Ouabain-Like Compound Changes Rapidly on Physical Exercise in Humans and Dogs
Effects of β-Blockade and Angiotensin-Converting Enzyme Inhibition


Abstract—Ouabain, an inhibitor of the sodium pump, has been identified as a constituent of bovine adrenal glands. We were interested whether the release of this cardiotonic steroid is stimulated by physical exercise. Hence, athletes and healthy dogs were subjected to ergometry. Ouabain-like compound (OLC) was measured in venous blood by enzyme-linked immunosorbent assay as well as by 86Rb+ uptake inhibition (as ouabain equivalents). OLC increased in venous blood of athletes after 15 minutes of ergometry from 2.5±0.5 to 86.0±27.2 nmol/L (n=51; P<0.001), as did the concentration of a circulating inhibitor of the sodium pump from 7.3±1.7 to 129.8±51 nmol/L (ouabain equivalents, P<0.05). Half-maximal increase in heart rate and systolic blood pressure occurred at 5.1±1.2 nmol/L and at 30±1 nmol/L OLC, respectively. On rest, OLC decreased in humans and dogs with a half-life of 3 to 5 minutes. In beagles exposed to moderate exercise on a treadmill for 13 minutes, levels of OLC increased 46-fold (from 3.7±0.8 to 166.9±91.8 nmol/L; n=6; P<0.005). This effect was suppressed when the dogs had been treated for 3 weeks with the β1-adrenergic receptor blocker atenolol or the angiotensin-converting enzyme inhibitor benazepril. We conclude that OLC changes rapidly during exercise and is under the control of norepinephrine and angiotensin II. (Hypertension. 2005;45:1024-1028.)

Key Words: angiotensin-converting enzyme inhibitor ■ β-blocker ■ circulation ■ endogenous ouabain ■ hypertension ■ sodium pump hypertension

Ouabain or its isomer has been isolated from blood, adrenals, and hypothalamus1–3 as one of the endogenous cardiac glycosides circulating in blood plasma.4 Evidently, ouabain is synthesized in adrenal glands,1,5,6 but it may also be accumulated there after resorption from the gut.7 Bovine adrenocortical cells in tissue culture release ouabain on exposure to epinephrine, angiotensin II, or corticotropin.1,6,8 Whether this in vitro finding translates into the in vivo situation is unclear. If so, physical exercise associated with the increase in epinephrine and norepinephrine should consequently increase endogenous ouabain. Previous studies showed ambiguous results. An increase9 as well as a decrease of plasma concentrations of ouabain-like compounds (OLCs)10,11 have been reported. Here, we investigated the effect of physical exercise on the endogenous ouabain plasma concentration in several experimental settings as well as the influence of β-blockade and angiotensin-converting enzyme (ACE) inhibition.

Materials and Methods
All chemicals were of the highest purity available. Anti-ouabain antibodies from sheep (CN2710) were from B.R.A.H.M.S. Arzneimittel (Dr A. Bergmann), Henningsdorf, Germany. The antibodies showed cross-reactivities with k-strophanthin 42%, ouabagenin 27%, dihydro-ouabain 0.3%, digitoxin 0.07%, and proscillaridin (<0.1%), and no cross-reaction (<0.01%) with strophanthinid, digoxin, digitoxigenin, oleandrin, marinobufagin, bufalin, cinobufagin, cinobufotalin, and 19 other steroid hormones.

Human Patients and Controls
Written informed consent was received from athletes (12 female and 39 male) and nonathletes (male only) (for details, see online supplement at http://hyper.ahajournals.org). The investigation was approved by the Ethics Committees of the Medical Faculties of the Universities of Tübingen and Cologne.

Ergometry
Athletes were studied in the course of their 15-minute training program either on a treadmill (n=34) or on a bicycle ergometer (n=17). Running started at 6 km/h, which was increased every other minute.
minute by 2 km/h up to an individual maximal speed (mean maximal speed: 16.85 ± 0.42 km/h). Bicycle ergometry started at 50 W, which was increased every third minute by 50 W. Maximal load was 245.3 ± 17.2 W within 15 minutes. Electrocardiograms and blood pressure (measured by sphygmomanometry in supine position) were monitored throughout the experiments. Nonathletes performed bicycle ergometry, which started at 25 or 50 W depending on the subject’s condition and was increased every other minute by 25 W. The exercise test was conducted until a heart rate of 220 bpm minus age was reached. Heart rate and blood pressure were monitored every minute. Before starting the ergometry, an ECG was obtained to exclude any conductance disturbances of the heart. Venous blood samples were taken from the cubital vein at the times indicated in Figure 1. Plasma was stored at −20°C until analysis.

