Kidney International, Vol. 40 (1991), pp. 418–424

Binding inhibitors restore furosemide potency in tubule fluid containing albumin

KENT A. KIRCHNER, JAMES R. VOELKER, and D. CRAIG BRATER

Department of Medicine, University of Mississippi Medical Center, Jackson, Mississippi and Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana

Binding inhibitors restore furosemide potency in tubule fluid containing albumin. We have previously suggested that albumin in tubule fluid at concentrations found in the nephrotic syndrome (NS) binds furosemide, thereby diminishing diuretic effect. This mechanism may contribute to diuretic resistance in NS. If this hypothesis is correct, displacement of albumin from furosemide should restore diuretic response in tubule fluid containing albumin. To test this supposition, in vivo loop microperfusion was performed in rats using perfusates containing 6 μM furosemide in the presence or absence of 3.8 μM albumin, or furosemide and albumin to which 12 μM warfarin or 5.4 mM sulfisoxazole had been added. These drugs are inhibitors of albumin-furosemide binding in plasma. Albumin in the perfusate impaired furosemide effect on loop chloride reabsorption (1248 ± 59 vs. 886 ± 65 pEq/min; $P < 0.05$). Addition of warfarin or sulfisoxazole to perfusate containing albumin normalized furosemide's effect. Neither drug affected furosemide response in the absence of albumin. Dansylsarcosine, a probe that binds albumin at a different site than furosemide, failed to normalize furosemide response in albumin perfusates. These data suggest that albumin in tubule fluid reduces diuretic response through a diminution in the free furosemide concentration. In as much as this mechanism contributes to diuretic resistance observed clinically in NS, displacement of furosemide from albumin binding sites may be a therapeutic strategy warranting study.

An attenuated response to loop diuretics is a frequent observation in patients with nephrotic range proteinuria [1, 2]. Dose response analysis comparing urinary excretions rates of diuretic and sodium demonstrate that nephrotic patients are less responsive to a given dose of drug than healthy subjects [2]. Thus, tubular resistance to the natriuretic effect of loop diuretics has been proposed to explain the blunted response. The mechanism of this tubular resistance is unknown. A contributing mechanism for diuretic resistance in patients with nephrotic syndrome (NS) may relate to the fact that furosemide, which is highly bound to albumin in serum, may also bind to albumin in the urine. As the protein bound fraction of a drug is considered to be inactive, furosemide binding to urinary albumin would therefore decrease response by reducing the unbound (that is, active) drug concentration at its site of action in the thick ascending limb of Henle's loop (TALH). In support of this hypothesis, we have shown that the addition of albumin to tubule fluid perfusing Henle's loop markedly reduces the ability of furosemide to inhibit loop segment chloride reabsorption [3]. This effect appears to occur through a specific interaction between albumin and furosemide in that albumin alone has no effect on loop segment chloride transport, and other proteins such as IgG do not blunt furosemide's response. Although the results of that study are consistent with a reduction in concentration of free furosemide in tubule fluid, the study design did not allow for a direct examination of this putative mechanism. In an effort to more specifically study the influence of protein binding in renal tubule fluid on diuretic response, we conducted a series of experiments designed to displace furosemide from its albumin binding site. An improvement in diuretic response following these maneuvers would further support the correctness of our hypothesis and might also lead to future therapeutic strategies to improve diuretic responsiveness in this disorder.

In in vitro systems, albumin has been found to contain at least three specific and discrete binding sites for drugs [4]. The extent of a drug's binding to albumin can be modified by the presence of another xenobiotic or endogenous substance which competes for binding at the same site. We reasoned that if the attenuated response to furosemide observed in albumin containing perfusates is due to albumin-furosemide binding, then drugs that displace furosemide would thereby restore the diuretic's potency. In contrast, compounds binding at albumin sites that do not affect furosemide's binding should not alter the diuretic response. The current study tested this hypothesis using in vivo loop segment microperfusion techniques to ensure precise delivery of drugs and solutes to the loop segment.

Methods

Male Sprague-Dawley rats maintained on standard rat chow and ad libitum fluids were anesthetized with Inactin (100 mg/kg/body wt i.p.) and prepared for micropuncture. The specific preparative surgical procedures used in our laboratory have been described previously [3, 5]. Following a 40-minute surgical recovery period, in vivo loop segment microperfusion was performed over a 90-minute experimental period. Blood was sampled periodically for determination of inulin, sodium and chloride concentrations. A timed urine sample was collected for determination of inulin and sodium concentrations from the experimental kidney. At the conclusion of study, the kidneys were removed and weighed.

