New Ouabain-Conjugated Peptide Found From Phage Displayed Peptide Library

Ming-juan Zhang, Jun Yang, and Zhuo-ren Lu

Background: Recently, numerous investigations have shown that endogenous ouabain plays an important role in primary and secondary hypertension. The purpose of this study was to find ouabain-conjugated peptides (OCP) to block or to antagonize actions between endogenous ouabain (EO) and sodium pump, to serve as theoretical and experimental bases of EO in hypertension.

Methods: The study involved screening the phage displayed 12-peptide library by biopanning for OCP. The DNA sequence of each selected peptide was determined and the amino acid sequences were deduced. The highest consistent of the polypeptide was synthesized by peptide synthesizer. Synthetic OCP was identified, its binding activity determined by radioligand binding assay, and bioactivity of ouabain conjugated peptide was measured by erythrocyte ^{86}Rb uptake.

Results: Three kinds of peptides were identified. Peptide A (12 peptide, Leu-Leu-Ala-Asp-Thr-Thr-His-His-Arg-Pro-Trp-Thr) was the highest consistence of peptide sequences, occupied in 66.7% (8/12). Peptide A was synthesized. The results verified that there was binding activity between synthetically OCP and ^{3}H-ouabain, and that OCP was capable of suppressing the inhibition action between ouabain and sodium pump on the surface of erythrocyte. The results showed that efficacy was in a dose-dependent manner.

Conclusion: It is important that the results not only obtain distinctive OCP, but also supply valuable experimental data in detection of ouabain and therapy in hypertension. Am J Hypertens 2004;17:619–623 © 2004 American Journal of Hypertension, Ltd.

Key Words: Ouabain, peptide library, sodium pump.

The search for endogenous digitalis has led to the isolation of ouabain as well as several additional cardiac steroids of the cardenolide and bufadienolide type from the blood, adrenals, and hypothalamus.[1,2] Much evidence suggests that endogenous sodium transport inhibitors circulate in amphibians and mammals and are present in elevated amounts in the blood of hypertensive animals.[3,4] Furthermore, diminished sodium pump activity may be important in the etiology of hypertension.[5–7] Recently, an endogenous sodium pump inhibitor of probable adrenal origin has been isolated from human plasma and was identified as ouabain or a closely related isomer. Although ouabain shares structural similarities with the digitalis glycosides, several characteristics differentiate them from the latter.[8] Ouabain-like compounds have been described in many animal species, and may counterbalance their actions within a regulatory framework of water and salt metabolism. Studies showed that infusion over several weeks of low concentrations of ouabain, but not of digoxin, induces hypertension in rats.[9] Therefore, elevated plasma concentrations of endogenous ouabain (EO) have been linked repeatedly with high blood pressure (BP).

In the past 5 years, researchers have found that by blocking the action between ouabain and sodium pump, BP could be significantly reduced.[10,11] In 1998, a new ouabain antagonist PST2238 allowed new possibilities for hypertension therapy. Recently, our group prepared an antiouabain polyclonal antibody, and experimental results showed that it could markedly reduce BP.[12] However, there still were some limits to its use, such as allergy. According to the results of past studies, our group suggested that ouabain-conjugated peptide (OCP), by specifically binding with EO, seals the binding site of sodium pump (Na,K-ATPase) and antagonizes higher plasma EO levels in ouabain-induced hypertension.

Methods

Biopanning Procedure

A solution of 10 μg/mL of ouabain-ovalbumin compound in 0.1 mol/L $NaHCO_3$ (pH 9.6) is first prepared. A quantity of 150μL of this solution is added to each well, and the solution is incubated overnight at 4°C in a humidified container. Phage displayed peptide library (New England

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From the Department of Cardiology, the Second Hospital and School of Medicine, Xi'an Jiao Tong University, Xi'an, China.

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Address correspondence and reprint requests to Dr. Ming-juan Zhang, Department of Cardiology, the Second Hospital, Xi'an Jiao Tong University, Xi'an, China 710004; e-mail: zhangmingjuanyj@yahoo.com

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Biolabs, Inc., Beverly, MA; 1.5×10^{13} pfu/mL, complexity: 2.7×10^9 transformants) is diluted with TBST, pipetted onto a coated plate, and rocked gently for 10 to 60 min at room temperature. Nonbonding phage is discarded by pouring off and slapping the plate face down onto a clean paper towel. The plate is washed 10 times with TBST. Bound phage is eluted with 100 μL 0.2 mol/L Glycine-HCl (pH 2.2), 1 mg/mL BSA, rocked gently for no more than 10 min, and neutralized with 15 μL 1 mol/L Tris-HCl (pH 9.1) for each microtiter well. Eluate is then pipetted into a microcentrifuge tube. The eluate is added to 20 mL of ER2738 culture (should be early-log at this point) to amplify phage. Three rounds of panning are then carried out. Finally, the unamplified third round eluate is added to the plate, coated with ovalbumin, and then panned for three rounds. The eluate is titered after each round.

