasma sample obtained from the tail artery. Then as described previously, bilateral renal artery ischemia induced by clamping. Then either the vehicle or AP-III was infused for 1 hr. Animal treated acutely with AP-III had lower serum creatinine concentration at 24 and 48 hr after the 60 min of ischemia than controls (Figure 9 and Table 6).

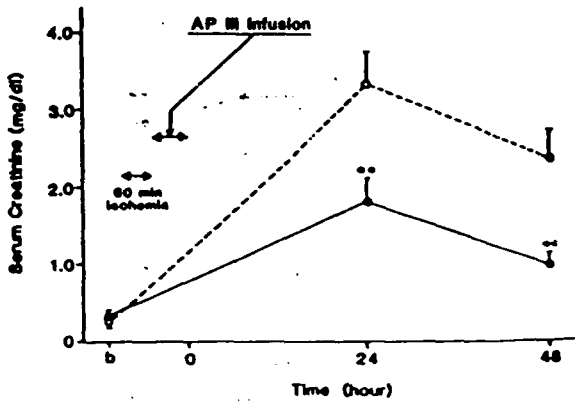


Figure 9. Effect of AP-III on serum creatinine after ischemia in vivo. AP-III was infused at 0.2 μg/kg/min (solid circles) (n=12) for 60 min. Control (open circles) (n=12) received no AP-III. b, blood taken before onset of ischemia. Results expressed as mean±SEM. *p<.05; **p<.01 compared with control.

Table 6. Effect of AP-III infusion on recovery of renal function over 48 hr after 60 min of ischemia in vivo

	Time	Control	AP-III
	h	*n = 12*	*n = 12*
C_{Cr} (*μl/min/100 g*)	0–24	60±23	110±18*
	24–48	126±34	219±34*
FR_{Na} (%)	0–24	94.1±1.8	98.3±0.3*
	24–48	93.6±3.0	99.3±0.2*
T_{Na} (*μmol/min/100 g*)	0–24	7±3	15±3*
	24–48	12±3	38±6‡
$U_{Na}V$ (*μmol/min/100 g*)	0–24	0.127±0.013	0.145±0.021
	24–48	0.173±0.024	0.153±0.031
FE_K (%)	0–24	279±49	173±44*
	24–48	115±27	50±11*
U_KV (*μmol/min/100 g*)	0–24	0.42±0.05	0.51±0.06
	24–48	0.40±0.06	0.47±0.03

FE_K, fractional potassium excretion; C_{Cr}, creatinine clearance. Results expressed in mean±SEM. *p<.05; ++p<.01 vs control.

Therefore, using P-31 NMR, enzymatic assay coupled with isolated rat kidney perfusion and in vivo functional studies, we have demonstrated the beneficial effect of AP-III in the protection of ischemic ARF in the in vitro as well as in vivo models (4). We have also shown the degree of protection afforded by AP-III was better than that accomplished by verapamil in the same model. Several groups have confirmed the beneficial effect of AP-III since the presentation of our paper at the American Society of Nephrology, 1986, and also after the submission of our paper to the Journal of Clinical Investigation.

Effect of Lowering Intracellular Phosphate and ATP and the Role of Exogenous Metabolic Substrates

Effect of lowering intracellular phosphate and ATP

Phosphate restriction has been shown to be protective against functional deterioration in animal models of chronic renal failure. Using NMR and other metabolic studies, we demonstrated that remnant kidney is associated with a high metabolic rate and phosphate restriction resulted in the lowering of nephron hypermetabolism in this model. To extend this finding to ARF, we examined the critical role of low phosphate in the pathogenesis of ARF. Studies were first performed in the isolated perfused rat kidney and perfused with phosphate free perfusate, no functional deterioration was observed in

control as well as during reperfusion after 30 min of ischemia. Since ATP was well preserved in kidneys perfused in the absence of phosphate, experiments were carried out in models of ATP depletion with glycerol or fructose (40 mM) added to the control perfusate. Similarly, renal function was observed during control periods without ischemia or following 30 min of clamp ischemia. Addition of glycerol or fructose resulted in lowering ATP levels to 60% and 30%, respectively (Figures 10 and 11). Figure 10 shows tissue levels of ATP following 70 min of non-ischemic perfusion and Figure 11 shows tissue levels of ATP after 70 min of reperfusion following 30 min of ischemia. Following ischemia glycerol and fructose reduced ATP to 51% and 37% and total adenine nucleotide to 67% and 65%, respectively, of values seen with control perfusate.

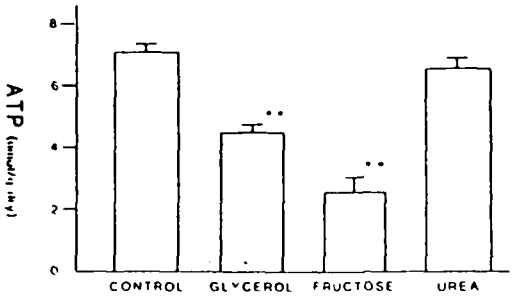


Figure 10. Tissue levels of adenosine triphosphate (ATP) following 70 min of non-ischemic perfusion. Large bars represent mean of 3-5 determinations for each group; small bars represent standard error of the mean. *p<.05, **p<.01 compared to control.