Received for publication October 15, 1990
and in revised form April 30, 1991
Accepted for publication May 1, 1991

The microperfusion technique was performed as follows: A pipette filled with FD&C green (Keystone Aniline and Chemical, Chicago, Illinois, USA) tinted nonradioactive perfusate was inserted into a random proximal tubule and a small bolus injected to identify both latest surface proximal and earliest surface distal segments of that nephron. The loop segment studied included a short terminal segment of a proximal convoluted tubule, the proximal straight tubule, the thin descending limb of Henle's loop, the TALH and a short segment of the distal convoluted tubule. A perfusion pipette attached to a microperfusion pump (WPI Instruments, New Haven, Connecticut, USA) calibrated previously in vitro for 20 nl/min was inserted into the latest accessible proximal tubule and perfusion of the nephron begun. The perfusate was an artificial tubule fluid-like (ATF) solution and contained (in millimolar) NaCl, 123; KCl, 5; $MgSO_4$, 1; $NaHCO_3$, 10; Na_2HPO_4, 1; $CaCl_2$, 1; urea, 4.2. Dialyzed (methoxy ^3H) inulin (New England Nuclear, Boston, Massachusetts, USA) was added to yield 100 to 150 cpm/nl. The pH was adjusted to 7.4 with 5% CO_2-95% O_2. Next a pipette was inserted proximal to the perfusion pipette and a large caster oil block introduced. The tubule segment proximal to the block was then vented and the glomerular filtration allowed to escape onto the kidney surface. Two to three minutes of perfusion were allowed to insure good flow. A collection pipette filled with Sudan black stained mineral oil was then inserted into the earliest surface distal tubule segment. An oil block was injected and a complete timed distal tubule fluid sample obtained.

Eleven groups of rats were studied. The first group (N = 8) served as furosemide controls. Loop segments in these animals were perfused with ATF to which 6.0 μM furosemide (Hoechst-Roussel Pharmaceuticals, Somerville, New Jersey, USA) had been added. This concentration of furosemide was chosen to approximate the concentration having a half maximal effect to inhibit chloride reabsorption (IC_{50}) as determined in the isolated perfused rabbit TALH [6]. In the second group of rats (N = 7) loop segments were perfused with the ATF furosemide perfusate to which 3.8 μM human serum albumin (fat free fraction V, Sigma Chemical Co., St. Louis, Missouri, USA) was added. The concentration of albumin was chosen to approximate the concentration measured at late proximal tubule puncture sites 142 to 144 hours after induction of aminonucleoside nephrosis in the rat [7]. In the third group of rats (N = 7) loop segments were perfused with the albumin-furosemide ATF containing 12.0 μM warfarin (Dupont Pharmaceuticals, Wilmington, Delaware, USA). Warfarin is the prototypic drug that binds to the same site on albumin as furosemide [4]. To exclude the possibility that warfarin itself potentiates furosemide response, loop segments in a fourth group of rats (N = 6) were perfused with ATF containing furosemide and warfarin but no albumin. A fifth group of rats (N = 5) were warfarin controls. Loop segments in these rats were perfused with ATF which contained only 12.0 μM warfarin. In a sixth group of rats (N = 7) loop segments were perfused with the furosemide-albumin perfusate containing 5.4 mM sulfisoxazole (Hoffman-LaRoche, Nutley, New Jersey, USA). Sulfisoxazole binds at the same site as furosemide on the albumin molecule [4]. Prior unpublished work by our laboratory indicated that displacement of furosemide from albumin by sulfisoxazole might occur with urine concentration of the drug that can be obtained clinically. Thus, sulfisoxazole was chosen for study because it might prove useful in future clinical tests of the role of intratubular binding of furosemide in man. As with warfarin, the possibility that sulfisoxazole itself could potentiate furosemide's response was examined in a seventh group of rats (N = 6) where loop segments were perfused with ATF containing furosemide and 5.4 mM sulfisoxazole but no albumin. In an eighth group of rats (N = 6) loop segments were perfused with the furosemide-albumin ATF to which 12 μM dansylsarcosine (Sigma Chemical Co.) had been added. Dansylsarcosine binds to the albumin molecule at a site separate from that which binds furosemide [4]. The concentrations of warfarin and dansylsarcosine employed were based on the finding that the binding constants for these drugs to their primary site on the albumin molecule are each less than 10 μM [4]. Dansylsarcosine, however, also binds at the furosemide site above concentrations of approximately 40 μM [4]. The concentration of sulfisoxazole chosen was based on our prior work and that by others in man showing urinary drug concentrations in the mM range following a standard size dose [8].

To provide additional evidence that albumin blunted furosemide's response by decreasing the unbound or pharmacologically active fraction of the drug, the effects of increasing the concentrations of furosemide and/or albumin on loop furosemide response were determined in three additional groups. In a ninth group of rats (N = 6) furosemide concentration was increased to 60 μM in ATF containing 3.8 μM albumin to determine if the presumed increase in free drug concentration would normalize diuretic response. In a tenth group of rats (N = 5) the albumin concentration was doubled to 7.6 μM in ATF containing 60 μM furosemide to see if diuretic response would again be blunted. Finally an eleventh group of rats (N = 6) were controls for the larger furosemide dose. Loop segments in these animals were perfused with ATF which contained only 60 μM furosemide.

