Inhibition of Erythrocyte Na,K-ATPase Activity During Anticipatory Hypoventilation in Micropigs

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Previous studies with micropigs showed that conditioned suppression of respiration preceding the onset of an avoidance task was associated with increased pCO₂, decreased plasma pH, decreased hematocrit, and increased blood pressure with no change in heart rate. Voluntary hypoventilation by humans, which evoked similar effects, was found to elicit increases in plasma endogenous digitalis-like factors (EDLF) and decreases in erythrocyte Na,K-ATPase. The present study investigated plasma EDLF and Na,K-ATPase activity in micropigs preceding and during avoidance sessions. Compared with levels in a controlled environment, 1 h of quiet waiting for the onset of a 30-min avoidance task was associated with hypoventilation, acidification of the plasma, and a decrease in hematocrit with progressive increases in plasma EDLF, and decreases in erythrocyte Na,K-ATPase activity (1.67 ± 0.35 vs 2.73 ± 0.24 μmol Pi/mL er/h). Systolic blood pressure increased (126.5 ± 5.7 vs 121.7 ± 4.2 mm Hg) during preavoidance periods, with no changes in heart rate (89.5 ± 3.9 vs 89.4 ± 4.0 beats/min). During the avoidance sessions, plasma EDLF, systolic blood pressure (126.7 ± 4.5 mm Hg), and heart rate (107.3 ± 4.8 beats/min) were elevated above the first 10 min of preavoidance, whereas Na,K-ATPase activity returned toward control values (2.46 ± 0.83 μmol Pi/mL er/h). These findings are consistent with the view that elevation of blood pressure during behaviorally induced hypoventilation in micropigs is mediated in part by inhibition of Na,K-ATPase by increases in plasma EDLF due to expanded plasma volume.

The findings that endogenous digitalis-like factors (EDLF) or inhibitors of Na,K-ATPase are increased in clinical and experimental hypertension suggest that the Na,K pump may be involved in the elevation of blood pressure. Plasma EDLF concentrations are known to increase and Na,K-ATPase activity to decrease acutely during saline infusion procedures that expand plasma volume. Saline infusion alone does not normally produce hypertension unless renal functions have been compromised. However, saline infusion for 14 days did produce experimental hypertension in laboratory dogs on avoidance schedules. The development of hypertension in saline-infused animals on avoidance schedules was not prevented by sustained adrenergic blockade. Significantly, however, the hypertension developed only in animals showing conditioned respiratory suppression during the intervals between avoidance sessions.

More recent research with micropigs has shown that conditioned suppression of respiration preceding the
onset of a well-learned avoidance task is accompanied by sustained increases in pCO₂, decreases in plasma pH, and decreases in hematocrit during preavoidance periods, indicating possible expansion of plasma volume under these conditions. In this regard, studies with humans have shown that voluntary increases in end-tidal CO₂ for 30 min was associated with decreases in plasma pH, decreases in renal excretion of Na and fluid volume, increases in plasma EDLF, decreases in erythrocyte Na,K-ATPase activity, and increases in blood pressure but not in heart rate.

The purpose of the present experiment was to determine whether respiratory suppression in micropigs during 60 min preceding the onset of a well-learned avoidance task was also associated with increases in plasma EDLF and decreases in erythrocyte Na,K-ATPase activity, and accompanying increased blood pressure during preavoidance and avoidance periods.

METHODS

Subjects and Avoidance Conditioning Procedures
Each of six adult 15 to 25 kg micropigs (Charles River, Boston, MA) was studied for 1.5 h/day under partial restraint in a sound-attenuated chamber. During the first 60 min of each session, no behavior was required (ie, preavoidance period). During the last 30 min, an avoidance contingency was installed (AST Premium 286, AST Research Inc., Ardsley, CA). The avoidance schedule required the micropig to press a response panel on the front chamber wall to prevent the occurrence of a constant voltage stimulus (2 to 5 mA for 0.5 sec), applied through electrodes taped to the rump. Each response reset a recycling 20-sec timer to postpone the occurrence of shock for another 20 sec. Through training over several weeks, each micropig learned to lay quietly in the chamber during the preavoidance period, then stand up when the ceiling light went off, and engage in panel pressing at rates averaging 10 responses per minute, thereby avoiding the occurrence of 98% of the potential shocks.