Rapid Purification of Sequencing Templates and DNA Sequencing

The plaque amplification procedure was then carried out. Rapid purification of sequencing templates was performed according to Watston's handbook for light M13 DNA isolation and purification; the kit was a product of Hua Shun Biological Engineering Co., Ltd. (Shang Hai, China). The −96 primer (New England Biolabs, Inc.; −96 gIII sequencing primer: 5′-^{HO}CCC TCA TAG TTA GCG TAA CG-3′, 1 pmol/μL) was used for automated sequencing. The sequences were determined by automated cycle sequencing with dye-labeled dideoxynucleotides (Sangon Biological Engineering Technology Co. Ltd., Shang Hai, China). The amino acid sequences were deduced from the anticodon strand of the read sequences and were analyzed by their homogeneous protein in Genbank.

Synthetic of Ouabain-Conjugated Peptide

The highest consistent of the polypeptide was added the spacer sequence Gly-Gly-Gly-Ser to the C-terminus, which was the sequence of synthetic peptide. Ouabain-conjugated peptide (OCP) was then synthesized with peptide synthesizer (Mei Lian Co., Beijing, China). The synthetically derived product was purified by high-performance liquid chromatography (HPLC). Purity is up to 98%.

Binding Ability of Ouabain-Conjugated Peptide With Ouabain Detected by Radioligand Binding Assay

A fixed concentration of OCP (2 mg/mL) and various concentrations of ^{3}H-ouabain (0.25, 0.5, 1.0, 2.0, and 4.0 nmol/L) were added to each tube, and 0.02 mol/L of ouabain was added to a nonspecific tube. Mixtures were then incubated for 90 min, after which ice-cold Tris buffer was added to stop the reaction. Bound and free radioactive products were separated by rapid filtration through glass fibers, and the fibers were heated for 30 min at 80°C. Finally, the fibers were put into scintillant, and retained radioactivity was counted in a scintillator (FJ-2107; 262 Co., Xi'an, China).

Erythrocyte ^{86}Rb Uptake Assay

Heparinized fresh venous blood was centrifuged and the plasma removed. The erythrocytes were washed three times and suspended with Ringer's buffer (NaCl 140 mmol/L, $MgSO_4$ 1 mmol/L, Na_2HPO_4 5 mmol/L, glucose 5.6 mmol/L, KCl 5 mmol/L, and $CaCl_2$ 1 mmol/L, pH 7.4) for measurement of ^{86}Rb uptake. Washed erythrocytes from healthy donors were incubated for 3 h at 37°C in Ringer's solution with 0.1–100 nmol/L ouabain. After a further incubation for 1 h with 1 μCi per tube of ^{86}RbCl (Pharmacia Biotech, New York, NY), the cells were washed with ice-cold isotonic saline, and radioactivity taken up by the cells was determined by a γ-counter. The ^{86}Rb uptake inhibition was calculated as a percentage of pump activity on Ringer's solution. Washed erythrocytes from healthy donors were incubated for 3 h at 37°C in Ringer's solution with 80 nmol/L ouabain with differential dose of OCP. The previously mentioned procedure was then repeated. The suppressing efficiency was worked out.

Results

The results showed that the efficiency of screening phage display peptide library for OAC with OVA-OUA combination was 6.7×10^{-6}% in the first round, 1.1×10^{-5}% in the second round, and 94% in the third round. After three times biopanning, the specific conjugated peptide was highly enriched with OVA-OUA combination. The phage peptide library (1.5×10^{11} pfu/mL) was then absorbed by OVA three times, and a more specific peptide library (2.0×10^2 pfu/mL) was obtained.

The results of DNA sequence analyses showed that three kinds of peptides were found. Peptide A (12-peptide sequence, Leu-Leu-Ala-Asp-Thr-Thr-His-His-Arg-Pro-Trp-Thr, Pic1) was occupied in 66.7% (8/12), Peptide B (8-peptide) in 16.7% (2/12), and peptide C (12-peptide) in 8.3% (1/12). There was only one case without an insert. The analysis of protein showed that there were no homogeneous between peptides A, B, C, and other proteins in Genbank.

The peptide A was synthesized by peptide synthesizer, and the product was purified by HPLC. Purity was up to 98%. The results of radioligand binding assay (RBA) showed that there were some binding activity between synthetically derived OCP and ^{3}H-ouabain. The dissociation constant (Kd) was 1.087 nmol/L, and the receptor density was 120 fmol/mg protein (Fig. 1).

