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Research Article

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Bumetanide Decreases Canine Cerebrospinal Fluid Production

In Vivo Evidence for NaCl Cotransport in the Central Nervous System

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Abstract

Na⁺/K⁺/2Cl⁻ cotransport carrier plays an important role in fluid absorption and secretion in many epithelial tissues. The role of the carrier, however, in mammalian choroidal cerebrospinal fluid (CSF) production has been controversial. We used ventriculo-cisternal perfusion (VCP) labeled with blue dextran with or without bumetanide and measured choroidal CSF production in anesthetized, and paralyzed, mechanically ventilated dogs. During 3 h of VCP, mean intracerebroventricular and arterial pressures, PaCO₂, pH, [HCO₃⁻], and serum osmolality remained normal in both groups ($n = 9$ in each group). Beginning 90 min after the start of VCP, choroidal CSF production was measured every 15 min. In group I (control group), values for CSF production (means±SD) were 49±20, 49±21, 51±21, 51±23, 48±20, 56±24, and 48±20 μ l/min, at 90, 105, 120, 135, 150, 165, and 180 min, respectively. These values did not differ significantly from each other. In group II (bumetanide group), after baseline control CSF production had been determined at 90 and 105 min, bumetanide (10^{-4} mol/liter) was added to VCP. Mean values for CSF production were 54±15 and 52±17 μ l/min before, and 39±25, 34±19, 28±10, 30±17, and 30±18 μ l/min after addition of bumetanide at 90, 105, 120, 135, 150, 165, and 180 min, respectively. Comparing the two groups, baseline values for CSF production measured at 90 and 105 min did not differ significantly. After addition of bumetanide (group II), however, decrements in CSF production varied from 30±27% at 120 min to 47±14% at 150 min, which were significantly different from changes in group I. The results of this study indicate that NaCl cotransport carrier is involved in secretion of CSF in dogs, and inhibition of the transporter results in ~ 50% reduction in CSF production. (*J. Clin. Invest.* 1993. 92:2257–2261.) Key words: cerebral edema • choroid plexus • hydrocephalus • ion transport • loop diuretics

Introduction

In the central nervous system (CNS),¹ cerebrospinal fluid (CSF) is continuously formed and reabsorbed at a slow rate. The choroid plexus (CP) located in the four cerebral ventricles

is thought to be the main site of CSF production (one third of CSF is produced by extrachoroidal tissue; for review, see references 1–5). A choroid plexus cell is shown diagrammatically in Figure 1. These specialized secretory cells are equipped with enzymatic machinery necessary for formation and secretion of CSF.

Chemical analysis of ionic composition of CSF shows that Na⁺ and Cl⁻ are the two ions present in the highest concentrations. Therefore, transport of Na⁺ and Cl⁻ across CP cells is of primary importance to CSF formation. As depicted in Fig. 1, the apical (CSF) side of the CP cell membrane contains the Na, K-ATPase pump (6), which results in active secretion of Na⁺ into CSF. Appropriate amounts of Cl⁻ and HCO₃⁻ follow secretion of Na⁺ into CSF to satisfy electroneutrality, and H₂O to satisfy isotonicity.

Continuous secretion of Na⁺ and Cl⁻ into CSF across the apical membrane of the CP cells requires continuous inward flux of the ions across the basolateral membrane (plasma side) (Fig. 1). There are several mechanisms which may mediate the transport of Na⁺ and Cl⁻ into CP cells across the basolateral membrane including the carrier which cotransports NaCl (Fig. 1).

Although NaCl cotransport carrier has not been isolated, its function has been well studied outside the CNS (7, 8), and it is known that the carrier plays a major role in NaCl cotransport and H₂O absorption in the ascending loop of Henle in the mammalian nephron. The loop diuretics, e.g., furosemide and bumetanide, combine with the carrier at the Cl⁻ site (9, 10) and consequently inhibit salt transport by the carrier.