Analytical techniques

The volume of tubule fluid collected from the distal tubule was measured in constant bore glass tubing with a slide comparator (Gaertner Scientific, Chicago, Illinois, USA). An aliquot of sample was then withdrawn and the chloride concentration determined using electrometric titration as previously described [5]. Perfusate chloride was also measured for direct comparison. The remaining volume was measured and the sample counted for 10 minutes in a liquid scintillation counter (Model LS7500, Beckman Instrument Co., Irvine, California, USA) to determine ^3H inulin radioactivity. Sodium concentration in serum and urine was measured by flame photometry (Instrumentation Laboratory, Model 943, Lexington, Massachusetts, USA). Inulin concentration in urine and plasma was determined as previously described [5].

Analysis of data

Determination of the concentrations of inulin and sodium in blood and urine and urinary flow rate permitted calculation of whole kidney glomerular filtration rate and urinary excretion rate of sodium according to standard expressions. In vivo perfusate rate (PR) was calculated as:

Table 1. Systemic and whole kidney variables in the experimental groups

	MAP *mm Hg*	C_{In} *μl/min/g kidney wt*	$U_{Na}V$ *nEq/min/g kidney wt*	FE_{Na} *%*	P_{Na} *mEq/liter*
6.0 μM Furosemide					
Furosemide ($N = 8$)	119 ± 3	1040 ± 74	374 ± 197	0.21 ± 0.09	153 ± 1
Furosemide-albumin ($N = 7$)	122 ± 3	986 ± 64	257 ± 115	0.16 ± 0.06	155 ± 2
Furosemide-albumin-warfarin ($N = 7$)	117 ± 3	968 ± 57	338 ± 88	0.24 ± 0.07	152 ± 2
Furosemide-warfarin ($N = 6$)	116 ± 3	940 ± 78	212 ± 119	0.15 ± 0.07	150 ± 2
Furosemide-albumin-sulfisoxazole ($N = 7$)	118 ± 2	1151 ± 95	449 ± 217	0.24 ± 0.11	154 ± 2
Furosemide-sulfisoxazole ($N = 6$)	123 ± 3	971 ± 66	233 ± 97	0.18 ± 0.09	150 ± 4
Furosemide-albumin-dansylsarcosine ($N = 6$)	122 ± 2	1128 ± 65	692 ± 359	0.40 ± 0.20	155 ± 3
60 μM Furosemide					
Furosemide ($N = 6$)	121 ± 2	1192 ± 102	113 ± 28	0.07 ± 0.02	155 ± 3
Furosemide-albumin ($N = 6$)	124 ± 2	1250 ± 63	148 ± 31	0.08 ± 0.02	157 ± 1
Furosemide-7.6 μM albumin ($N = 5$)	120 ± 2	998 ± 137	237 ± 116	0.13 ± 0.05	147 ± 1
Warfarin control ($N = 5$)	124 ± 4	1282 ± 211	420 ± 199	0.21 ± 0.10	152 ± 3

Values are means ± SE. Abbreviations are: Numbers in parenthesis, number of animals studied; MAP, mean arterial pressure; C_{In}, inulin clearance; $U_{Na}V$, absolute urinary sodium excretion; FE_{Na}, fractional sodium excretion; P_{Na}, plasma sodium concentration.

Table 2. Loop perfusion rate, absolute and fractional loop segment fluid reabsorption

	Calculated PR *nl/min*	$TF_{In}/Perf_{In}$	Absolute fluid reabsorption *nl/min*	Fractional fluid reabsorption
6.0 μM Furosemide				
Furosemide ($N = 8$)	19.6 ± 0.4	1.51 ± 0.04	6.47 ± 0.39	33.0 ± 2.2
Furosemide-albumin ($N = 7$)	19.2 ± 0.5	1.45 ± 0.05	6.00 ± 0.55	29.7 ± 2.2
Furosemide-albumin-warfarin ($N = 7$)	18.3 ± 0.5	1.41 ± 0.05	5.11 ± 0.43	27.1 ± 2.6
Furosemide-warfarin ($N = 6$)	20.9 ± 0.6	1.49 ± 0.05	6.50 ± 0.95	30.6 ± 3.4
Furosemide-albumin-sulfisoxazole ($N = 7$)	19.0 ± 0.4	1.48 ± 0.04	6.26 ± 0.45	32.0 ± 2.0
Furosemide-sulfisoxazole ($N = 6$)	19.2 ± 0.5	1.44 ± 0.04	5.77 ± 0.40	29.6 ± 1.8
Furosemide-albumin-dansylsarcosine ($N = 6$)	19.1 ± 0.9	1.60 ± 0.05	7.09 ± 0.62	36.8 ± 1.9
60 μM Furosemide				
Furosemide ($N = 6$)	19.1 ± 0.3	1.36 ± 0.03	4.71 ± 0.34	24.5 ± 2.3
Furosemide-albumin ($N = 6$)	19.6 ± 0.4	1.43 ± 0.05	5.75 ± 0.56	29.2 ± 2.8
Furosemide-7.6 μM albumin ($N = 5$)	20.1 ± 0.9	1.53 ± 0.04	6.79 ± 0.18	34.1 ± 1.7
Warfarin control ($N = 5$)	19.0 ± 0.9	1.49 ± 0.06	5.99 ± 0.63	31.4 ± 2.9