The result of erythrocyte ^{86}Rb uptake showed that low-dose ouabain was probably able to inhibit the sodium pumps on the surface of healthy erythrocytes, and the ratio of the suppression of erythrocyte uptake was up to 50.3% (Fig 2). The experiment verified that OCP was capable of suppressing the inhibition action between ouabain and sodium pump on the surface of erythrocyte, which improved the erythrocyte ^{86}Rb uptake from 60% to 80%. The

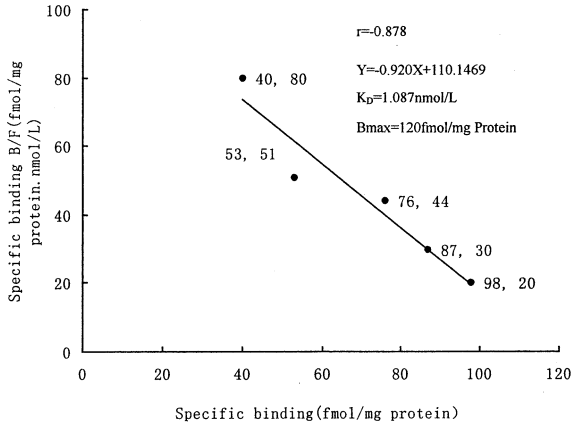

FIG. 1. Scratchard plot showing specific binding capability.

results showed that the efficacy of OCP was in a dose-dependent manner (Fig. 3).

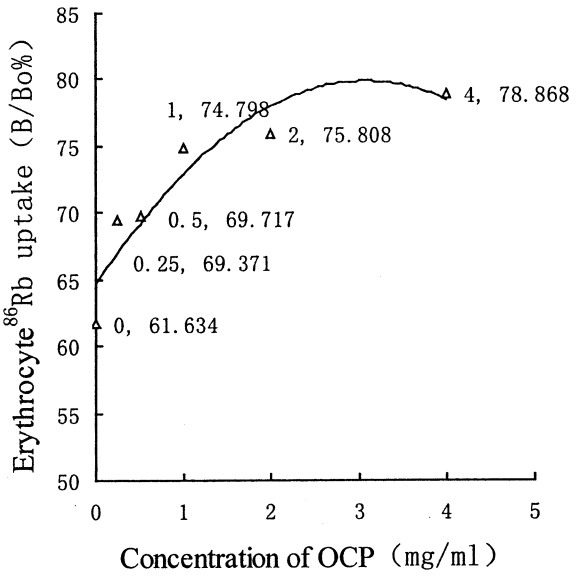

FIG. 3. Antagonistic effect of ouabain-conjugated peptides (OCP) on inhibition of erythrocytes by ouabain.

Discussion

Several reports have identified endogenous ouabain (EO) as a recently discovered adrenal hormone. A number of investigations have reported that higher plasma levels of EO were found in patients with various types of hypertension. Plasma levels of EO are higher in 30% to 45% of patients with essential hypertension and in normotensive individuals with family histories of hypertension.[6,7] Elevated EO levels were found in the plasma of rats with DOCA-saline hypertension and reduced renal mass–saline hypertension, and in Milan hypertensive rats.[13] Furthermore, plasma levels of EO were also found to be elevated in patients with primary aldosteronism, diabetes, and some secondary hypertension.[4,6,7] Multiple regression analysis showed a significant relationship between mean BP and plasma EO. These results suggest that EO probably plays a role in the etiology of the abnormally increased BP in these diseases, either directly or indirectly, by increasing the resistance of blood vessels and leading to elevated BP.

Ouabain and related cardiac glycosides are specific inhibitors of Na^+,K^+-ATPase, the enzyme that carries out the active transport of Na^+ and K^+ across the plasma membrane of most animal cells. First, interaction of cardiac glycoside drugs with the Na^+,K^+-ATPase regulates contractility of cardiac myocyte.[14] Second, infusion ouabain raises vascular resistance.[15] Third, increased sympathetic outflow from the central nervous system has been implicated in ouabain-dependent hypertension.[16] Fourth, prolonged administration of ouabain raises renal vascular resistance and affects renal function to a significant extent[17–19] (Fig. 4).

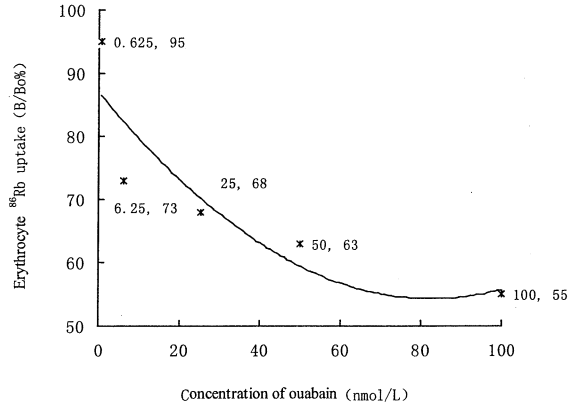

FIG. 2. Inhibition effect of ouabain on erythrocyte ^{86}Rb uptake.