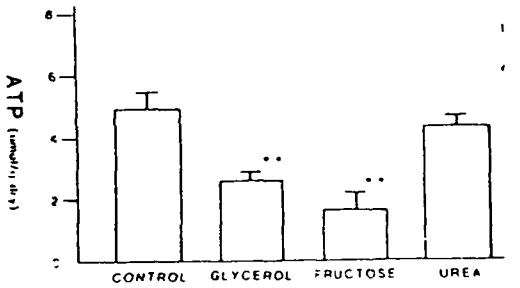


Figure 11. Tissue levels of adenosine triphosphate (ATP) after 70 min of reperfusion following 30 min of ischemia. Large bars represent mean of 3-5 determinations for each group; small bars represent standard error of the mean. *p<.05, **p<.01 compared with control.

Despite the difference in ATP, physiological parameters of renal function were unaffected by the addition of either glycerol or fructose (Table 6 and 7). Thus, a 33-60% reduction of renal ATP does not appear to have a deleterious effect on organ function (5).

Table 7. Renal function during non-ischemic perfusions

Experimental Group	RPF (ml/min/g)	C_{In} (µl/min/g)	FE_{Na} (%)	V (µl/min/g)
Control media (n=7)	38.5 ±1.8	492 ±63	1.5 ±0.3	14 ±3
Media with 40 mM glycerol (n=5)	36.3 ±4.9	490 ±70	7.2 ±2.7*	102 ±36*
Media with 40 mM fructose (n=5)	40.5 ±2.4	507 ±44	10.6 ±4.2*	121 ±22**
Media with 40 mM urea (n=5)	39.6 ±2.9	483 ±32	12.1 ±1.4**	105 ±5**

Abbreviations: RPF = renal plasma flow, C_{In} = inulin clearance, FE_{Na} = fractional sodium excretion, V = urine flow
Results expressed as mean ± SEM.
* or ** difference between adjacent value and control value is p<.05 and p<.01.

Table 8. Renal function following 30 min of ischemia

Experimental Group	RPF (ml/min/g)	C_{In} (µl/min/g)	FE_{Na} (%)	V (µl/min/g)
Control media (n=5)	34.0 ±0.8	146 ±43	8.1 ±1.8	18 ±4
Media with 40 mM glycerol (n=5)	36.8 ±1.5	125 ±30	29.6 ±4.0**	72 ±14**
Media with 40 mM fructose (n=5)	36.8 ±1.5	163 ±38	22.1 ±1.5**	64 ±15*
Media with 40 mM urea (n=5)	34.0 ±6.4	92 ±15	25.2 ±3.1**	34 ±6*

Results expressed as mean ± SEM.
* or ** difference between adjacent value and control value is $p<.05$ and $p<.01$.

Effect of lowering ATP in the in vivo models

The technique of isolated perfused rat kidney as used in the previous study, however, limited the amount of reflow time following the ischemic period that could be observed. It is also not optimal for the histologic evaluation of ischemic injury. For these reasons, we conducted the in vivo studies in conjunction with P-31 NMR. Administration of glucose or fructose IP did not appreciably effect renal physiologic functions assessed 24 hr after sham operation compared with the control group. The data from these three groups (C+G and F) has therefore been pooled and is listed as Group C'. Twenty-four hr following 30 min of ischemia, rats that received no IP fluids (C+I) had approximately a 48% reduction in C_{In} and T_{Na} compared with C' (Table 9).

Table 9. Renal physiologic data in experimental animals.

Group	(N)	Cin (µl/min)	V (µl/min)	FE_{Na} (%)	T_{Na} (µmol/min)
C'	(18)	888±35	3.0±0.4	0.11±0.02	120±4
C+I	(6)	467±46**	2.7±0.4	0.07±.02	66±7**
G+I	(7)	732±39*++	2.5±0.3	0.05±0.01	99±7*++
F+I	(6)	701±42*++	3.7±0.5	0.08±0.04	94±6*++

Results expressed as mean±SEM. *p<.05, **p<.01 compared with C'.
+p<.05, ++p<.01 compared with C+I.

The concentration of ATP and other phosphate metabolites were monitored by P-31 NMR. In Figure 9 are shown a typical control spectrum (1A), and a spectrum obtained immediately after fructose injection (1B). The concentration of phosphomonoesters (peak 1) increased immediately after the fructose injection. A fall in ATP is also observed. The increased phosphomonoester peak is primarily due to an increase in the concentration of fructose-1-phosphate. P-31 NMR spectra are shown in Figure 12 and the time course of the changes are shown in Figure 13.