Animals
Healthy beagle dogs (3 males, 4 neutered females, mean body weight 17.9 ± 1.3 kg, mean age 68.8 ± 0.8 years) were kept at room temperature with natural light in cages of 2.3 × 1.2 m. They received Hill’s canine maintenance diet and water ad libidum. Before ergometry, a catheter was placed into the vena jugularis. For randomized studies, 1-adrenergic blocker atenolol or the ACE inhibitor benazepril on ouabain release, the dogs were medicated for another 5 days, and 1.4 mg/kg thereafter). At day 16, when ergometry was performed. During medication, blood pressure (measured by sphygmomanometry in supine position) were monitored throughout the experiments. Nonathletes performed bicycle ergometry, which started at 25 or 50 W depending on the subject’s condition and was increased every other minute by 25 W. The exercise test was conducted until a heart rate of 220 bpm minus age was reached. Heart rate and blood pressure were monitored every minute. Before starting the ergometry, an ECG was obtained to exclude any conductance disturbances of the heart. Venous blood samples were taken from the cubital vein at the times indicated in Figure 1. Plasma was stored at −20°C until analysis.

Experiments With Dogs on a Treadmill
Catheterized, fasted dogs were encouraged to run on a treadmill at 7.3 or 6 km/h. After 3 minutes, the treadmill’s slope was increased by 4° every 2 minutes up to 20°. Blood samples were taken before and immediately after the 15-minute exercise period and thereafter at the times indicated in Figure 2. Heart rate was recorded constantly by telemetry. The experiment was approved by the Regierungspräsidium Gießen (Gi/20/15-2/99).

Analysis of Blood Parameters
Clinical chemical analyses in dogs were performed using a Cobras Mira auto analyzer (Roche, Basel). Enzymatic lactate analysis was performed with capillary blood taken from the athletes’ ear lobe.

Quantitation of Endogenous Ouabain and Sodium Pump Inhibitors
Plasma was separated on C18 disposable columns and the fraction eluted with 25% acetonitrile/0.1% trifluoro acetic acid was collected. Parallel samples of this fraction known to contain ouabain but not marinobufagin or digitoxin were transferred to an enzyme-linked immunosorbent assay for ouabain, as well as to a bioassay testing the inhibition of the sodium pump. For the enzyme-linked immunosorbent assay, anti-ouabain antibodies from sheep were allowed to bind to ouabain-C6-trypsin conjugate (0.1 µg/well) attached to a microtiter plate. After competition with ouabain within the extract bound anti-ouabain-IgG was detected with biotinylated anti-sheep-IgG and streptavidin-phosphatase, which hydrolyzed p-nitrophenylphosphate. The assay detected ouabain at a concentration of as low as 0.001 nmol/L (Figure 3). All measurements rely on concentration dependence of the fractions tested.

Statistical Analysis
Data were analyzed by 1-way ANOVA, Kruskal-Wallis test, followed by Dunn’s or Bonferroni’s multiple comparison test, the paired t test, and linear regression analysis.

Results
Effect of Bicycle and Treadmill Ergometry on Endogenous Ouabain in Human Volunteers
Maximal exercise of 51 athletes led to an increase in heart rate and plasma lactate and produced an 18-fold increase of a sodium pump inhibitor (129.8 ± 51 versus 7.3 ± 1.7 nmol/L ouabain equivalents; P < 0.05). In parallel, endogenous ouabain immunoreactivity (OLC) increased 36-fold from 2.5 ± 0.5 to 86.0 ± 27.2 nmol/L (P < 0.001). To test whether regular physical training may affect OLC release, 26 healthy nonathletes were tested. Their OLC increased from 2.5 ± 0.5 to 176 ± 68 nmol/L (P < 0.001). However, the values obtained at maximal exercise did not differ statistically (P = 0.498). During rest, elevated OLC declined with a half-life of 2.8 ± 0.1 minute to baseline levels (Figure 1, inset). Blood lactate increased along with OLC in a log-linear fashion (log lactate = [0.34 ± 0.06]+[1.41 ± 0.09]×log OLC; P < 0.0001; r² = 0.64) (not shown), whereas the correlation between heart rate and the blood lactate concentration was hyperbolic (R² = 0.93; at half-maximal increase of...
heart rate, lactate concentration was 2.5 ± 0.2 mmol/L). Heart rate and systolic arterial blood pressure increased with rising concentrations of OLC (Figure 4). Diastolic arterial blood pressure remained unaltered (Figure 4B). Half-maximal increase in heart rate was seen at 5.1 ± 1.2 nmol/L OLC (Figure 4A), whereas that of arterial systolic blood pressure was reached at 30 ± 1 nmol/L (Figure 4B).