Cardiovascular Monitoring and Experimental Protocol
After acquisition of stable avoidance performance, a Tygon catheter was implanted aseptically into the aorta. During experimental sessions, blood pressure and heart rate were monitored through a pressure mini-transducer connected to a computer (model 466D2, Hyundai, Litchfield, CT). Patency of the catheter was maintained during experimental sessions by slow infusion of heparinized saline (0.42 mL of saline/min for 90 min) through a peristaltic pump (model 607, Harvard Apparatus, Natick, MA).

Previous studies showed that these behavioral procedures produce a typical cardiovascular response in well-trained animals, characterized by progressive increase in total peripheral resistance and blood pressure but not heart rate or cardiac output during preavoidance intervals, followed by sustained increases in blood pressure mediated by increases in heart rate and cardiac output during avoidance sessions.

Cardiovascular and Metabolic Measures
Systolic and diastolic blood pressure and heart rate were averaged over successive 10-min intervals of each session in a computer system (Acquire Plus, Kent Scientific Co., Litchfield, CT). Ten-milliliter blood samples were obtained remotely during the first 10 min of preavoidance, during the last 10 min of preavoidance, and during the middle of the avoidance performance interval. A control blood sample was also obtained in the home kennel, typically 2 h before the experimental session. This procedure was adopted after it had been determined with two untrained micropigs that no systematic variations in Na,K-ATPase activity occurred as a function of the time of day between 9 AM and 5 PM. Plasma pO₂, pCO₂, pH, bicarbonate, Na and K concentrations, and hematocrit were measured immediately in a blood gas analyzer (model 288, CIBA Corning Diagnostics, Norwood, MA).

Erythrocyte Na,K-ATPase Activity, Plasma EDLF and Cortisol
Blood samples were immediately centrifuged for 10 min at 300 g. Plasma was stored at -80°C for analysis of digoxin-like, ouabain-like, and marinobufagenin-like immunoreactivity and cortisol measurement. Activity of Na,K-ATPase in the intact erythrocytes was measured immediately and determined as the difference between total ATPase activity and ouabain-insensitive ATPase activity, as described in detail elsewhere. The production of inorganic phosphate (Pi) from ATP under each condition was measured spectrophotometrically (model DU-65, Beckman, Chicago, IL) and activity of Na,K-ATPase was expressed as micromoles of inorganic phosphate per milliliter of erythrocyte per hour.

The plasma was extracted with 80% acetonitrile on C-18 reverse-phase columns (Sep-Pak, Millipore Corp., Milford, MA). Measurement of digoxin-like, marinobufagenin-like, and ouabain-like immunoreactivity was based on the competition between immobilized conjugated antibodies and a sample of EDLF for rabbit antidigoxin, antimarainobufagenin, or antiouabain polyclonal antibody as described in Bagrov et al. Cortisol in the plasma was measured with a commercial kit (Wallac, Gaithersburg, MD), based on the competition of the cortisol in the samples with immobilized cortisol for the europium-labeled polyclonal antibody. Plasma EDLF and cortisol were expressed in nanomoles per liter.
Data Analysis  Means of systolic and diastolic blood pressures, heart rate, $\text{pO}_2$, $\text{pCO}_2$, plasma pH and bicarbonate ion concentrations, plasma Na and K, hematocrit, cortisol, digoxin-like, marinobufagenin-like, ouabain-like immunoreactivity, erythrocyte Na,K-ATPase activity were determined for each micropig during the first and last 10 min of preavoidance and during avoidance. Measures from blood samples obtained in the home kennel were also analyzed. Data were available from 13, 8, 3, 3, 2, and 1 sessions for each pig, respectively (n = 30). Inspection of the data indicated no systematic changes in any measure across sessions.