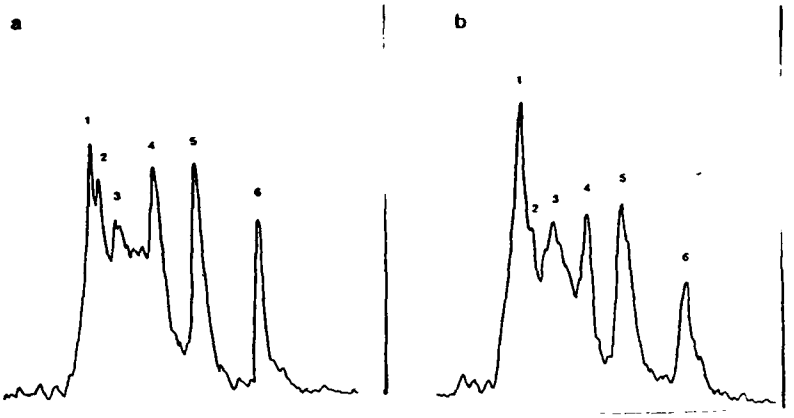


Figure 12. P-31 NMR spectrum at 32.6 MH_2 of rat kidney in vivo. The spectrum represent the sum of 512 scan at 60° pulses with a delay of 2 sec between pulses; array size of 1K. The spectrum was obtained by Fourier transformation with a line-broadening of 10 Hz. Peak assignment: 1) phosphomonoesters (sugar phosphate, AMP, and the 3-phosphate of 2,3-DPG); 2) inorganic phosphate and 2-phosphate of 2,3-DPG; 3) phosphodiesters (including urinary phosphate); 4) γ-phosphate of ATP; 5) α-phosphate of ATP; and 6) β-phosphate of ATP

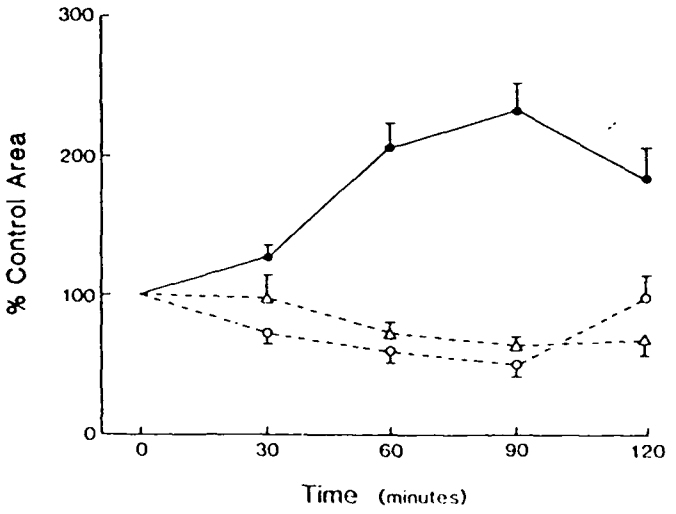


Figure 13. Changes in peak areas of phosphomonoesters closed circles), inorganic phosphate (open circles) and β-phosphate of ATP (open triangles). Results are expressed as mean±SEM. *p<.05 and **p<.01

Histologic evaluation of the same study was also performed. The percentage of S3 necrosis in rats that were not exposed to ischemia was 0% in all kidneys studied (Groups C, G and F). These data are pooled together and shown in Figure 14. Rats exposed to ischemia without IP fluids (I) had approximately 11% of S3 segments that were necrotic. Rats exposed to glucose and ischemia (G+I) and fructose and ischemia (F+I) despite their marked differences in ATP and adenine nucleotide levels in the early post-ischemic period had comparable numbers of S3 segments necrotic.

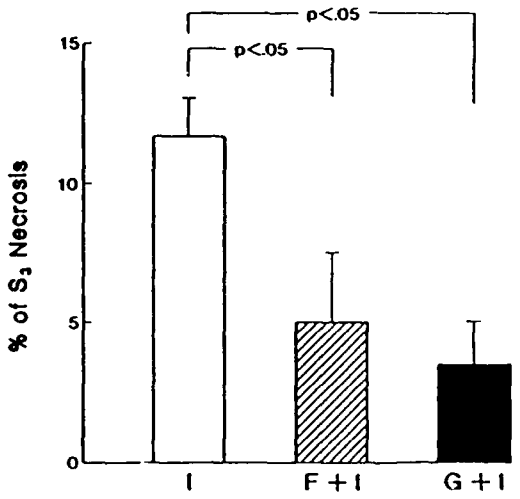


Figure 14. Percentage of necrotic S_3 tubules. Results expressed as mean±SEM of 3-5 kidneys. G+I, rats exposed to glucose and ischemia (30 min); F+I, fructose and ischemia (30 min); and I, ischemia alone.