Values are means ± SE. Abbreviations are: PR, in vivo perfusion rate; $TF_{In}/Perf_{In}$, tubule fluid to perfusate inulin.

$$PR = CR \times \frac{cpm_2}{cpm_1} \text{ (nl/min)}$$

where CR is the distal collection rate, and cpm_1 and cpm_2 are the cpm/nl of ^3H inulin in the perfusate and collected fluid, respectively. Only perfusions having a calculated perfusion rate between 15 and 25 nl/min were accepted for analysis. Tubular response to furosemide was determined by calculating the absolute amount of chloride reabsorption (AR_{Cl}). This parameter was calculated as:

$$AR_{Cl} = (Perf_{Cl} \times PR) - (ED_{Cl} \times CR)$$

where $Perf_{Cl}$ and ED_{Cl} are chloride concentrations in the perfusate and fluid collected from the early distal tubule, respectively. Values are expressed as mean ± SE. The results from one to seven tubules (mean ± SE, 3.7 ± 0.5) were used for each rat and an "N" representing the number of rats (not tubules) was used to calculate group means.

Analysis of variance was used to determine statistical significance. If analysis of variance indicated that a statistical significance existed, Bonferroni's modification of the t-test was used to determine differences among groups. Statistical significance was set at the $P < 0.05$ level.

Results

Mean arterial pressure, inulin clearance, and absolute as well as fractional urinary sodium excretions were not different between any of the study groups (Table 1). Plasma sodium concentrations were also not different between groups.

Loop segment perfusion rate determined in vivo was not different between groups (Table 2). Neither absolute nor fractional loop segment fluid reabsorption was different between groups (Table 2). Thus, the respective solute and total furosemide deliveries to the loop segment were equivalent in all groups. In agreement with our previous studies, addition of 3.8 μM albumin to the perfusion solution markedly ($P < 0.05$) impaired the inhibitory effect of furosemide on loop chloride reabsorption (Fig. 1). Loop chloride reabsorption increased from 886 ± 65 pEq/min in segments perfused with ATF containing furosemide to 1248 ± 59 pEq/min in loop segments perfused with ATF containing furosemide and albumin (Fig. 1). The addition of warfarin to perfusate which contained albumin

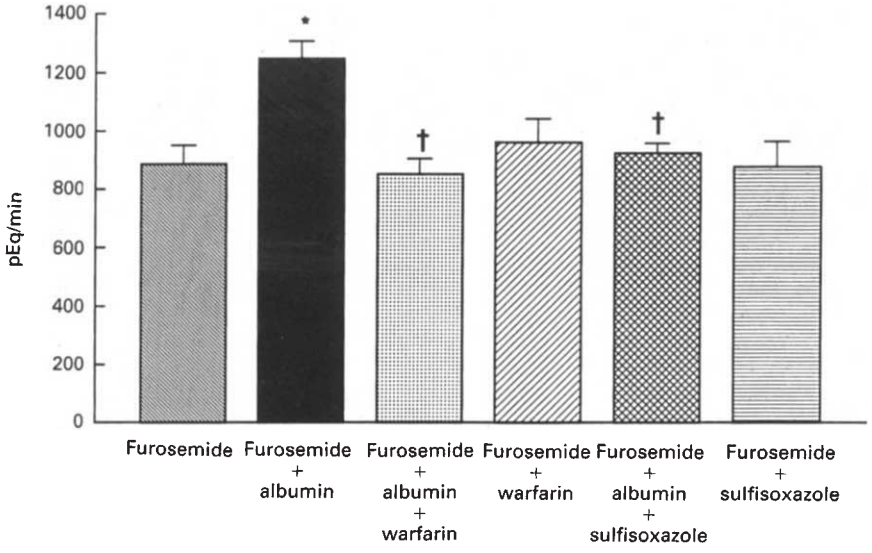

Fig. 1. *Absolute chloride reabsorption during loop segment perfusion with competitive inhibitors of furosemide-albumin binding.* * $P <$ 0.05 vs. furosemide; + $P < 0.05$ vs. furosemide and albumin.

and furosemide restored loop chloride reabsorption to 851 ± 54 pEq/min. This value was significantly less ($P < 0.05$) than the amount of chloride reabsorbed by loop segments perfused with albumin and furosemide, but not statistically different from the amount reabsorbed by loop segments perfused by furosemide alone. Of note, warfarin per se appeared to have no intrinsic effect on solute transport by the loop segment. Loop segment chloride reabsorption was 1684 ± 137 pEq/min during perfusion with ATF containing 12 μM warfarin alone. This reabsorptive rate is not different (P = NS by unpaired Student's t-test) from the reabsorptive rate of 1375 ± 86 pEq/min previously reported from our laboratory during perfusion of loop segments with ATF alone [5]. Furthermore, the addition of warfarin to perfusates which contain furosemide without albumin did not alter furosemide's effect on loop chloride reabsorption (986 ± 81 vs. 886 ± 65 pEq/min; P = NS). Thus, warfarin's inhibition of chloride transport by the loop segment occurred only in the presence of furosemide *and* albumin.