The significance of the differences in systolic and diastolic pressure and heart rate between the first 10 min of preavoidance and last 10 min of preavoidance or the avoidance sessions were determined by one-tailed t tests for difference scores.

The significance of the differences between home kennel control, the first and last 10 min of preavoidance, and the avoidance sessions for each blood sample measure was determined with the analysis of variance and Newman Keuls multiple comparison tests.

RESULTS

Cardiovascular Responses  Mean systolic blood pressure during the last 10 min of preavoidance (126.5 ± 5.7 mm Hg) and during the avoidance sessions (126.7 ± 4.5 mm Hg) were increased above the mean for the first 10 min of preavoidance (121.7 ± 4.2 mm Hg; P < .01). Mean diastolic blood pressure during the last 10 min of preavoidance (86.8 ± 5.0 mm Hg) and during avoidance (86.5 ± 3.4 m Hg) were not significantly increased above the level at the first 10 min of preavoidance (83.9 ± 3.9 mm Hg).

Mean heart rate during the last 10 min of preavoidance was not significantly different from mean heart rate during the first 10 min of preavoidance (89.5 ± 3.9 vs 89.4 ± 4.0 beats/min). Mean heart rate during the avoidance sessions (107.3 ± 4.3 beats/min) was increased substantially above preavoidance intervals (P < .01).

Blood Gases, Plasma pH, Bicarbonate, Na, and K During Preavoidance and Avoidance  Table 1 shows means and standard errors of $\text{pO}_2$, plasma pH, bicarbonate, Na and K concentrations under control conditions, during the first and last 10 min of preavoidance, and during the avoidance sessions. Mean $\text{pO}_2$ showed no significant differences between conditions. Figure 1 shows that mean $\text{pCO}_2$ was increased above control levels during both preavoidance intervals and during the avoidance sessions (P < .02). Mean plasma pH was lower during the first 10 min of avoidance than during control conditions and during the avoidance sessions (P < .02). Mean plasma bicarbonate concentrations were higher during both preavoidance periods and during the avoidance sessions than under control conditions (P < .01). No significant differences in mean plasma Na concentrations were observed between conditions. Mean plasma K concentrations were lower during the first 10 min of preavoidance than under control conditions, but increased during late preavoidance to levels that were not significantly different from control. Plasma K returned to control levels during avoidance sessions (P < .005).

Hematocrit, EDLFs, Cortisol, and Erythrocyte Na,K-ATPase Activity During Preavoidance and Avoidance  Figure 1 shows that mean hematocrit was decreased below control levels during the first 10 min (P < .01) and last 10 min (P < .01) of the preavoidance periods, and increased during avoidance sessions above preavoidance levels (P < .01).

Figure 1 also shows that mean plasma digoxin-like (P < .05), marinobufagenin-like (P < .05), and ouabain-like (P < .05) immunoreactivity during the last 10 min of preavoidance were all significantly increased above control levels and those during the first 10 min of preavoidance, and remained increased during the avoidance sessions.

Figure 1 also shows that mean erythrocyte Na,K-ATPase activity during the first 10 min of preavoidance was not significantly different from that observed under con-