Therefore, the rat kidney can withstand rather low levels of ATP without appreciable effect on organ function. Our data is seemingly at odds with an extensive body of compiled work suggesting a benefit to infusions of ATP-$MgCl_2$ following ischemia. However, there are multiple reasons for this apparent discrepancy. ATP per se may not be important since the half-life of ATP in vivo or during perfusion is very short. ATP is rapidly broken down to other intermediary metabolite. The effect of fructose on the rat kidney concentration of ATP can be explained as governed by the law of mass action which is entirely consistent with the view that the reaction of ADP+Pi to ATP catalyzed by adenylate kinase is near equilibrium in vivo. If fructose does not effect net energy turnover, as assessed by oxygen consumption, then its effect on ATP concentration would be expected to effect physiologic function only when a limiting ATP concentration became substrate limiting for key enzymatic reactions.

The role of metabolic substrate

It is clear from the above reasoning that the critical factor for cell viability is the provision of substrates for ATP synthesis or metabolism. To explore this possibility, fructose-1,6-diphosphate (FDP), a metabolite in the glycolytic pathway, was infused to the isolated kidney perfusion after 40 min of clamping and perfused for 60 min. FDP infusion improved inulin clearance (Figure 15), urine flow and tubular reabsorption of Na. FDP concentration in the perfusate decreased with a proportionate increase in dihydroxyacetone phosphate (Figure 16 and Table 10). Kidney ATP shows no difference during ischemia but is higher after 60 min reperfusion in the treated group. These functional and metabolic findings are substantiated in the in vivo models. The changes in high energy phosphate were monitored in vivo by P-31 NMR (Figure 17). ATP resynthesis as determined by NMR was greater following ischemia in FDP-treated kidneys (2.2±0.3 vs 1.1±0.4 mM, $p<0.05$). This study has been presented in part at the Xth International Congress of Nephrology in London, July 26-31, 1987. Further in vivo studies are in progress. The use of FDP has several advantages over ATP. It is less expensive, more stable and has less vasodilation and hemodynamic effect. Finally, FDP appears to ameliorate ischemic damage by a cellular mechanism, possibly by providing essential substrates for glycolysis and enhancing oxidative metabolism during reperfusion.

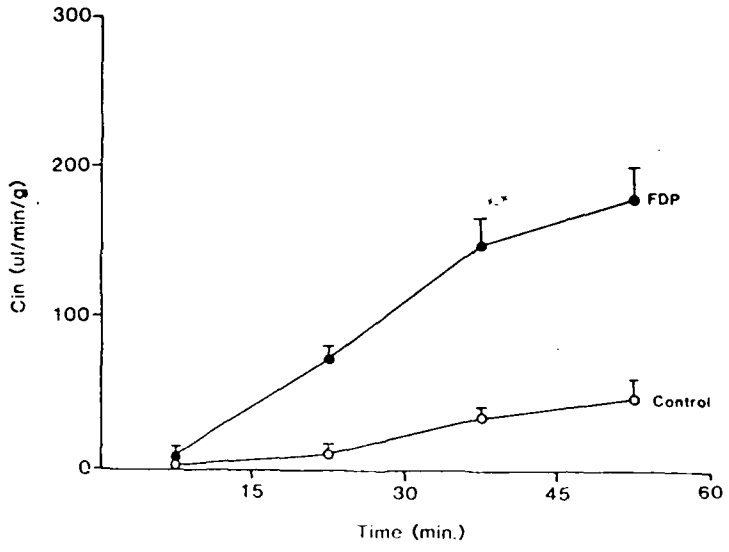


Figure 15. Effect of FDP on inulin clearance following 40 min of ischemia in the isolated rat kidney.

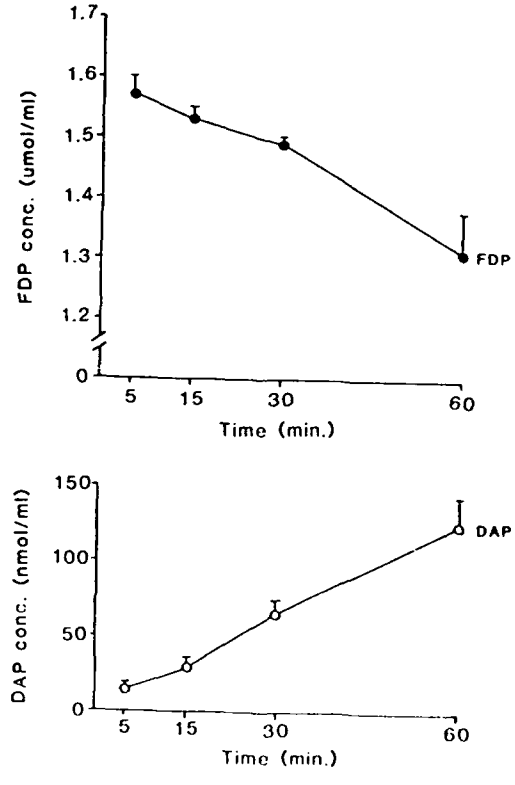


Figure 16. FDP and DAP concentration of perfusate in the isolated, ischemic rat kidney. DAP, dihydroxyacetone phosphate

