Original Article

Endogenous Ouabain and Its Binding Globulin: Effects of Physical Exercise and Study on the Globulin's Tissue Distribution

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Ouabain, that has been isolated from bovine adrenals and hypothalamus, is a new cardiotonic steroid hormone, which is either synthesized in the adrenals or stored there after it has absorbed from the diet. Little is known *in vivo* which events may lead to the release of ouabain into blood. Moreover, a binding protein for cardiotonic steroids exists in blood, which binds cardiac glycosides with high affinity. It may affect the action of endogenous ouabain on heart and circulation, but the physiological function of this protein is unclear. To realize, which physiological stimuli *in vivo* may affect blood concentrations of endogenous ouabain and which function the cardiotonic binding protein may have in modulating ouabain effects, the effect of physical exercise on endogenous ouabain was studied and the tissue distribution of its binding protein was investigated. We found that endogenous ouabain changes rapidly in blood upon physical exercise and behaves like expected for a hormone of circulation. The cardiotonic steroid binding globulin shows the highest concentration in the kidney, which suggests that sodium pumps of the kidney are protected against its inhibition by ouabain which would lead not only to natriuresis but also to a deleterious loss of glucose, amino acids and phosphate. (*Hypertens Res* 2000; 23 Suppl: S93-S98)

Key Words: endogenous ouabain, cardiac glycoside binding globulin

Introduction

Consistent with the postulate of Szent-Gyorgyi in 1953 on the existence of an endogenous digitalis in mammals (1), several laboratories reported on the isolation of endogenous ouabain as a cardiotonic steroid from bovine adrenals and hypothalamus (2, 3) and from human blood, although the definitive proof is missing yet that the substance in human blood is not an isomer of ouabain (4). Endogenous ouabain-immunoreactive material has been found to be increased in blood plasma in low renin hypertension (5, 6), when blood pressure was increased due to

a tumor producing ouabain (7), in anoxia (8) and in congestive heart failure (9). About 50% of Caucasians with essential hypertension show elevated levels of ouabain-immunoreactivity (5). These observations, and the finding that angiotensin II releases endogenous ouabain from *zona glomerulosa cells* in tissue culture (10) point to the possibility that endogenous ouabain is a new hormone controlling the contractility of heart and arterial vessels. Further support for the concept, that ouabain is a new cardiotonic steroid hormone regulating heart function and circulation, comes from the finding that a binding globulin for cardiotonic steroids can be isolated from bovine and human plasma (11, 12).

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Although a big body of evidence is existing that besides ouabain also other cardiotonic steroids (like marinobufagenin (13) and 19-norbufalin (14)) are involved in the regulation of salt and water metabolism of mammalian tissues, there is not much information available on the physiological conditions leading to the release of endogenous ouabain and its analogs. It is also unclear, which physiological task the cardiac glycoside binding globulin in blood may fulfill; due to its high affinity for cardiac glycosides (11) it should act as a buffer for cardiotonic steroids in blood serum. To learn which physiological conditions may release ouabain to blood and which role the cardiac glycoside binding protein may have, humans were subjected to mild physical exercise and tissue distribution of the cardiac glycoside binding globulin was investigated in porcine tissues. It became evident that ouabain is increased rapidly with mild exercise of circulation and behaves like expected for a hormone of circulation. Kidneys contain a high amount of the cardiac glycoside binding protein. It is assumed that the binding globulin may protect sodium pumps of the kidneys against its inhibition by cardiac glycosides which would lead to a loss of the body of glucose, lactate, amino acids, phosphate and sulfate in the urine.

Material and Methods

Materials

All chemicals were of the highest purity available and purchased from E. Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Bio-Rad (Munich, Germany), Sigma Chemicals, Molecular Probes (Eugene, OR, USA) and Boehringer Mannheim (Mannheim, Germany). Colloidal Coomassie blue from ICN (Costa Mesa, USA). Complete™ protease inhibitor tablets, a cocktail of various proteases, were purchased from Boehringer (Mannheim, Germany). CNBr-activated Sepharose was from Pharmacia (Freiburg, Germany) or made according to (15); protein-A Sepharose was from Zymed (San Francisco, USA). Molecular weight markers were from Pharmacia (Freiburg, Germany) or Bio-Rad (Munich, Germany). Goat anti-rabbit IgG, anti-rabbit IgG coupled to alkaline phosphatase and streptavidin coupled to phosphatase were from Jackson Immuno Research Dianova, and C18 Isolute™ disposable SPE columns (500 mg×1.0 ml XL with column reservoir) were purchased from ict (Bad Homburg). Microtiter plates were from NUNC (Wiesbaden). Antisera against the cardiac glycoside binding protein from bovine plasma were prepared as described earlier (11).

