Hemodynamic Effects of Bufalin in the Anesthetized Dog

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Studies in Lichstein's laboratory suggest that the endogenous digitalislike substance implicated in low renin hypertension might be a steroidal dienolide derivative. If this is true, the bufadienolides should block potassium vasodilation and enhance norepinephrine vasoconstriction, constrict blood vessels, raise blood pressure, and produce natriuresis and diuresis. We have therefore examined these parameters while infusing bufalin (aglycone), a bufadienolide, intrabrachially and intravenously in the anesthetized dog. Intrabrachial infusion of 5–25 μg/min with brachial arterial blood flow held constant at 100 ml/min produced a dose-dependent increase in perfusion pressure with rapid onset and offset, a progressive decrease in the vasodilator response to intrabrachial injection of 1 ml iso-osmotic potassium chloride solution (but not to acetylcholine), and an increase in the vasoconstrictor response to intrabrachial injection of 0.1 μg norepinephrine. Intravenous infusion at 5–50 μg/min produced a dose-dependent increase in systemic arterial blood pressure, rate of change of ventricular pressure (dP/dt), and after the highest dose, cardiac irregularities. Natriuresis and diuresis were not observed. Thus, bufalin does in fact have some of the physiological properties required to be considered a candidate for the digitalislike substance found in low renin hypertension. (Hypertension 1989;13:690–695)

Cardiotonic substances (bufadienolides) structurally related to the cardiac glycosides have long been known to exist in the poison glands of toads.1–3. High concentrations of digitalislike activity in the skin of several species of amphibia have been identified on the basis of a sensitive radioreceptor assay, inhibition of K+ transport, and inhibition of sodium-potassium adenosine triphosphatase (Na+K+-ATPase).4 Furthermore, serum from Bufo marinus has been shown to cross-react strongly in a digitalis immunoassay.5 Shimoni et al6 have partially purified a compound from toad skin that binds to the glycoside (ouabain) receptor, inhibits Na+,K+-ATPase activity, and increases the force of contraction of cardiac tissue. The compound is also present in toad plasma. The toad skin compound appears to be the steroidal dienolide derivative resibufagenin (1-hydroxy-14,15-epoxy-20,22-dienolide glycoside)7 (Figure 1). Tal et al8 found ouabainlike activity in bovine plasma, retained on columns for times almost identical with the retention of the toad skin ouabainlike compound,9 suggesting that the bovine plasma compound may also be a steroid resembling the structure of the toad skin compound. Recently, Brownlee et al10 have shown that bufalin (aglycone), a commercially available bufadienolide almost structurally identical to resibufagenin (Figure 1), is a potent inhibitor of canine renal Na+,K+-ATPase but, judging by studies in the rat, is only weakly natriuretic.

Some of these findings make the bufadienolides, like other steroids,1–11 candidates for the circulating endogenous digitalislike compounds implicated in the pathogenesis of low renin hypertension in experimental animals and in humans.12,13 If this is true, they should, like the cardiac glycosides, block potassium chloride (KCl) vasodilation,14 potentiate norepinephrine (NE) vasoconstriction,15 constrict blood vessels,16 and raise blood pressure.17–20 We have therefore examined the effects of bufalin on forelimb vascular resistance, KCl vasodilation, and NE vasoconstriction in the dog. We have also examined its effect on arterial pressure, rate of change of left ventricular pressure (dP/dt), and urinary sodium and water excretion.

Materials and Methods

Three groups of male dogs (average weight 22 kg) were used in these studies. All animals were fasted, anesthetized with pentobarbital, and intubated for...
mechanical ventilation. Blood gases were monitored using a Radiometer Model ABL3 (Radiometer Copenhagen, Copenhagen, Denmark) and were normalized through adjustments in respiratory rate and volume. Body temperature was maintained at 37°C with a Gaymar Solid State T-Pump (Gaymar Industries, Orchard Park, New York).

**Forelimb Study**

In the first series of experiments \((n=7)\), a forelimb preparation was used that maintains major veins and nerves intact. The skin of the right forelimb was sectioned circumferentially approximately 3-5 cm above the elbow. The right brachial artery was dissected free, and tourniquets were applied to skin and muscles to constrict collateral blood vessels.