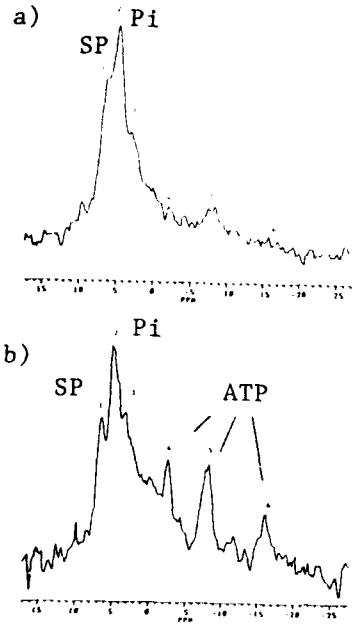


Figure 17. ^{31}P NMR spectra of rat kidney in vivo. a) Ischemic rat kidney following 60 min of reflow, and b) FDP-treated ischemic rat kidney with 60 min of reflow. ATP peaks are profoundly increased by FDP treatment. Pi, inorganic phosphate; SP, sugar phosphates (including FDP)

Table 10. Effect of fructose-1,6-diphosphate on renal function over 30 min following 40 min of ischemia in the isolated kidney.

	FDP (n=6)	Control (n=6)	p Value
RPF (ml/min/g)	35.6±2.2	35.0±1.3	NS
C_{In} (µl/min/g)	164.6±17.8	39.9±9.4	.001
UV (µl/min/g)	20.8±2.1	5.9±1.6	.001
T_{Na} (µmol/min/g)	24.1±2.7	5.5±1.3	.001
$U_{Na}V$ (µmol/min/g)	2.5±0.4	0.6±0.2	.005
FE_{Na} (%)	7.5±1.0	7.2±1.9	NS
O_2 Consumption (µmol/min/g)	2.46±0.18	1.86±0.18	.025
Tissue ATP (µmol/g d.w.)	4.78±0.19	3.67±0.12	.005

Results are expressed as mean±SEM.

Conclusion

These varied experimental maneuvers suggest the different pathogenetic mechanisms of ischemic ARF and the various possible methods of intervention, namely calcium entry blocker, AP-III and an intermediary metabolite such as FDP. AP-III appears to be the most promising agent so far. Further in vivo studies of the route, timing and duration of administratin should be performed to establish guidelines that might be amenable to clinical studies.

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Recent Abstracts:

1. Nakamoto M, Shapiro JI, Schrier RW, Chan L: Mechanism for the protective effect of fructose-1,6-diphosphate: A perfusion and in vivo P-31 nuclear magnetic resonance study. Xth Int Cong Nephrol, London, 1987, pp 476.
2. Shapiro JI, Harris DCH, Schrier RW, Chan L: Analysis of nephron hypermetabolism in remnant kidney by nuclear magnetic resonance: Potential factors in the progression of chronic renal failure. Xth Int Cong Nephrol, London, 1987, pp 518.
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4. Chan L, Shapiro JI, Schrier RW: P-31 nuclear magnetic resonance in the investigation and differential diagnosis of experimental acute renal failure. Xth Int Cong Nephrol, London, 1987, pp 454.

PROJECT 2

Introduction

In vivo studies of the pathogenesis and prevention of ischemic ARF are associated with both vascular and tubule (epithelial) damage. In the in vivo setting it is difficult, if not impossible, to separate the direct effects of oxygen deprivation on tubule cell function from those effects associated with altered renal flow and its sequelae. This project has examined, in a freshly isolated population of enriched proximal tubules from the rat, the direct effects of hypoxic injury.

Preparation of Tubules

In the early part of this contract year, we questioned the viability of our isolated tubules, seeking to increase the integrity of the preparation. Our goals were to reduce lactate dehydrogenase (LDH) release to <10% throughout 2 hr of incubation; to improve ATP levels to values of 6-12 nmol/mg protein; to improve morphologic appearance of the tubules; and to normalize cellular potassium and Ca content (in nmol/mg dry weight) of tubules.

Since our previous results (during contract year 01) were characterized by values for these parameters that were less than optimal, we have spent several months reexamining our preparative and incubation techniques to determine how such improvements might be accomplished. The following summarizes our new, improved technique and where appropriate contrasts it with our previous procedure.