A pattern similar to the warfarin experiments was observed following addition of sulfisoxazole. This displacing agent also restored responsiveness to furosemide (Fig. 1). Thus, chloride reabsorption was significantly lower in loop segments perfused with ATF containing the combination of furosemide, sulfisoxazole and albumin compared to loop segments perfused with ATF containing furosemide and albumin (925 ± 34 vs. 1248 ± 59 pEq/min; $P < 0.05$) (Fig. 1). The addition of sulfisoxazole to perfusates which contain furosemide but no albumin did not increase furosemide's effect on loop chloride reabsorption (878 ± 87 vs. 886 ± 65 pEq/min; P = NS).

In contrast to the results obtained with warfarin and sulfisoxazole, addition of dansylsarcosine to ATF containing furosemide and albumin did not restore response to furosemide (Fig. 2). Thus, a compound that binds to albumin at a site different from that which binds furosemide did not alter the inhibitory effect of albumin on furosemide response.

Chloride concentrations determined in perfusates collected from the early distal tubule site were lower ($P < 0.05$) during loop perfusion with ATF containing furosemide and albumin than during loop perfusion with ATF containing furosemide

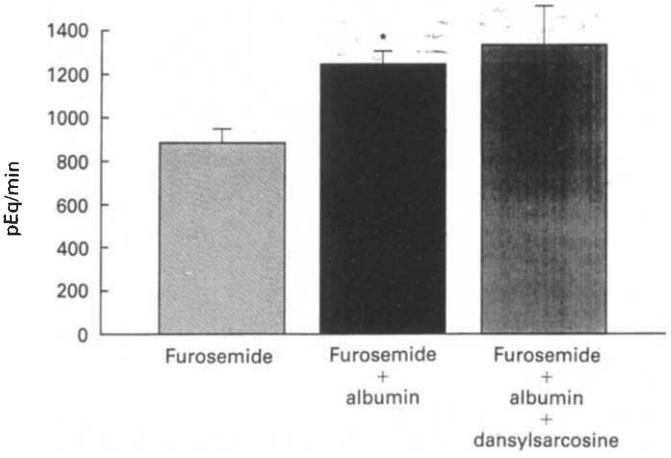

Fig. 2. *Absolute chloride reabsorption during loop segment perfusion with various combinations of furosemide, albumin and dansylsarcosine.* Dansylsarcosine binds to albumin at a site different from that binding furosemide. * $P < 0.05$ vs. furosemide.

alone (Fig. 3). Addition of either warfarin or sulfisoxazole to the furosemide-albumin perfusate restored early distal tubule chloride concentration to values not different from those measured during perfusion with furosemide alone, and significantly greater than values measured during perfusion with furosemide and albumin. The addition of warfarin or sulfisoxazole to ATF containing furosemide did not affect the early distal tubule chloride concentration. Early distal tubule chloride concentration was not different between loop segments perfused with furosemide and albumin and loop segments perfused with a combination of dansylsarcosine, furosemide and albumin (Fig. 4). However, both were significantly less then the group infused only with furosemide.

The addition of 3.8 μM albumin to ATF containing 60 μM furosemide had little effect on furosemide's ability to inhibit loop chloride reabsorption (Fig. 5). Loop chloride reabsorption was 655 ± 33 pEq/min in segments perfused with ATF containing 60 μM furosemide alone, and 784 ± 61 pEq/min in segments

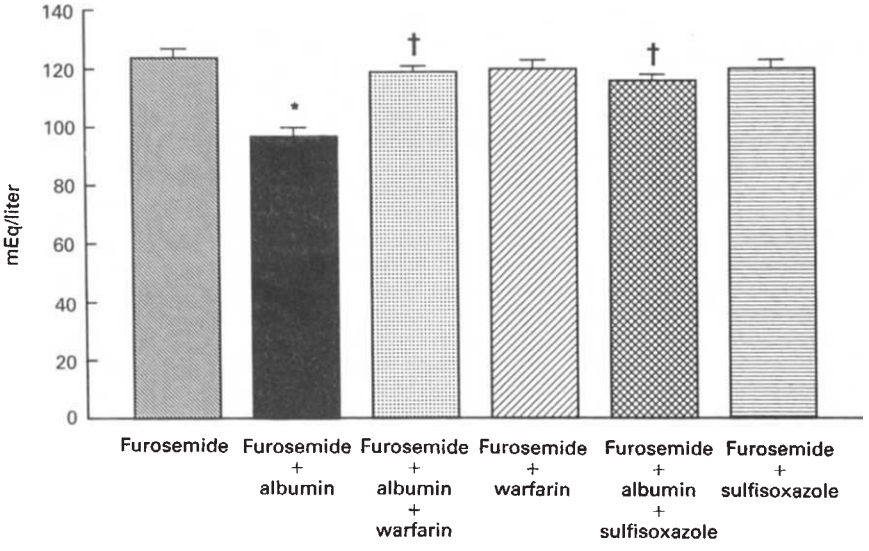

Fig. 3. *Tubular fluid chloride concentration at early distal tubule collection site during loop perfusion with competitive inhibitors of furosemide-albumin binding.* * $P < 0.05$ vs. furosemide, + $P < 0.05$ vs. furosemide and albumin.