| TABLE 1. MEANS AND STANDARD ERRORS OF PLASMA pH, PLASMA BICARBONATE, pO2, SODIUM, POTASSIUM, AND CORTISOL CONCENTRATIONS DURING CONTROL, FIRST 10 MIN OF PREAVOIDANCE, LAST 10 MIN OF PREAVOIDANCE, AND DURING AVOIDANCE SESSIONS, AVERAGED FOR 30 SESSIONS FOR SIX MICROPIGS |
|-----------|-------------|-------------|-------------|-------------|
| CTL       | PAV1        | PAV2        | AVD         |
| Plasma pH | 7.456 ± 0.006 | 7.447 ± 0.004* | 7.457 ± 0.006 | 7.470 ± 0.005* |
| Plasma $\text{HCO}_3^-$ (mEq/L) | 29.1 ± 0.1 | 33.0 ± 1.2* | 34.0 ± 1.6* | 34.6 ± 2.1 |
| Plasma $\text{pO}_2$ (mm Hg) | 85.2 ± 2.0 | 87.9 ± 2.1 | 90.8 ± 2.4 | 89.2 ± 1.5 |
| Plasma Na$^+$ (mEq/L) | 141.6 ± 1.2 | 140.9 ± 1.1 | 141.1 ± 1.1 | 142.1 ± 0.7 |
| Plasma K$^+$ (mEq/L) | 4.70 ± 0.10 | 4.22 ± 0.05* | 4.39 ± 0.08 | 4.56 ± 0.02† |
| Plasma cortisol (nmol/L) | 42.5 ± 8.0 | 51.0 ± 18.1 | 44.2 ± 8.2 | 112.6 ± 15.0* |

* P < .05 compared with control; † P < .05 compared with PAV1.

CTL, control; PAV1, first 10 min of preavoidance; PAV2, last 10 min of preavoidance; AVD, avoidance sessions.
FIGURE 1. Means and standard errors of pCO₂, hematocrit, erythrocyte Na,K-ATPase activity, plasma digoxin-like, marinobufagenin-like (MBG-like), and ouabain-like immunoreactivity in the home kennel (CTL), during the first (PAV1) and last 10 min (PAV2) of the 60-min preavoidance periods, and during the 30-min avoidance sessions (AVD), averaged for 30 sessions with six micropigs. *P < .05 compared with CTL.

trol conditions, but was decreased by almost 40% during the last 10 min of preavoidance (P < .03). Na,K-ATPase activity during avoidance sessions increased to levels that were not significantly different from control.

Table 1 shows that plasma cortisol levels during the first and last 10 min of preavoidance were comparable to control levels, and that they increased during avoidance sessions above control and preavoidance levels (P < .01).

DISCUSSION

The results of this study confirmed previous findings of respiratory acidosis, decreased hematocrit, and increased blood pressure preceding the onset of a well-learned avoidance task, and also found that plasma EDLF, assessed by digoxin-like, marinobufagenin-like, and ouabain-like immunoreactivity, were increased, whereas erythrocyte Na,K-ATPase activity was decreased. During the avoidance performance periods, blood pressure and plasma EDLF remained elevated, heart rate increased above preavoidance levels, and the Na,K-ATPase activity recovered to control levels.

Previous studies with dogs showed that the progressive blood pressure increases during preavoidance periods were not prevented by administration of adrenergic antagonists. Inhibition of Na,K-ATPase in vascular smooth muscle by EDLF during preavoidance periods might have contributed to the elevations in blood pressure under these conditions. In this regard, previous
studies in normal rats have shown that administration of a bufodienolide compound (bufalin) increased blood pressure acutely, but not chronically, whereas administration of equimolar doses of ouabain had no acute effects on blood pressure, but produced hypertension over periods of weeks. It is well established that plasma EDLF can be increased in human and experimental hypertension, but whether they are a cause or a consequence of the hypertension remains to be determined.

Hypercapnia is known to produce respiratory acidosis, followed by increased renal excretion of hydrogen ions and increased renal reabsorption of sodium and water. The decrease in hematocrit observed during the preavoidance period suggests an increase in plasma volume through this mechanism. That the hematocrit decreased at the beginning of preavoidance suggests the participation of extrarenal mechanisms in increasing plasma volume (but not the effect of slow infusion of saline needed to maintain catheter patency during the session). This finding complements the previous report of reduced respiratory acidosis and urinary sodium excretion during voluntary hypercapnic breathing by human subjects. It is well documented that expansion of plasma volume by rapid infusion of saline increases concentrations of EDLF and inhibits sodium pump activity.