The role of NaCl cotransport in mammalian CSF secretion, however, has been controversial (for review see reference 5). Investigators have used loop diuretics and measured CSF production (by various methods) to study the function of the carrier in the CNS (11–18). The interpretation of the results of these studies is subject to controversy because of the methodological reasons detailed elsewhere (5). Briefly, these include (a) the renal effect of the diuretics when given systemically resulting in volume depletion, (b) the carbonic anhydrase (CA) inhibitory activity of furosemide within CNS (a and b, per se, may decrease CSF production), (c) lack of measurements of serum and CSF concentrations of furosemide or bumetanide and their CA inhibitory activity, (d) lack of appropriate control, since with time CSF production usually decreases, and (e) lack of careful control of hemodynamics, ventilation, arterial blood acid-base balance, and electrolytes, which can affect CSF production.

With these considerations, the present study was designed to directly measure CSF production under carefully controlled standardized conditions in dogs. The results show that NaCl cotransport carrier mediates CSF production in this mammalian species, and inhibition of the carrier results in ~ 50% reduction in production rate.

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1. Abbreviations used in this paper: CA, carbonic anhydrase; CNS, central nervous system; CP, choroid plexus; CSF, cerebrospinal fluid; VCP, ventriculocisternal perfusion.

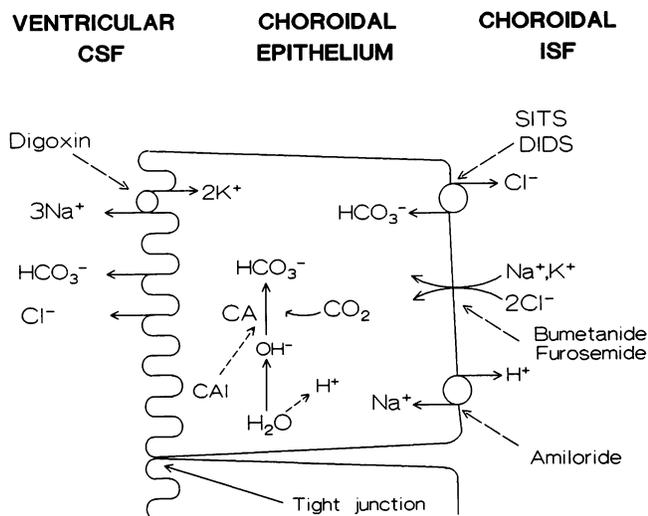


Figure 1. A simplified model of choroidal transepithelial ion transport for CSF production. Various antiporters and Na, K, 2Cl cotransporter with their inhibitors are shown. CAI, carbonic anhydrase inhibitor; SITS and DIDS are two disulfonic stilbene derivatives; ISF, interstitial fluid. For references to various transporters, see references 1–5.

Methods

General. Mongrel dogs (7.5–13.5 kg) were anesthetized with intravenous pentobarbital sodium (30 mg/kg intravenously, followed by 4.5 mg/kg every 1.5 h intramuscularly) and paralyzed with succinylcholine (2 mg/kg every 1.5 h intramuscularly). An endotracheal tube was placed and the animals were ventilated with a respirator (model 607, Harvard Apparatus, South Natick, MA), with room air enriched with oxygen to maintain P_{aO_2} between 100 and 150 mmHg. Respiratory rate was set at 12–14/min and tidal volume was adjusted to maintain normal arterial CO_2 tension (P_{aCO_2}) of about 35 mmHg. Tracheal PCO_2 was monitored by an infrared CO_2 analyzer (medical gas analyzer LB-2, Beckman Instruments, Inc., Fullerton, CA). Polyethylene catheters were placed in the femoral artery to monitor arterial pressure (Statham transducer) and in the femoral vein for injections. Rectal temperature was monitored (multipurpose thermometer, BAT 8, Bailey Instruments, Saddle Brook, NJ) and kept constant by a heating blanket at the initial dog's temperature.