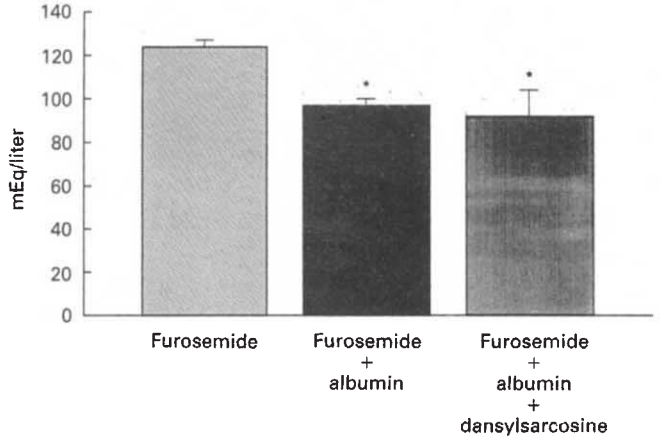

Fig. 4. *Tubular fluid chloride concentration at early distal tubule collection site during loop perfusion with various combinations of furosemide, albumin and dansylsarcosine.* Dansylsarcosine binds to albumin at a site different from that binding furosemide. * $P < 0.05$ vs. furosemide.

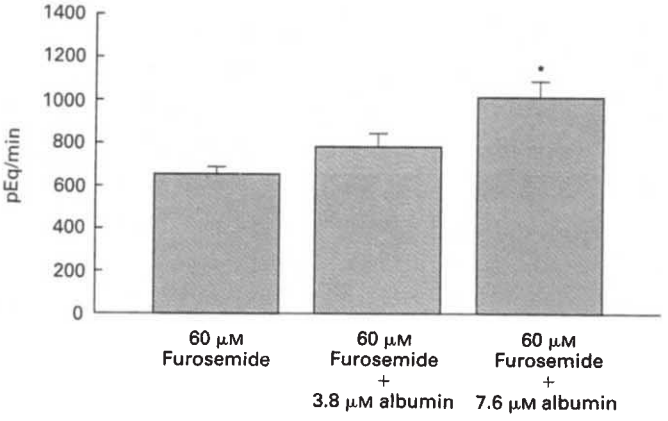

Fig. 5. *Absolute chloride reabsorption during loop segment perfusion with ATF containing 60 μM furosemide; 60 μM furosemide and 3.8 μM albumin; or 60 μM furosemide and 7.6 μM albumin.* * $P < 0.05$ vs. 60 μM furosemide.

perfused with ATF containing 60 μM furosemide and 3.8 μM albumin. Increasing the albumin concentration to 7.6 μM in the 60 μM furosemide containing perfusate, however, significantly impaired diuretic activity of 60 μM furosemide on loop chloride uptake. Loop chloride reabsorption was 655 ± 33 pEq/min in segments perfused with ATF containing 60 μM furosemide and 1015 ± 74 pEq/min in segments perfused with ATF containing 60 μM furosemide with 7.6 μM albumin ($P < 0.05$).

Discussion

The current study confirms that albumin in tubule fluid can impair the loop segment response to furosemide. This study additionally demonstrates that the extent of this impairment depends on the relative concentrations of both agents in the fluid entering the loop. Thus, a greater concentration of furosemide reduces the inhibition produced by a constant concentration of albumin. Conversely a greater concentration of albumin impairs the loop segment response to increased concentrations of furosemide. Furthermore, both warfarin and sulfisoxazole, drugs which competitively inhibit binding of furosemide to albumin in plasma, restore diuretic response in tubule fluid containing albumin. However, neither warfarin nor sulfisoxazole potentiates furosemide's tubular response in the absence of albumin and warfarin alone did not reduce loop chloride reabsorption. Thus, the most plausible explanation for warfarin's and sulfisoxazole's ability to restore furosemide response in tubule fluid containing albumin is that these agents displace furosemide from binding sites on albumin. This binding appears to be a specific interaction as dansylsarcosine, a compound which also binds to albumin but at a different site from furosemide, failed to restore furosemide response in tubule fluid containing albumin. Taken together, these data argue that the reduction in furosemide response in the presence of albumin is due to furosemide-albumin binding. The net effect

of this interaction is a decrease in the amount of pharmacologically active drug present at the diuretic effector site in the TALH.