Patients

The two healthy male volunteers of 37 and 63 years of

age gave their consent before they were set on a ergofit 310 bicycle ergometer (Boso Ergofit, Pirmasens). Pulse frequency was measured constantly by telemetry and blood pressure by sphygmometry. A vein of the fore arm was punctured and blood was taken before and during bicycling for 15 min at 35 W, followed by a 20 min period of rest laying on a bed. The experiments were approved by the ethical committee of the Medical Faculty of the Justus-Liebig-University of Giessen, and the subjects gave their informed consent.

Analysis of Endogenous Ouabain with a Fluorescent Immuno Sorbent Assay (FIA)

All incubation steps were performed at room temperature on a rotary shaker. Microtiter plates were coated with 100 μ l goat anti-rabbit-IgG (1:5,000) per well in coating buffer (15 mmol/l Na_2CO_3 , 35 mmol/l NaHCO_3 , pH 9.6) for 12 h at 4°C. Free binding sites were blocked with 1% gelatin in TBS (10 mM Tris/HCl 7.8, 150 mM NaCl, 0.1% NaN_3 , 0.1% Tween 20) for 30 min. Thereafter, 20 μ l of rabbit anti-ouabain IgG (1:1,000) was added and incubated for 60 min. Biotinylated ouabain (100 μ l) (10^{-11} mol/l) was added to the wells and filled up with 100 μ l TBS to a total volume of 200 μ l. To some of the wells additionally various dilutions of ouabain (10^{-3} – 10^{-13} mol/l) or 10–100 μ l of the plasma sample extracts were given. Plasma extracts were purified on C18 disposable Isolute™ according to Harris *et al.* (16). Alkaline phosphatase coupled to streptavidin (100 μ l) was then added diluted by 1:500 in PBS for 30 min followed by 4 μ mol/l difluorescein diphosphate (dissolved in 10 mmol/l Tris, 150 mmol/l NaCl-buffer (pH 7.8)). The phosphatase reaction was allowed to proceed for 30 min, whereafter it was stopped by the addition of 50 μ l 3 mol/l NaOH. The fluorescein formed was determined in a LS 50 B Perkin Elmer fluorescence photometer equipped with a microtiter plate reader using 485 nm (band path 5 nm) for exciting and 530 nm (band path 10) for emitting light.

Between all incubation steps, microtiter plates were washed three times for 3 min with 200 μ l of 150 mmol/l TBS, pH 7.8. This modified procedure gave an extended calibration curve that allowed to measure with precision in the range of 10^{-8} to 10^{-11} mol/l ouabain.

ELISA for a Cardiac Glycoside Binding Protein

NUNC microtiter plates were coated over night at 37°C with 100 ng bovine cardiac glycoside binding globulin (CGBG) (11) per well. The plates were washed and free sites were blocked with 1% blocking solution (Boehringer, Mannheim) in TBS for 1 h at room temperature. To construct a calibration curve, increasing concentrations of CGBG (11) (in the range between 0.48 μ g to 1 ng) were added to the wells and an appropriate dilution of the anti-

Fig. 1. Effect of physical exercise of two healthy persons onto the concentrations of endogenous ouabain in venous blood, on pulse frequency and on blood pressure. Mean values $\pm$ SEM of 3 experiments are shown. The probands were loaded for 15 min with 35 W on a bicycle ergometer and subsequently allowed to rest.