Procedures for ventriculocisternal perfusion (VCP). The head of each animal was fixed in a stereotaxic instrument and the suboccipital cistern and the lateral cerebral ventricles were entered with 23-gauge spinal needles which were secured in the stereotaxic micromanipulators. Standard neurosurgical techniques were used for puncturing the lateral cerebral ventricles (19–21). The zero pressure was set with transducers placed at the level of metal ear bars of the stereotaxic device. A dual Harvard pump (model 555-2226, Harvard Apparatus) was used for perfusing the cerebral ventricles.

Intraventricular placement of the needles was confirmed by post-mortem examination at the end of the experiment. Data were discarded if the tips of both needles were not within the lateral ventricles.

Analyses of blood and cisternal and mock CSF. Arterial blood samples (3.0 ml) were obtained anaerobically in syringes (5.0 ml) whose dead spaces were filled with heparin solution. Arterial blood PO_2 , PCO_2 , and pH were measured by appropriate electrodes (Corning pH blood gas analyzer, model 158, Corning Medical Instruments, Medfield, MA) as detailed elsewhere (22, 23). Measurements of lactate, electrolytes, osmolality, ethanol, and blue dextran concentration were performed on centrifuged samples. Details for measurements of ions have been published previously (22, 23). Ethanol was measured by fluorescence inhibition technique (TDx FLx, Abbott Laboratories, USA, Abbott Park, IL) and osmolality by freezing point depression (Advanced Micro-Osmometer, Advanced Instruments, Inc., Needham Heights,

MA). The above variables were usually measured three times and the mean values were used.

Hematocrit was measured in a Readacrit Centrifuge (Clay Adams, Parsippany, NJ) initially and at the end of each experiment.

Technique of measurement of CSF production. CSF production was measured according to the VCP technique described originally by Pappenheimer and associates (19). Multiple factors have been shown to affect CSF production (20) and were considered in our methodology. Before VCP, a 0.4-ml sample of cisternal CSF was obtained for measurement of osmolality. Mock CSF of matching osmolality was prepared by mixing standard solutions (osmolality 260, 280, or 300 mosmol/kg) containing ethanol (Dehydrated 200 proof, Pharmco Products, Inc., Bayonne, NJ) and labeled with blue dextran (Sigma Chemical Co., St. Louis, MO) (1 g/liter mock CSF). Because blue dextran's molecular weight is 2×10^6 g, the final osmolality of CSF did not measurably change.

Bumetanide is barely soluble in aqueous solutions; therefore, 1.2 ml of ethanol was added to 1 liter of mock CSF to solubilize 40 mg of bumetanide (Hoffmann-LaRoche Inc., Nutley, NJ). This resulted in a bumetanide concentration of 10^{-4} mol/liter with a measured ethanol concentration of ~ 100 mg/dl.

The mock CSF had the following approximate concentrations for major ions: $[Na^+] = 150$, $[K^+] = 3$, $[Cl^-] = 133$, $[HCO_3^-] = 24$, $[lactate] = 2$, $[Ca^{++}] = 3$, and $[Mg^{++}] = 3$ meq/liter. Solutions were tonometered with CO_2 at $37^\circ C$ to $PCO_2 = 45$ Torr (the normal CSF PCO_2 in dogs in our laboratory).

In each animal, a heating lamp maintained the temperature of the syringes containing mock CSF at the rectal temperature of the animal.

Each sample of outflow perfusate was collected over a period of 5–7 min, and was centrifuged immediately for 12 min to remove debris. The measure of concentration of Blue dextran was its optical density which was measured spectrophotometrically (model 35, Beckman Instruments, Inc., Irvine, CA) at 610 nm. Blue dextran was measured three times, and usually two of the three measurements were similar; otherwise, the mean of the two closest values was used for calculations.

Cerebrospinal fluid production was calculated using the standard equation (19).

Experimental design. After completion of the surgery, all baseline samples were obtained and various pressures recorded (0 h). VCP started at the rate of 0.6 ml/min (through each ventricle) to enhance equilibration of blue dextran with cerebral extracellular fluids. After the first 45 min, VCP continued at 0.3 ml/min (through each ventricle) to the end of the experiment. In preliminary experiments we found that steady state was achieved within 45 min after 0.3 ml infusion (i.e., 90 min after 0 h; see results). At the 90th min, and every 15 min afterwards, outflow perfusate samples were collected for measurement of Blue dextran concentration.