The renal response to hypercapnia, although slower, is more powerful than the extracellular buffering mechanism in reversing the acidosis. It is known that hypercapnia produces a marked decrease in urine flow, glomerular filtration rate, and renal plasma flow. In addition, sodium retention during preavoidance might have been mediated by the renal Na-H exchange, augmenting urinary excretion of H and reabsorption of Na and fluid volume. The progressive increases in plasma bicarbonate concentrations observed during preavoidance are consistent with this view.

Substantial decreases in plasma K concentrations observed during the first 10 min of preavoidance, compared with control values, could also have been a consequence of increased renal Na-H exchange. Whether the initial decrease in plasma K concentrations in this experiment was due to extracellular events or to increased renal excretion remains to be clarified. The recovery of plasma K during the later part of preavoidance and during the avoidance sessions could have been a result of decreased sodium pump activity due to a progressive accumulation of circulating EDLF.

The results of this study are consistent with the view that vascular tone and blood pressure during hypercapnic breathing were influenced by concurrent increases in Na-H exchange and decreases in Na,K pump activity in vascular smooth muscle, resulting in increased intracellular Na, reduced Ca efflux, and/or increased Ca influx through the Na-Ca exchange. Accordingly, the effects of increased Na-H exchange should be predominant until the acidosis was buffered, whereas the EDLF effects should become more significant as plasma volume gradually increases. Ultimately, the natriuretic effects of EDLF should constrain further increases in plasma volume associated with sustained hypercapnia and increased Na-H exchange. In addition, ouabain can attenuate reuptake of norepinephrine into sympathetic nerves, resulting in increased plasma norepinephrine. The extent to which the vasoconstriction during preavoidance was attributable to the sodium pump inhibitory effects of EDLF or to other effects on sodium transport mechanisms remain to be clarified in future studies.

EDLF are a group of steroids and peptide substances that inhibit the sodium pump and cross-react with antibodies against several digitalis glycosides. Mammalian plasma contain EDLF that cross-react with antiouabain and antidigoxin antibodies. Recently, evidence has been obtained that a mammalian digoxin-like immunoreactive EDLF may represent an endogenous bufodienolide, termed marinobufagenin-like substance. Administration of marinobufagenin was shown to produce a rapidly developing and sustained vasoconstriction in isolated rat aorta.

The functional effects of various EDLF may be specific to different subunits of Na,K-ATPase in different tissues. In the present study, plasma concentrations of all three sodium pump inhibitors were elevated and Na,K-ATPase was inhibited after 50 min of preavoidance. The time course of these effects is consistent with the one found in a recent study that swimming stress increased plasma concentrations of a ouabain-like compound in rats after 50 min. In addition, voluntary hyperventilation by humans was found to increase the plasma marinobufagenin-like substance, decrease Na,K-ATPase activity, and increase blood pressure within 30 min, whereas plasma ouabain-like concentrations were not increased within that time span. Therefore, which specific EDLF inhibited Na,K-ATPase during preavoidance in the present study remains to be clarified.

Previous studies have documented that performance of avoidance behavior by laboratory animals is accompanied by sustained increases in adrenergic activity, plasma renin activity, and urinary cortisol and aldosterone concentrations. Swimming stress in rats increased adrenocortical concentrations of both corticosterone and the ouabain-like compound within 10 min, but plasma corticosterone increased more rapidly than plasma ouabain-like compound. In the present study, plasma cortisol remained at control levels during preavoidance periods, and increased only during avoidance performance periods. Previous research in rats showed that administration of adrenocorticotropic hormone increased plasma concentrations of a digoxin-like factor as well as blood pressure. Therefore, the concentrations of digoxin-like fac-
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REFERENCES