A central principle in pharmacology is that binding of a drug to protein in serum may importantly influence pharmacokinetic and hence dynamic properties of the drug [9]. Furosemide, for example, is highly bound to albumin in plasma (>90%) and the extent of this binding has been demonstrated to influence the drug's volume of distribution, total clearance and possibly its ototoxicity [1, 2, 10, 11]. The current study indicates that protein binding can also influence drug activity when binding proteins gain access to biologic fluids at the drug's site of action. Thus, the presence of albumin in ATF at a concentration similar to that measured in the aminonucleoside induced rat model of NS markedly attenuates the effect of furosemide on loop chloride reabsorption. Four lines of evidence suggest this effect results from inactivation of furosemide by binding to albumin. First, the addition of albumin alone to ATF does not affect loop chloride reabsorption [3]. Secondly, IgG, a protein that does not bind furosemide, has no affect on furosemide's response [3]. Third, as indicated by the current observations, drugs that competitively inhibit the binding of furosemide to albumin but have no intrinsic chloruretic effect restore furosemide's response in albumin containing ATF. Finally, a compound which binds to albumin but does not affect the binding of furosemide fails to restore response.

The clinical significance of binding of furosemide to albumin in the urine of patients with NS has been controversial. Green and Mirkin first hypothesized this mechanism was responsible for diuretic resistance in the NS [12]. Employing the aminonucleoside rat model of NS they noted that the response to furosemide was independent of serum protein concentration or glomerular filtration rate and was inversely correlated with the degree of proteinuria. Their findings have been disputed by other investigators. From a comparison of sodium excretion to total or unbound furosemide excretion rates in voided urine, Keller and associates and Smith and coworkers concluded that furosemide binding to filtered albumin had little effect on the magnitude of the diuretic response and did not contribute significantly to diuretic resistance in nephrotic individuals [1, 2]. In these latter two studies, the conclusions were based on measurements of sodium concentration and furosemide-albumin binding determined in voided urine. However, marked alterations in sodium reabsorption occur beyond the TALH in NS [13] and changes in ionic strength within the range encountered along the distal nephron may alter the amount of furosemide bound to albumin [14]. Consequently, assessment of the contribution of protein binding to the blunted diuretic response observed in NS may be erroneous if based on data obtained in voided urine.

The methods used in the current studies circumvent some of the problems inherent in the prior animal and clinical investigations. The microperfusion technique allows delivery of equivalent concentrations of both diuretic and solute to the loop segment in all groups of animals studied. These experimental conditions, coupled with the observation that loop segment fluid reabsorption was not different between groups, suggest that the changes in chloride uptake observed in the current study resulted from events primarily localized to the TALH. Thus, the current study confirms that albumin in tubule fluid at concentrations representative of those found in an animal model of NS attenuates furosemide activity in the renal tubule. The extent to which this mechanism contributes to the diminished diuretic response observed in nephrotic patients remains to be determined.

Although our work to date has been confined to in vivo microperfusion experiments in rats, the findings that displacement of furosemide from albumin normalizes diuretic response suggest potential strategies to study furosemide-protein binding as a mechanism for diuretic resistance in man. In humans, competitive inhibitors of drug binding to albumin in plasma have been shown to influence the pharmacologic response to the drug. For example, administration of sulfonamide antibiotics and tolbutamide may result in hypoglycemic episodes through this mechanism [16, 17]. Similarly, kernicterus can occur in premature infants receiving sulfisoxazole due to displacement of albumin bound bilirubin [18]. The observation that both warfarin and sulfisoxazole restore furosemide response in albumin containing tubule fluid suggests that displacement binding can have pharmacologic consequences in biologic fluids other than plasma. It is interesting to speculate whether reducing urinary binding of furosemide to albumin might be a useful therapeutic adjunct in nephrotic patients. Obtaining such a benefit would presumably depend upon the urinary concentration of the inhibitor, and the relative affinities of the inhibitor and primary drug (that is, furosemide) for albumin. Thus, while micromolar concentrations of warfarin restore furosemide potency in microperfused tubules, it is unlikely to have an effect clinically, since negligible warfarin is excreted in the urine. Sulfisoxazole, on the other hand, requires millimolar concentrations to restore furosemide's effect in vitro, but enters the urine in high concentrations after conventional clinical doses. Thus, if a major portion of the attenuated diuretic response observed in NS results from furosemide binding to filtered albumin, then studies to restore furosemide potency through binding displacement with drugs such as sulfisoxazole seem warranted. In fact, such studies may be the only clinical means to quantitatively assess the contribution of binding to diuretic resistance in this setting, since attempts to extrapolate binding measured in final urine to the free drug concentrations present at the TALH are subject to the caveats previously discussed.

Although our data provide compelling evidence for protein binding contributing to diuretic resistance in NS, the results must be considered indirect. It would have been desirable to provide a quantitative assessment of the binding between furosemide and albumin in our early distal tubule collectate under the various conditions studied. Unfortunately, direct measurement of unbound furosemide concentration in fluid obtained from this collection site is not technically possible due to the combination of both small sample volumes and a lack of radiolabeled furosemide with sufficient specific radioactivity to conduct binding measurements. Thus, while all data from this and our previous studies [3, 14] strongly suggest that binding between albumin and furosemide accounts for the attenuated diuretic response observed in albumin containing perfusates and that restoration of this response by sulfisoxazole and warfarin is due to displacement of furosemide from albumin, the evidence is not yet incontrovertible. Definitive data is extremely difficult to obtain with existing techniques.