Heparin \((10 \text{ mg/kg})\) was administered intravenously, a pump (pressure independent) was interposed between the femoral artery and brachial artery (Cole-Parmer Pump model 7520-20, Barnant Co., Barrington, Illinois), and arterial blood was shunted to the right forelimb at a constant flow rate of 100 ml/min. A side branch of the brachial artery was cannulated for measurement of brachial artery perfusion pressure (PP), and aortic blood pressure was measured via the proximal end of the brachial artery. Pressures were measured with low volume-displacement Statham transducers (Gould-Statham, Oxnard, California) and recorded on a Hewlett-Packard direct-writing oscillograph (model 7758B, Hewlett-Packard, Waltham, Massachusetts).

An equilibration period of at least 20 minutes was allowed before the experimental protocol. Bolus intrabrachial injections of bufalin (Sigma Chemical Company, St. Louis, Missouri) in an iso-osmotic solution of 10% EtOH and 90% saline \((0.1, 0.2, 0.4, \text{ and } 0.8 \text{ ml, } 20 \mu\text{g/ml})\) and the vehicle were given while recording PP. The maximal increase in PP (peak response, mm Hg), duration of the response (duration, seconds), and area below the PP response curve (area, square millimeters chart paper) were calculated.

After the transient responses to bolus injections of bufalin were complete, intrabrachial bolus doses of isotonic KCl \((1 \text{ ml})\), acetylcholine chloride (Ach) \((0.2 \text{ ml, } 1 \mu\text{g/ml})\), and NE \((0.05 \text{ ml, } 1 \mu\text{g/ml})\) were then injected while PP was recorded. After the response to the NE bolus subsided \((2-3 \text{ minutes})\), bufalin \((20 \mu\text{g/ml in } 10\% \text{ EtOH+90\% saline})\) was infused intrabrachially at three different rates \((0.247, 0.494, \text{ and } 1.23 \text{ ml/min})\) for approximately 5 minutes each. When PP reached a steady state with each infusion rate, the bolus injection of isotonic KCl was repeated. At the highest infusion rate of bufalin, Ach and NE were also injected. Ten minutes after the bufalin was discontinued, all three bolus injections were again repeated.

Twenty minutes after the bufalin infusion was discontinued, the vasoactive potency of the vehicle \((10\% \text{ EtOH+90\% saline})\) was tested by infusing it at the highest volume rate \((1.23 \text{ ml/min})\).

**Intravenous Administration**

A second group of animals \((n=7)\) was used to test the hemodynamic and urinary responses to intravenous infusion of bufalin. Systolic, diastolic, and mean aortic pressures, left ventricular pressure, and the first derivative of left ventricular pressure

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**Figure 1. Diagram of chemical structure of bufalin, resibufagenin, and ouabagenin.**
Bufalin and fig below the response with 16 jtg (0.8 ml) bufalin was produced stepwise increases in PP (area and peak duration). For example, the area difference from control. Of p<0.05 were considered to indicate a significant difference from control.

All animals were allowed to equilibrate for a minimum of 30 minutes until three consecutive 10-minute urine samples were equal in volume. Intravenous infusion of bufalin was then begun. Four 20-minute infusion periods were each alternated with a 20-minute postinfusion control period. Bufalin was infused in the following steps: 5 μg/min (20 μg/ml solution in 10% EtOH), 10 μg/min (40 μg/ml), 25 μg/min (100 μg/ml), and 50 μg/min (100 μg/ml). All parameters were measured at the end of each bufalin infusion and postinfusion control period.

Another group of dogs (n=7) were prepared in the same way as those described above with one addition. In these animals, the left renal artery was exposed retroperitoneally to allow placement of a flow probe for measurement of renal artery blood flow using an Electromagnetic Flowmeter (model 501D, Carolina Medical Electronics, Inc., King, North Carolina). Renal arterial blood flow was measured at the end of each 20-minute infusion and postinfusion control period. At the end of the experiment, electronically calculated renal blood flow was converted to actual blood flow by calibrating the flowmeter using a pump-perfused renal artery in situ. In all experiments, the relation between electronically derived blood flow and actual blood flow was linear, with correlation coefficients greater than 0.96.