CGBG from rabbits was allowed to interact with the sessile and free porcine CGBG at room temperature for 1 h (total volume 100 μ l). After removal of the reagents and washing, bound anti-CGBG was detected by incubation with anti-rabbit-IgG coupled to alkaline phosphatase for 1 h at room temperature. The activity of the alkaline phosphatase was detected by the hydrolysis of *p*-nitrophenyl-

phosphate (1 mg/ml) to *p*-nitrophenol, that was measured after alkalisation at 405 nm in an ELISA photometer. The data were analyzed by fitting the data to a one site competition curve using the PRISM™ software of GraphPad-Software, San Diego, USA.

Purification of the Cardiac Glycoside Binding Protein from Various Porcine Tissues for the Estimation of its Tissue Content

A cytosolic supernatant of various porcine tissues was obtained by homogenization of freshly slaughtered tissues in a Waring blender with a 10 fold excess (w/v) of 50 mM Tris/HCl pH 7.5 containing 150 mM NaCl and protease inhibitors (cocktail tablets Complete™ of Boehringer Mannheim). The homogenate was first centrifuged for 30 min at $9,000 \times g$ at 4°C and subsequently for the same time at $70,000 \times g$ in a 55.2 Ti rotor of the Beckman ultracentrifuge. The clear extract (100 ml) was given to a 25 ml anti-CGBG-Sepharose column (11) and was allowed to re-circulate for 15 h at 18°C. The column was then washed with TBS containing 0.04% NaN₃ to remove other proteins but CGBG which was then eluted by 0.1 M sodium acetate buffer (pH 4.5) containing 0.5 M NaCl to give the fraction containing the cardiac glycoside binding globulin.

Statistical Analysis

The one-way ANOVA statistical analysis was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, California, USA, "www.graphpad.com".

Results

Effect of Physical Exercise onto the Concentration of Endogenous Ouabain in Humans

It is now established that ouabain is a constituent of bovine adrenals and hypothalamus (2, 3) and most probably of human blood plasma as well (4). Like all other cardiotonic steroids, ouabain is known to induce bradycardia and inotropic response. Since ouabain has to be considered as a new steroid hormone (5, 17, 18), we asked ourselves under which physiological conditions inotropic response and bradycardia might be of physiological benefit for heart action. Physiological exercise increasing the beating rate of heart was a candidate for such an event. Therefore, 2 persons underwent submaximal bicycle ergometry at 35 W for 15 min and the endogenous ouabain was measured as well as heart frequency and blood pressure over this time, followed by a time of rest for 25 min. Figure 1 shows the mean value of 3 experiments $\pm$ SEM. It is evident that bicycle ergometry led to a

Fig. 2 Comparison of the concentrations of a cardiac glycoside binding protein in porcine kidney, blood and heart. Mean values of 2-4 determinations are shown. The non-parametric one-way ANOVA statistical analysis with Bonferroni's multiple comparison tests revealed that the mean values of endogenous ouabain in blood and heart differ statistically significantly from the mean value in the kidney ($p < 0.001$), however the difference between the concentration in heart and plasma is $p > 0.05$. For details see "Methods".

considerable increase of endogenous ouabain peaking at 10 min of exercise. Endogenous ouabain rapidly declined when the physical exercise stopped. The rise of endogenous ouabain seems to be preceded by the pulse frequency and the blood pressure which both peak at about 5 min.

Quantitation of the Cardiac Glycoside Binding Globulin in Porcine Tissues

A binding protein with high specificity for cardiotonic steroids has been reported recently to exist in bovine and human plasma (11, 12). Binding proteins for steroid hormones are mostly released from the liver and placenta (19-22). So far it is unclear, where the biosynthesis of the cardiac glycoside binding globulin may occur and from which tissue it is released to the blood. We, therefore, set up a competitive ELISA for the cardiac glycoside binding protein and measured the amount of the protein in some tissue homogenates. Figure 2 shows that the concentration of CGBG in porcine kidney is about 500 fold higher than in blood plasma and heart. Statistical analysis by the one-way ANOVA (and non-parametric) assay and Bonferroni's multiple comparison tests revealed that mean values of endogenous ouabain in blood and heart differ statistically significantly from the mean value in kidney ($p < 0.001$), however the difference between the concentration in heart and plasma is $p > 0.05$.