In the control group (group I, $n = 9$), mock CSF did not contain bumetanide; in the experimental group (group II, $n = 9$), bumetanide (10^{-4} mol/liter) was added to the infusate after 105 min of VCP. Therefore, in addition to the control group, each animal in the experimental group served as its own control as well.

End-tidal PCO_2 was monitored and kept constant by fine adjustment of respiratory rate when necessary. Every half hour, arterial blood samples and other measurements similar to those obtained at 0 h were repeated.

Because anesthesia and time affect CSF production, in the present study, the time of induction of anesthesia, the total dose of anesthesia (mg/kg), and the time when VCP started were similar for all animals. The total duration of each experiment from beginning of VCP (0 h) to the end was three hours. This period was chosen because in preliminary experiments we determined that CSF production remained stable in this model.

Statistical analysis of the data. The statistical analysis system (SAS) of the University of Cincinnati was used for calculations. Data were expressed in means \pm SD. Analysis of variance with repeated measures along with t tests and Bonferroni correction factor were used to determine statistical significance among the mean values (24).

Table I. Mean Values (\pm SD) of Arterial and Intracranial Pressures and Arterial Blood Acid-Base Data in Groups I and II

Time min	MAP	ICP		Paco ₂	pH	[HCO ₃] meq/liter
		RCV mmHg	LCV mmHg			
Group I						
0	162 \pm 14	2 \pm 2	1 \pm 2	36 \pm 4	7.39	22 \pm 3
30	164 \pm 13	2 \pm 2	2 \pm 2	35 \pm 2	7.40	21 \pm 2
60	165 \pm 17	2 \pm 2	2 \pm 2	35 \pm 2	7.40	21 \pm 2
90	165 \pm 17	2 \pm 2	2 \pm 2	35 \pm 3	7.39	21 \pm 2
120	165 \pm 15	2 \pm 2	2 \pm 2	35 \pm 2	7.39	21 \pm 2
150	165 \pm 17	2 \pm 4	2 \pm 4	38 \pm 4	7.36	21 \pm 1
180	160 \pm 18	2 \pm 4	2 \pm 4	38 \pm 5	7.37	21 \pm 2
Group II						
0	158 \pm 15	4 \pm 4	3 \pm 3	35 \pm 3	7.42	23 \pm 2
30	165 \pm 16	4 \pm 5	3 \pm 3	36 \pm 1	7.42	23 \pm 2
60	168 \pm 15	2 \pm 3	1 \pm 2	35 \pm 2	7.40	22 \pm 3
90	168 \pm 11	1 \pm 2	1 \pm 3	36 \pm 3	7.40	22 \pm 2
120	169 \pm 15	2 \pm 3	1 \pm 2	36 \pm 2	7.38	21 \pm 3
150	169 \pm 17	3 \pm 5	2 \pm 4	35 \pm 2	7.38	21 \pm 2
180	166 \pm 20	2 \pm 3	1 \pm 3	38 \pm 3	7.36	21 \pm 2

For each variable, there were no significant inter- or intragroup differences among the mean values. Abbreviations: ICP, intracranial pressure; LCV and RCV, left and right cerebral ventricles, respectively; MAP, mean arterial pressure.

Results

The mean arterial, left and right cerebroventricular pressures, arterial PCO₂, pH, [HCO₃]⁻ (Table I), electrolytes, and hematocrit (Table II) did not differ significantly with time or when the two groups were compared.