Recent studies have shown that the antiouabain polyclonal antibody was capable of reducing elevated BP to normal levels in animal models of hypertension. In addition, a new type of ouabain receptor blocker, PST2238 (identified by Ferrari et al), was able to compete with EO to bind with sodium pump, so that BP could be markedly reduced.[20,21] However, in recent years, many studies have suggested that sodium pump probably was not only an energy-transducing ion pump but a signal transducer as well.[22] Therefore, interaction of EO and Na^+,K^+-ATPase not only cause partial inhibition of Na^+,K^+-ATPase and increase the concentration of $[Ca^{2+}]i$, but also activate multiple signal transduction pathways, including activation of Src kinase and tyrosine phosphorylation of the epidermal growth factor receptor and other proteins, followed by the activation of Ras and the Ras/Raf/MEK/MARK cascade.[23] Therefore, PST2238 and other sodium pump inhibitors could only partially block the action of ouabain. The results of our study showed that OCP was able to antagonize higher EO in plasma to a greater extent, and also was able to seal the binding site of sodium pump, such that OCP may be able block the action of ouabain through different pathways. This recognition, in turn, will be helpful in developing therapy for hypertension.

With regard to activity of OCP, it is now widely

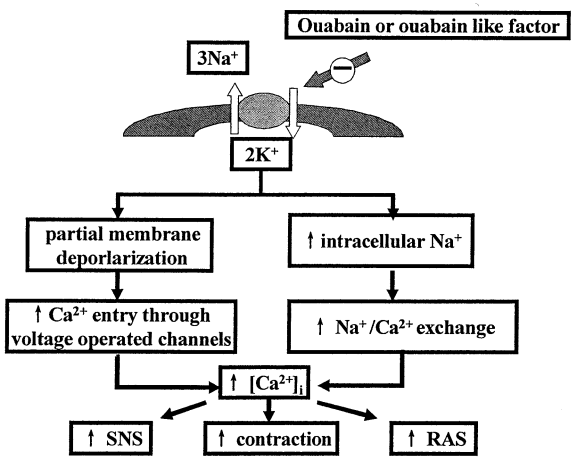

FIG. 4. The possible main mechanism of ouabain-inducing hypertension through inhibiting Na^+, K^+-ATPase activity. Inhibition of the Na^+, K^+-ATPase by ouabain leads to partial membrane depolarization and elevation of $[Na^+]_i$ that in turn leads to activation of voltage-gated Ca^{2+} channels, and Na^+/Ca^{2+} exchanger and elevation of $[Ca^{2+}]_i$. The increase of $[Ca^{2+}]_i$ can be further potentiated via Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum (SR). Elevation of $[Ca^{2+}]_i$ induces vascular smooth muscle contraction, activation of the sympathetic nervous system (SNS), and renin angiotensin system (RAS).

accepted that partial inhibition of the cells Na^+,K^+-ATPase by a cardiac glycoside causes a modest increase in $[Na^+]_i$, which in turn affects the plasma membrane Na^+/Ca^{2+} exchanger, leading to a significant increase in $[Ca^{2+}]_i$ and contractile force. Ouabain binds specifically to the Na^+,K^+-ATPase in cell membrane and inhibits active Na^+/K^+ transport. In extensive investigation, this binding has been demonstrated to occur with a 1:1 molar stoichiometry, and it is generally accepted that the number of specifically bound ouabain molecules closely reflects the number of Na^+/K^+pump units. Additionally, ^{86}Rb, similar to potassium, is actively transported into the cells by ATPase system and is accepted as a measure of its activity.

The substance OCP was discovered by screening phage displayed peptide library, without knowing the structure of OCP in advance.[24,25] The results verified that, first, OCP were capable of binding with ouabain, that Kd was 1.087 nmol/L between ^3H-ouabain and OCP, and that Bmax was 120 fmol/mg protein. Second, low-dose ouabain was capable of inhibiting sodium pump on the surface of membrane of erythrocyte, and the ratio of suppression of ^{86}Rb uptake amounted to 50%. Third, OCP could blocked the inhibition of ouabain to sodium pump on the surface of erythrocyte, and the erythrocyte ^{86}Rb uptake was increased to 80%, and the efficiency in a dose-dependent manner. These results suggested OCP could modify the activity of sodium pump and could also adjust the intracellular and extracellular concentrations of Na, K, and Ca. Thus, it may be useful in developing new antihypertensive agents in the future and for supplying valuable data regarding therapy for hypertension. (Fig. 4)

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