**Effect of β-Blockade and ACE Inhibition on Exercise-Induced Increase in Endogenous Ouabain Levels in Dogs**

Beagle dogs were encouraged to run on a treadmill at 7.3 km/h for 13 minutes (n=7). A ~500-fold increase in the concentration of OLC (6882 ± 1436 nmol/L versus 14.1 ± 5.0 nmol/L; P<0.0001), an increase in the heart rate (218 ± 9 versus 115 ± 6 bpm; P<0.0001), and venous lactate concentration (2.39 ± 0.24 mmol/L versus 0.84 ± 0.1 mmol/L; P<0.0001) were observed. As expected, plasma K⁺ increased significantly from 4.52 ± 0.12 to 4.87 ± 0.13 mmol/L (P<0.005). When the experiment was repeated at a lower velocity (6 km/h for 13 minutes, n=6), a 46-fold increase of OLC (from 3.7 ± 0.8 nm to 139 ± 55 nmol/L) was seen along with the increase of heart rate (from 101 ± 3 to 213 ± 5 bpm), lactate (from 0.83 ± 0.10 to 1.66 ± 0.21 mmol/L; P<0.05), and norepinephrine concentrations (from 0.68 ± 0.09 to 1.16 ± 0.19 nmol/L; P<0.05; Figure 2). The concentration of OLC (Y) rose linearly along with that of norepinephrine (X) as Y=209±38X (r²=0.65; P<0.0001).

On rest, OLC declined with a half-life of 3.9 ± 0.5 minutes (Figure 2). Dogs pretreated with the β-receptor blocker atenolol or the ACE inhibitor benazepril (n=6) showed at rest a significantly elevated norepinephrine concentration compared with control (both 1.54 ± 0.24 nmol/L; P<0.01), indicating a counter-regulation of the blood pressure–controlling system. As expected, the exercise-induced increase in heart rate was lowered by β-receptor blockade (from 93 ± 3 to 176 ± 3 bpm) but not by ACE inhibition (110 ± 3 to 210 ± 6 bpm). Interestingly, in both atenolol- and benazepril-pretreated dogs, no increase in OLC was observed in response to exercise, whereas increases in norepinephrine levels and plasma lactate were still present (Figure 2).

**Discussion**

The most striking observations in this study is the marked and rapid change of a plasma ouabain-like compound in humans and dogs during physical exercise. OLCs increase studied by 2 independent methods correlated positively with heart rate, systolic blood pressure, plasma lactate, and norepinephrine concentrations. Dogs treated with β-blockers or ACE inhibitors showed no increase in OLC on physical exercise.

The origin of the OLC and the mechanism of its rapid release during exercise or elimination after exercise are
OLC concentration in plasma is lower than that one determined after extraction (eventually caused by its binding to cardiac glycoside binding proteins in blood\textsuperscript{20,21}) or the sodium pump is not yet inhibited within the time interval studied because of the slow on-rate in forming the inhibitory and almost irreversible ouabain-Na\textsuperscript{+}/K\textsuperscript{+}-ATPase complex.\textsuperscript{22} A transient increase of endogenous ouabain may not lead to inhibition of the pump, although a rapidly dissociable ouabain-Na\textsuperscript{+}/K\textsuperscript{+}-ATPase complex might be formed. Noninhibitory ouabain concentrations activate an intracellular signaling cascade yielding inotropic response\textsuperscript{23,24} and release of endothelin-1 in endothelial cells.\textsuperscript{25} Thus, the integral of the local ouabain concentration over time rather than the peak concentration may be of inhibitory relevance. Moreover, exercise may increase K\textsuperscript{+} concentration locally, much higher than those levels observed in blood plasma.\textsuperscript{14} It is well known that K\textsuperscript{+} lowers ouabain affinity for Na\textsuperscript{+}/K\textsuperscript{+}–ATPase.

The present study provides only indirect evidence how the rapid release of OLC during exercise is mediated. Correlations were found between OLC and plasma concentrations of lactate, norepinephrine, as well as heart rate and systolic blood pressure. Such correlations were also seen under stress\textsuperscript{9,26,27} and when ouabain was infused intraventricularly to rats.\textsuperscript{28} These correlations may be causative, but they may also be purely coincidental. The fact that in dogs the increase in OLC release is completely abolished by β-blockade and ACE inhibition suggests that hypoxia, which clearly remained unaffected by these interventions (lactate unchanged), may not be the major cause of ouabain release.\textsuperscript{29,30} Instead, the results suggest that both β\textsubscript{1}-adrenergic stimulation and the renin-angiotensin system are immediately involved in OLC release during exercise.

**Perspectives**

OLC behaves like a rapidly regulated hormone that is released quickly from intracellular stores in response to physical exercise and stress\textsuperscript{9,27} The rapid changes of its plasma concentration suggest that OLC may play an immediate role for circulatory regulation. Our results indicate that epinephrine and angiotensin II are important for OLC release.\textsuperscript{1,6,15} Whether the main physiological impact of OLC is reduction of heart rate, induction of positive inotropy, increase in blood pressure by release of vasoconstrictors,\textsuperscript{25} or something else remains unknown to date. Prolonged elevated plasma ouabain concentrations lead to arterial hypertension.\textsuperscript{31} Because ≈50% of whites with uncomplicated arterial hypertension show elevated concentrations of OLC in blood plasma,\textsuperscript{32} the present observation may stimulate more detailed investigations of whether enduring stress, a defective elimination system, or both may lead to increased OLC concentrations in blood and hence to hypertension.

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