In group I cisternal CSF production remained stable and did not change significantly during the period of observation. Values for CSF production were 49 \pm 20, 49 \pm 21, 51 \pm 21, 51 \pm 23, 48 \pm 20, 56 \pm 24 and 48 \pm 20 μ l/min, at 90, 105, 120, 135, 150, 165, and 180 min, respectively, after beginning of VCP. Respective values for group II were 54 \pm 15 and 52 \pm 17 before and 39 \pm 25, 34 \pm 19, 28 \pm 10, 30 \pm 17, and 30 \pm 18 μ l/min after addition of bumetanide to infusate. In group II, after addition of bumetanide, CSF production progressively decreased, with maximum inhibition occurring at 150 min ($P = 0.003$), about 45 min after infusion with bumetanide.

Comparing the two groups, values of baseline CSF production at 90 and 105 min (i.e., before addition of bumetanide in group II) were similar and did not differ significantly (see above). However, afterwards, the mean values were consistently lower in bumetanide treated dogs than in control animals and statistical significance was achieved at 150 and 165 min ($P = 0.02$).

Fig. 2 shows percent change in CSF production from baseline; the latter was calculated for each group, as the mean of all values at 90 and 105 min. In group I, these changes were not significant during the period of observation. In group II, however, the percent decrement in CSF production was significant within 15 min after addition of bumetanide and progressively increased with time. The maximum decrease in CSF production was $\sim 47\%$.

Discussion

The results of the present study are the first in vivo demonstration of a significant decrease ($\sim 47\%$) of directly measured CSF production under strict standardized conditions in the presence of an appropriate intracranial concentration of bumetanide. These results, therefore, are consistent with NaCl cotransport carrier playing an important role in CSF production in a mammalian species.

Previous studies on mammalian CSF production in the presence of loop diuretics have revealed controversial results primarily because of methodological reasons as briefly stated in the Introduction and detailed elsewhere (5). Among the major issues discussed was the use of furosemide in most studies of CSF production (11–18). In addition to inhibiting NaCl cotransport, furosemide at concentrations of 10⁻³ mol/liter, also inhibits CA (16) (Fig. 1) which, per se, may decrease CSF production (18). Bumetanide is up to 40 times more potent in inhibiting NaCl cotransport and only seven times more potent in inhibiting CA than furosemide (16, 18). Therefore, for inhibiting choroidal NaCl cotransport, bumetanide is superior to furosemide; yet systematic studies with bumetanide are lacking.

In two important negative studies (16, 18), Vogh and associates used bumetanide in a limited number of rats and rabbits and concluded that bumetanide had no effect on CSF production. In experiments with nephrectomized rabbits ($n = 3$), bumetanide (50 mg/kg) was dissolved in ethanol and injected into the peritoneum (16). Bumetanide is barely soluble in aqueous solutions and could have in part precipitated within the peritoneum, and, therefore, may not have been adequately

Table II. Initial and Final Values (Means \pm SD) of Arterial Blood Serum Electrolytes in Groups I and II

Time min	[Na ⁺]	[K ⁺]	[Cl ⁻]	[La]	Osmolality mosmol/kg	Hct %
	meq/liter					
Group I						
0	145 \pm 1	3.7 \pm 0.2	115 \pm 2	2.0 \pm 1.0	300 \pm 6	46 \pm 4
180	144 \pm 2	3.7 \pm 1.0	116 \pm 3	2.0 \pm 1.0	298 \pm 10	49 \pm 6
Group II						
0	145 \pm 1	3.6 \pm 0.3	115 \pm 1	2.0 \pm 1.0	307 \pm 9	43 \pm 8
180	144 \pm 3	3.4 \pm 0.4	112 \pm 2	3.0 \pm 1.0	304 \pm 7	52 \pm 6

For each variable, there were no significant inter- or intragroup differences among the mean values.