Specifically, and as originally described in our contract, thin slices containing tubules from the cortex of both kidneys of 5-6 rats are digested for 30 min in 30 ml of a collagenase solution (200 U/ml) in an oxygenated Krebs-Henselite buffer (KHB) media. Following the addition of 120 ml of cold KHB and

a 10 min period with gentle stirring on ice to permit further digestion, the tubules are currently being pressed gently through a tea strainer rather than squeezed through the nylon mesh previously used. They then are centrifuged at 30xg for 30 sec. This more gentler handling of the tissue digest was employed to lessen the likelihood of mechanical damage to the tubules. After 2 subsequent washes with cold, oxygenated KHB, the tubules are suspended in 10 ml of 5% bovine serum albumin (in KHB) for 5 min at 4°C. After centrifugation at 30xg for 1 min, the tubules are suspended in cold, 40% Percoll, previously gassed for 20 min with 95% O_2/5% CO_2. The tubule suspension is centrifuged at 10,000 rpm for 30 min in a Sorvall Refrigerated Centrifuge. This procedure allows 4 bands of tissue to be visualized. The lowest (fourth band) is removed with a plastic pipette and washed twice in 30 ml of cold, oxygenated KHB.

At this point the second major change in our procedure compared to our previous studies was achieved. Previously, we added approximately 80 ml of 37°C, oxygenated KHB to the tubules yielding therefore a suspension of about 1 mg/protein per ml of media. We had previously separated this 80 ml suspension into two containers containing 40 ml of tubules each. These were gassed at 0.5 L/min for 30 min, the nominal equilibration period.

Currently, we add 60 ml of cold, oxygenated media to the tubules and subdivide the resultant suspension into 4 cups of 15 ml each and gas them at 3.0 L/min.

The major changes are listed below:

Previous	Current
Nylon Mesh	Tea Strainer
37°C KHB	4°C KHB
2 cups of 40 ml each	4 cups of 15 ml each
O_2/CO_2 gas rate 0.5 L/min	Gas rate 3.0 L/min

The result of these changes can be seen below with mean values obtained after 30 min of equilibration in a shaking water bath set at 37°C.

	Previous	Current
ATP (nmol/mg protein)	3.8	7.3
LDH release (%)	28	8

Conclusion

We believe that the addition of 37°C media to tubules at 4°C after Percoll separation, as done previously, induced thermal shock to the proximal tubules in suspension leading to high morphologic injury (approximately 40%), low ATP content and high LDH release even after 30 min of equilibration in 95% O_2. In addition, the low flow rate of O_2 (0.5 L/min) while sufficient to maintain O_2 content of the tubule suspension at reasonably high values during equilibration, may have compromised our subsequent studies of "anoxia". Figure 1 below demonstrates, even when gassed at 0.5 L/min, that pO_2 of media with tubules falls during incubation in collagenase from approximately 500 mmHg at time - the start of incubation - to 225 mmHg at 10 min, to 180 mmHg at 20 min and to 140 mmHg at 30 min, just prior to placing the tubules on ice and stirring them for 10 min. Furthermore, during stirring the pO_2 drops to undetectable levels and is immeasurable at the end of the 10 min cold digestion period. The pO_2 of the media during the first 30 min incubation (equilibration) period does not change, however, but it must be noted that there was extensive tubular injury assessed morphologically and by LDH release and ATP content. Moreover, the amount of tissue (proximal tubules only) is much smaller during equilibration than was present during digestion of the entire cortex.

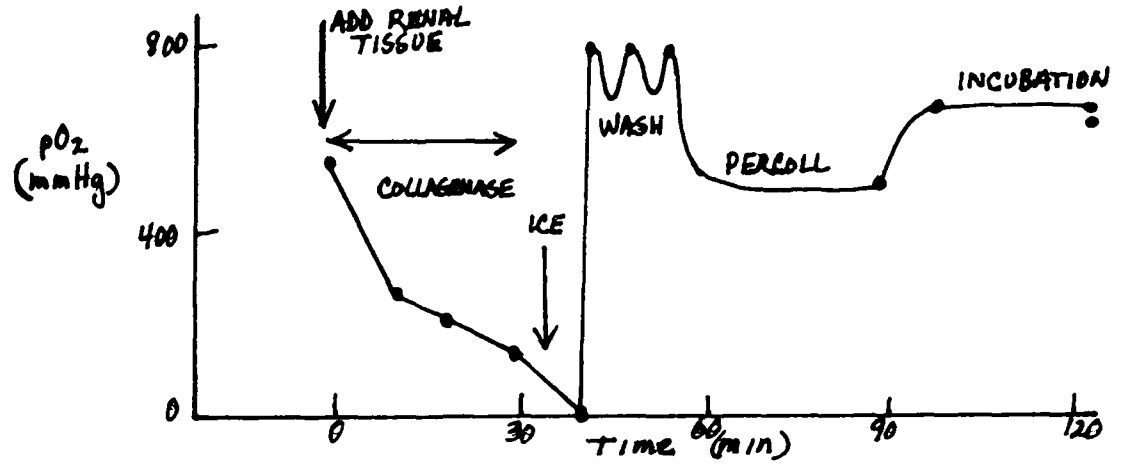

Figure 1. pO_2 versus time in media containing proximal tubules.

The benefit of gassing a small volume of KHB not containing tubules is shown in Figure 2.