Statistical Procedure

In all series, the data were evaluated statistically with both the paired Student's t test and the analysis of variance with repeated measures design (using the Duncan procedure as the post hoc test). For each parameter, using either statistical test, the mean value during the experimental period was compared with its respective control period. Values of p≤0.05 were considered to indicate a significant difference from control.

Results

Forelimb Study

Bolus injections of 2, 4, 8, and 16 μg bufalin produced stepwise increases in PP (area and peak response but not duration). For example, the area below the response with 16 μg (0.8 ml) bufalin was 5.7 times greater than with 2 μg (0.1 ml) bufalin and 6.2 times greater than with 0.8 ml of the vehicle.

Intrabrachial infusion of bufalin over the range 5-25 μg/min produced a dose-dependent increase in PP (Figure 2, top) and hence vascular resistance. The onset of the response was rapid and the response reached a steady state within 4 minutes of starting the infusion. The rate of PP return to control was also rapid; when the bufalin infusion was discontinued, vascular resistance returned toward control within 1 minute. The drug vehicle (10% EtOH in saline) was not responsible for a significant portion of this vasoconstrictor response; infusion of the vehicle at the same volume infusion rate used to deliver bufalin at 25 μg/min (1.23 ml/min) produced only one fifth of the increase in PP. During and after the intrabrachial infusion of bufalin, heart rate (not shown) and mean arterial blood pressure were unchanged from control.

The KCl vasodilator response (area above the PP response curve) decreased significantly after 4 minutes of drug infusion at 5 μg/min. The response decreased further with the higher rates of bufalin infusion so that at the highest rate the response was eliminated. In contrast, the vasodilator response to Ach increased during infusion of bufalin at 25 μg/min, as did the vasoconstrictor response to NE (Figure 2, bottom). Ten minutes after discontinuation of the infusion, the KCl and Ach vasodilation responses returned to control levels, whereas the NE constrictor response remained somewhat elevated. Both the duration of the response and the peak change in PP were altered in a similar manner to the area under the curve with all agents injected. Thus local administration of bufalin produced vasconstriction in the dog forelimb, and this was accompanied by decreased KCl vasodilation and increased NE vasoconstriction, as occurs during infusion of ouabain.14,15

Intravenous Administration

Intravenous infusion of bufalin at 5 μg/min was without effect (Figure 3). Infusion at 10 μg/min increased aortic and left ventricular systolic pressure and left ventricular dP/dt. Soon thereafter, mean and diastolic aortic pressures also increased significantly. These parameters remained elevated throughout the remainder of the experiment. Ventricular tachycardia and ST segment elevation occurred in three of the seven dogs toward the end of the 50-μg/min infusion period. These changes also occurred in the remaining four animals shortly after stopping this infusion. Heart rate was significantly elevated before and after the 50-μg/min infusion. Blood pressure and dP/dt returned toward control levels after stopping the infusion (Figure 3).

Bufalin did not cause significant diuresis or natriuresis at any time during the experiment. Urine flow, urine osmolality (not shown), and sodium, potassium, and chloride (not shown) excretion were unchanged during the experimental protocol. To explore the possibility that the absence of diuresis and natriuresis resulted from disproportionate renal...
vasoconstriction, bufalin was administered in the same manner to another series of dogs while left renal arterial blood flow was measured. Control renal blood flow was 170 ml/min and did not change significantly with any infusion rate of bufalin or after the infusion was discontinued.