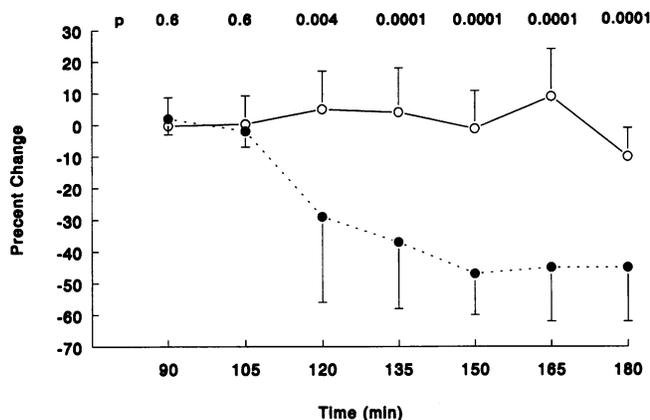


Figure 2. Percent changes in CSF production. In group I (○; control) changes (%) in CSF production from baseline were minimal. However, in group II, bumetanide (●), which was added to the ventricular infusate after baseline sample had been collected (at 105 min), resulted in considerable decrease in CSF production from baseline. *P* values show statistical significance when respective means between groups I and II were compared.

absorbed into the circulation. In the other study (18), Vogh and Doyle injected bumetanide at 150 mg/kg intravenously into a limited number of rats (the exact number of animals was not reported) and concluded that the influx of radioactive Na from plasma into CSF remained unaffected. Details of these limited experiments were not given and with bumetanide being insoluble in H₂O, it is unclear how it was dissolved. Furthermore, rat is known for considerable extrarenal metabolism of the drug (25, 26). Therefore, failure to see an effect may have been in part related to low concentration of bumetanide at the active sites (these authors did not report serum or CSF concentration of bumetanide in the two studies).

To our knowledge, there is only one study in which bumetanide was injected into the cerebral ventricles. Using VCP, Johnson et al. (27), showed that in anesthetized, mechanically ventilated dogs, bumetanide at a concentration of 10⁻⁵ mol/liter decreased the flux of the radioactive Cl⁻ from blood into CSF perfusate by about 50%. In the present study we directly measured canine CSF production, and bumetanide decreased CSF production by ~47%, the same amount as radioactive chloride entry.

Most recently, in a number of important *in vitro* studies (28–30) using rat isolated CP, Johanson and colleagues showed that choroidal cellular uptake of radioactive K⁺ (⁸⁶Rb as the marker), Na⁺ and Cl⁻ was diminished up to ~45–50% by pretreatment with bumetanide (10⁻⁵ to 10⁻⁴ mol/liter), indicating the presence of a bumetanide-inhibitible Na, K, 2Cl cotransporter. Johanson and colleagues (30) also showed up to 30% reduction of radioactive Cl⁻ flux from blood to CSF in rats pretreated with ethacrynic acid (50 mg/kg intravenously), another loop diuretic.

The results of the present study do not allow us to localize the exact sites of the cotransporter. However, *in vitro* studies of Johanson et al. (28, 29) using rats, and of Saito and Wright (31) using bullfrogs localize the cotransporter in CP cells. Studies of Betz (32) suggest that the cotransporter may also exist in cerebral capillaries. It is noted that Saito and Wright (31) localized the cotransporter at the basolateral membrane of bullfrog

CP cells, whereas, the experiments of Johanson et al. (30) on rat CP suggest an apical localization of the cotransporter.

In summary, data obtained in the present study along with the radioactive ion flux studies in dogs (27) and both *in vivo* (30) and *in vitro* CP studies (28, 29) in rats strongly indicate the presence of NaCl cotransport in mammalian CNS. Furthermore, the data from this study showing that bumetanide decreases CSF production, along with our previous data (33–35) showing that CSF ionic composition and CSF [Na⁺-Cl⁻] are not affected by bumetanide, indicate that NaCl cotransport is involved primarily in volume regulation of CSF. These findings are in contrast to ionic changes induced by loop diuretics in CP intracellular fluid (30). Lack of effect of bumetanide on CSF acid–base regulation is important, since ionic composition of cerebral fluids plays a major role in control of breathing and in neuronal activity.

It is to be hoped future research should result in isolation of the carrier and synthesis of new chemical compounds that preferentially decrease CSF production without having a major renal effect. Such compounds could be used to decrease cerebral edema which commonly accompanies various CNS pathological processes and impairs CNS functions.

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