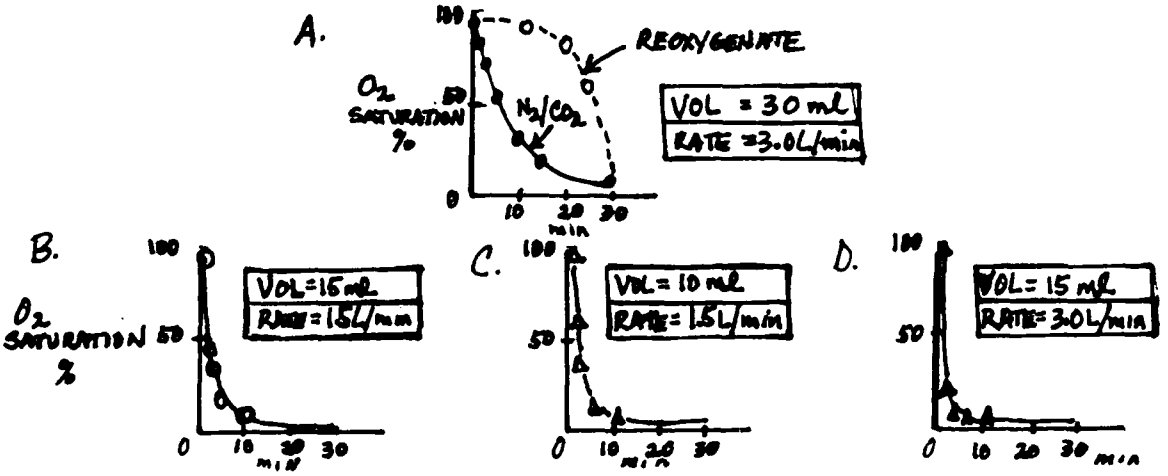

Figure 2. Effect of different rates of gassing with N_2/CO_2 on the % O_2 saturation in media of different volumes.

At the top, % O_2 saturation is on the Y-axis. When the pO_2 of a fully gassed (3.0 L/min) cup of KHB at 37°C is measured, the value is approximately 600 mmHg. If the gas mixture is changed to one containing 95% N_2/5% CO_2 and % saturation is measured, it can be seen that it takes >28 min to reach an O_2 content of 4%. Direct measurements of pO_2 confirm and corroborate these findings. Shown at the bottom are three subsequent studies. The open circles and closed triangles are studies in which the gas rate was lower, i.e. 1.5 L/min. The cup indicated by the open circle contained 15 ml of KHB whereas the one indicated by the closed triangle contained 10 ml of KHB. As can be seen, the O_2 content falls much more quickly and 5%O_2 content is reached in about 5-7.5 min. We also took a 15 ml of KHB and gassed it at 3.0 L/min; this is shown in closed triangles and in this study 5% O_2 is achieved within 2 min. The critical difference in the study shown at the top and those at the bottom relates to the surface area to volume ratio (SA/V). The diameter of these containers is 47.6 mm. The formula for SA is πr^2. For a radius of 23.8 mm (or 2.38 cm), the SA is 17.78 cm^2. Shown below is the SA/V for different volumes of KHB.

Volume	SA/V
30 ml	0.592 cm^{-1}
15 ml	1.18 cm^{-1}
10 ml	1.778 cm^{-1}

Thus, the larger the SA/V ratio, the more rapidly will O_2 (or N_2) displace the existing gas.

Our previous studies during the first year of the contract using 30-40 ml of media gassed at 0.5 L/min of 100% N_2 probably created a modest hypoxic injury which was, however, attended by several metabolic and morphologic changes (Figure 3).

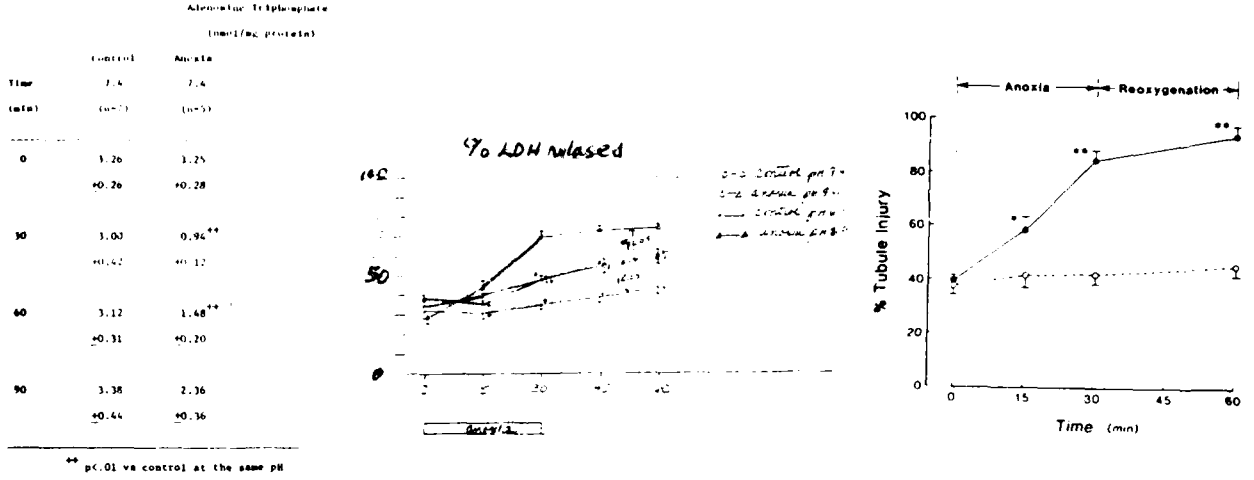

Figure 3. ATP, LDH and morphologic injury in hypoxia.