Discussion

These studies show that, among other things, bufalin constricts the forelimb vascular bed and raises arterial blood pressure in the anesthetized dog. Bufalin is the fifth Na⁺,K⁺-ATPase inhibitor we have encountered with these properties (the others are ouabain,16 methylguanidine,21 vanadate,22 and hypokalemia23-25). Bufalin is of special interest because it belongs to a class of steroids with ouabain-like activity of animal rather than plant origin and it is closely related structurally to resibufagenin, a compound recently identified in toad skin and plasma and suggested to be a physiological regulator of Na⁺,K⁺-ATPase.7 Figure 1 shows that the bufadienolides bufalin and resibufagenin are structurally similar to the cardenolide ouabagenin and that bufalin differs from resibufagenin only in one H⁺. Bufalin administered into the arterial blood perfusion line of the forelimb produced dose-dependent vasoconstriction and suppression of KCl vasodilation. KCl vasodilation results from stimulation of Na⁺,K⁺-ATPase activity in the sarcolemma of the vascular smooth muscle cell.14 Ach vasodilation and NE vasoconstriction also increased. This combination of changes also occurs during intrabrachial infusion of ouabain.14-16 The decreased KCl and increased NE responses would be expected if the Na⁺,K⁺-ATPase were specifically inhibited, whereas the increase in the Ach response is the type of nonspecific change in the response to vasodilators that results from an increase in initial resistance. The vasoconstriction and suppression of KCl vasodilation occurs more quickly with bufalin than with ouabain; bufalin vasoconstriction reaches a plateau in about 3 minutes (Figure 2) whereas it takes approximately 30 minutes for ouabain vasoconstriction (6.4 μg/min) to reach its plateau.16 The bufalin response also wanes very quickly when the infusion is discontinued; PP decreased substantially within 1 minute (Figure 2, top).
Bufalin inhibits purified Na⁺,K⁺-ATPase to the same extent as ouabain.¹⁰ The mechanism of bufalin vasoconstriction is therefore likely to be the same as that seen with ouabain or hypokalemia.¹⁴ Inhibition of Na⁺,K⁺-ATPase reduces active Na⁺ and K⁺ transport. Since this transport is electrogenic in vascular smooth muscle, bufalin, by blocking active Na⁺ and K⁺ transport, could lead to membrane depolarization, increased Ca²⁺ influx through voltage sensitive Ca²⁺ channels, increased intracellular free calcium concentration ([Ca²⁺]), and hence contraction of the smooth muscle cell. Alternatively or simultaneously, the increased intracellular sodium concentration ([Na⁺]) could lead to increased intracellular free [Ca²⁺] via the Na⁺-Ca²⁺ exchange mechanism.¹³ It is also possible that bufalin could constrict in part by influencing Na⁺,K⁺-ATPase-dependent NE transport in the sympathetic nerve terminal and hence its concentration in the neuromuscular cleft.²⁶,²⁷

Intravenous administration of bufalin increased left ventricular systolic pressure, left ventricular dP/dt, and aortic pressure, upon which were superimposed cardiac arrhythmias with the higher doses. These changes are also seen during intravenous infusion of cardiac glycosides. In normal conscious dogs and humans,¹⁷-²⁰ the increase in blood pressure during infusion of the cardiac glycosides results from an increase in total peripheral resistance; cardiac output does not increase. We have not compared the pressor effect of bufalin with that of ouabain in the anesthetized dog, but, in the anesthetized rat, bufalin raises blood pressure more than ouabain.²⁸

Diuresis and natriuresis would be expected with an agent that inhibits Na⁺,K⁺-ATPase and raises blood pressure. However, these changes were seen in only three of seven animals on stopping the bufalin infusion, and mean urine flow and sodium excretion during and after bufalin infusion were not significantly different from those during the control period. We considered the possibility that diuresis and natriuresis were prevented by a decrease in renal blood flow, as occurs during intravenous vanadate in the anesthetized dog,²² but renal blood flow did not change during intravenous infusion of bufalin. The lack of diuresis and natriuresis in the dog is puzzling, especially since it has been observed in the rat. Brownlee et al¹⁰ reported mild postinfusion natriuresis in conscious sedated rats, and Pannani et al²⁸ observed massive diuresis and natriure-
sis in the anesthetized rat. The mechanism of this species difference awaits additional study.

Bufalin administration blocks potassium vasodilation, potentiates NE vasoconstriction, increases vascular resistance when administered locally, and results in hemodynamic changes characteristic of the cardiac glycosides and other Na⁺,K⁺-ATPase inhibitors when given intravenously. Thus bufalin has some of the properties required to be considered a candidate for the circulating endogenous digitalislike compound seen in low renin hypertension.

References

Key Words • cardiac glycosides • bufadienolides • sodium-potassium ATPase • ouabainlike compounds
Hemodynamic effects of bufalin in the anesthetized dog.
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Hypertension. 1989;13:690-695
doi: 10.1161/01.HYP.13.6.690

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1989 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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