Discussion

Ouabain is synthesized in adrenal cortical cells and released there from *in vitro* upon hormonal stimuli like ACTH, angiotensin II and epinephrine (10, 23-25); about 3-5 % of ouabain found in the diet may also be absorbed and stored in the adrenocortical cells (26). It is unclear so far which physiological stimuli except anoxia (8) may *in vivo* release ouabain from its intracellular stores. Like all cardiotonic steroids, the hydrophilic ouabain is known to induce bradycardia and inotropic response. Inotropy and bradycardia may be physiologically beneficial when brain and periphery are demanding for increased blood supply, as it is seen under the conditions of physical exercise. Hence, the effect of physical exercise on the levels of endogenous ouabain was studied. Consistent with this hypothesis a rapid increase of ouabain in venous blood was seen in healthy volunteers upon physical exercise (Fig. 1). It went back rapidly to normal values, when exercise stopped. This observation is consistent with the observations that endogenous ouabain was found to be elevated in hypertension (5, 6, 27). However, the rapid decline of endogenous ouabain after rest was somewhat unexpected. Most medical textbooks report a $t_{1/2}$ value of the action of ouabain of 12-19 h (28). In fact, it has been reported that the effect of ouabain on atrial fibrillation dissipates with a half-life of 23 h (29) and on myocardial contractility with a half-life of 22 h (30) and that these effects relate to the half-life of elimination of ouabain from plasma of 18 h (31). Unfortunately, Selden and Smith did not consider from their studies on ouabain pharmacokinetics in dog and man a very short half-life visible in their publication as important (31). This short half-life is also visible from the rapid decline of endogenous ouabain in Fig. 1. It is also described in a recent study of Harashima *et al.* on the clearance of radioactive ouabain from plasma in guinea pigs. Several compartments of ouabain with half-lives in the range of minutes and below were found (32).

Hence, endogenous ouabain behaves like a rapidly acting hormone; It increases rapidly upon endogenous stimuli and rapidly falls back to normal values if the stimulus is switched off.

Yet, it is unclear so far, which hormones may release *in vivo* ouabain from endogenous sources to the blood. Since the pulse frequency increases faster upon onset of physical exercise than endogenous ouabain (Fig. 1), epinephrine may induce the release of ouabain. Epinephrine has been shown *in vitro* to release ouabain from bovine adrenal glomerulosa cells in tissue culture (25). It will be necessary in future experiments to verify this possibility.

Measurements of endogenous ouabain under the above conditions refer to the total concentration of ouabain.

Free ouabain cannot be measured so far, although it is known that a specific binding globulin for cardiotonic steroid hormones circulates in blood (11, 12), because neither a specific test for the binding protein is existent, nor it is possible to separate the free ouabain out. Therefore, as a first step, we developed an ELISA to determine the tissue concentration of the cardiac glycoside binding globulin (Fig. 2). It became evident that the cardiac glycoside binding globulin can be found in other tissues of the body. This phenomenon is analogous to the binding proteins of other steroid hormones which are not only found in blood plasma but also in many tissues of mammals (19, 23).

Interestingly, the concentration of the cardiac glycoside binding globulin was highest in the kidney and very low in the heart (Fig. 2). Hence, it seems possible that the cardiac glycoside binding globulin circulating in blood stems from the kidney. If so, the secreted cardiac glycoside binding globulin may protect the sodium pump of kidneys against inactivation by circulating cardiac glycosides. It is well known that toxic concentrations of cardiac glycosides are needed to inhibit the sodium pump of kidneys *in vivo*. Inhibition of the sodium pumps in kidneys by cardiac glycosides would not only lead to natriuresis but also to a loss of all substrates which are taken up in the kidneys by sodium dependent processes like glucose, amino acids, lactate, phosphate and sulfate (34). Such a loss would be deleterious. Eventually, the observations in Fig. 2 also explain why cardiotonic steroids acts preferably onto the heart and not onto other tissues although they may contain high concentrations of sodium pumps. The tissue concentration of the heart in the cardiac glycoside binding globulin seems to be lower than the concentration in blood. One may expect that a more careful analysis of the tissue concentration of the cardiac binding globulin will not only reveal considerable new insights into the physiology of action of cardiotonic steroids and its regulation but also into its pathophysiological aspects like the role of the cardiotonic binding globulin in hypertension.

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