In summary, the current study demonstrates that competitive

inhibitors of furosemide-albumin binding restore the blunted response to furosemide observed in tubule fluid which contains albumin. In contrast, a substance which binds to albumin at a separate location does not restore furosemide response. These findings indicate that reductions in free furosemide concentration by binding to albumin in tubule fluid could be one mechanism accounting for the blunted diuretic and natriuretic response to loop diuretics in NS. Future clinical assessment of the contribution of this mechanism to overall diuretic resistance NS can employ the principles demonstrated herein.

Acknowledgments

Portions of these data were presented at the annual meeting of the American Federation for Clinical Research in Washington, DC, April 1990 and were published in abstract form in *Clinical Research* 38:417A, 1990. This work was supported in part by a grant from Kidney Care, Inc. The authors thank Ms. Bridget Crosby and Mrs. Tamara Walton for technical assistance and Ms. Tammy Blakeney for secretarial help.

Reprint requests to Kent A. Kirchner, M.D., University of Mississippi Medical Center, Department of Medicine, 2500 North State Street, Jackson, Mississippi 39216-4505, USA.

References

1. KELLER E, HOPPE-SEYLER G, SCHOLLMEYER P: Disposition and diuretic effect of furosemide in nephrotic syndrome. *Clin Pharmacol Ther* 32:442–449, 1982
2. SMITH DE, HYNECK ML, BERARDI RR, PORT FK: Urinary protein binding, kinetics and dynamics of furosemide in nephrotic patients. *J Pharm Sci* 74:603–607, 1985
3. KIRCHNER KA, VOELKER JR, BRATER DC: Intratubular albumin blunts the response to furosemide-A mechanism for diuretic resistance in the nephrotic syndrome. *J Pharmacol Exp Ther* 252:1097–1101, 1990
4. SJOHOLM I, EKMAN B, KOBER A, LJUNGSTEDT-PAHLMAN I, SEIVING B, SJODIN T: Binding of drugs to human serum albumin XI. The specificity of three binding sites as studied with albumin immobilized in microparticles. *Mol Pharmacol* 16:767–777, 1979
5. KIRCHNER KA: Prostaglandin inhibitors alter loop segment chloride uptake during furosemide diuresis. *Am J Physiol* 248(Renal Fluid Electrol Physiol 17):F698–F704, 1985
6. SCHLATTER E, GREGER R, WEIDTKE C: Effect of "high ceiling" diuretics on active salt transport in the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pflügers Arch* 396:210–217, 1983
7. LEWY JE, PESCE A: Micropuncture study of albumin transfer in aminonucleoside nephrosis in the rat. *Pediatr Res* 7:553–559, 1973
8. NELSON E, O'REILLY I: Kinetics of sulfisoxazole acetylation and excretion in humans. *J Pharmacol Exp Ther* 129:368–372, 1960
9. BENET LZ, SHEINER LB: Pharmacokinetics: The dynamics of drug absorption, distribution and elimination, in *Goodman and Gilman's, The Pharmacological Basis of Therapeutics* (7th ed), edited by AG GILMAN, LS GOODMAN, TW RALL, F MURAD, New York, McMillan Publishing Co., pp. 3–34
10. INOUE M, OKAJIMA K, ITOH K, ANDO Y, WATANABE N, YASAKA T, NAGASE S, MORINO Y: Mechanism of furosemide resistance in analbuminemic rats and hypoalbuminemic patients. *Kidney Int* 32:198–203, 1987
11. IKEDA K, MORIZONO T: Effect of albumin-bound furosemide on endocochlear potential of the chinchilla. *Arch Otolaryngol Head Neck Surg* 115:500–502, 1989
12. GREEN TP, MIRKIN BL: Resistance of proteinuric rats to furosemide: Urinary drug protein binding as a determinant of drug effect. *Life Sci* 26:623–630, 1980
13. ICHIKAWA I, RENNKE H, HOYER JR, BADR KF, SCHOR N, TROY JL, LECHENE CP, BRENNER BM: Role of intrarenal mechanisms in the impaired salt excretion of experimental nephrotic syndrome. *J Clin Invest* 71:91–103, 1983
14. VOELKER JR, JAMESON DM, BRATER DC: In vitro evidence that urine composition affects the fraction of active furosemide in nephrotic syndrome. *J Pharmacol Exp Ther* 250:772–778, 1989
15. GUTSCHE H-U, MULLER-OTT K, BRUNKHORST R, NIEDERMAYER W: Dose-related effects of furosemide, butmetanide and piretanide on the thick ascending limb function in the rat. *Can J Physiol Pharmacol* 61:159–165, 1983
16. BRODIE BB: Displacement of one drug by another from carrier or receptor sites. *Proc Roy Soc Med* 58:946–955, 1965
17. BUTTNER H, PORTWICH F: Kompetitionsphanomene bei der binding von pharmaka an albumin. *Klin Wochschr* 45:225–230, 1967
18. SILVERMAN WA, ANDERSON DH, BLANE WA, CROZIER DN: A difference in mortality rate and incidence of kernicterus among premature infants alloted to two prophylactic antibacterial regimens. *Pediatrics* 18:614–625, 1956