^{45}Ca Uptake

Based on the description above which contrasts the two preparations used over the last 2 years, some insight into the question of whether Ca uptake plays a role in ischemia injury can be obtained. The curve of control and anoxic studies done previously with the somewhat more labile preparation as compared to the more recent preparation with enhanced viability are contrasted below (Figure 4).

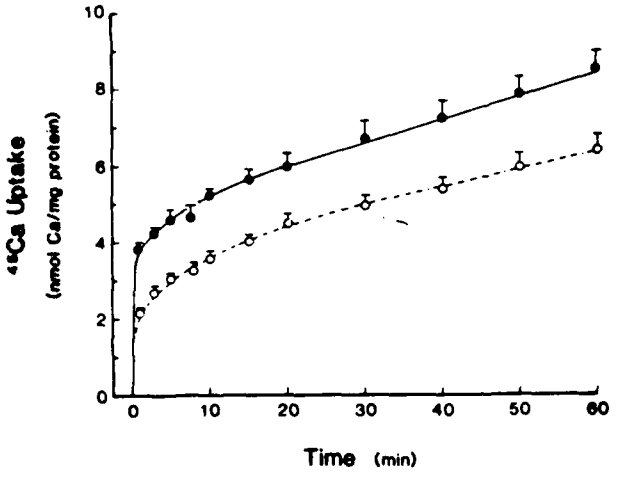

Figure 4. On the left it can be seen that Ca uptake during control is lower than that seen after hypoxia. On the right, using the higher flow rate of 3.0 L/min of 95% N_2/5% CO_2, it will be noted that this degree of hypoxic injury is not associated with increased ^{45}Ca uptake.

Moreover, our ATP levels and adenylate energy charge decrease only modestly with hypoxic injury and LDH rises only marginally (even though we presumably now achieve an even greater degree of hypoxia than we did previously) (Figure 3).

We have also determined that the slow rise in LDH release is not evidence of further cell injury but relates in large part to evaporation (and therefore an enhanced concentration of LDH which remains in the media in the incubation cups). We performed a control study to evaluate this phenomena. We added an LDH standard to freshly prepared KHB media and then gassed and incubated the mixture at 37°C. At the appropriate times, which coincided with the sampling times of a normal experiment, we removed "pseudo samples". This maneuver had the effect of decreasing the media volume with time. We found that LDH concentration rose about 50% between 30-60 min and 105 min of continued incubation. Based on this observation, we now incubate containers of media and tubules for fixed periods of time, i.e. 30, 60 or 105 min, and sample for K^+, Ca^{2+}, LDH, ATP, and morphology from the separate containers at only that one time period. Thus, evaporation occasioned by: 1) opening and closing the containers and 2) repetitive sampling is eliminated.

Finally, our KHB maintains pH during a study quite well and we have therefore eliminated the Hepes which was previously used to attempt to hold pH at 7.4 during gassing at 0.5 L/min with 100% N_2. Curiously the ATP values in paired experiment performed on tubules (from the same rats) incubated in a Hepes-free KHB buffer are higher than are those in buffer with Hepes (8.0 vs 5.0 nmol/mg).

Conclusions and Summary

Isolated tubule studies, while providing a vehicle to study anoxic cell injury in vitro present certain problems in themselves for the investigative team. Using the techniques detailed above and indicated below, we feel that a great deal of precise information can be achieved in understanding the pathogenesis and prevention of cell injury. It appears especially important that the $CaCl_2$ concentration be high (>1.53 mM) during incubation in collagenase or during metabolic studies, since the BSA (albumin) will bind some of the calcium.

	Composition of KHB
NaCl	115 mM
KCl	2.1 mM
KH_2PO_4	2.4 mM
$MgSO_4$	1.2 mM
$MgCl_2$	1.2 mM
$CaCl_2$	1.5 mM
Glucose	5.0 mM
Lactate	4.0 mM
Glutamine	1.0 mM

Collagenase Digestion Solution
30 ml KHB, gassed at 4°C with 95% O_2/5% CO_2
200 U/ml collagenase
750 mg BSA

Percoll

Percoll	24 ml	
KHB (10X concentrated)		6 ml
H_2O		30 ml

Incubation Solution (per container)

KHB	15 ml	
BSA		2.5 g%

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