NEIYUANXING WABAYIN YANJIU WENJI

内源性哇巴因

研究文集

主编　吕卓人

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＊ 内源性哇巴因课题研究组成员名单 ＊

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序　言

吕卓人教授和他的课题研究组所编写的《内源性哇巴因研究文集》反映了该研究小组成员多年来刻苦钻研所取得的成果，真实地记载了一大批青年科研人员成长的历程。本书内容丰富，不仅涉及生理和病理生理研究，并且也注意到临床应用，是广大基础与临床工作者可贵的参考资料。

正如作者所说，内源性哇巴因研究的意义不仅是发现和阐明一种新激素，还可能为“心血管内分泌学”这一新的边缘学科的诞生做出贡献，这是内源性哇巴因研究组的理想。我相信在他们的不断努力下一定能成为现实。

由于这个研究组开展了广泛的国际交流与协作，因此他们始终活跃在国内外科研的第一线，不曾遭遇思路的枯竭。尤其值得提出的是这个研究组的学风，那就是追求真理的执着精神、求实严谨的科学态度和刻苦勤奋的工作热情。在当前学术界正被浮躁所困扰之时，这种朴实的学风就更值得提倡与推崇。

中国医学科学院阜外医院教授

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刘力生

2003 年 3 月 11 日

前　言

我的梦……

在硕士研究生期间，导师杨鼎颐教授为我定了“洋地黄”的研究方向，钠泵、洋地黄受体以及以后文献上报道的“内源性类洋地黄物质”（endogenous digitals－like substances, EDLS），使我产生了浓厚的兴趣。1987年，我获得了国家教委公派赴意大利进修的机会，毫不犹豫地选择了在EDLS研究方面十分活跃的Bologna大学心血管病研究所。记得刚到Bologna大学的第一天，实验室主任Boschi博士问我：“你为什么选择内源性类洋地黄物质作为研究方向？有没有思想准备到最后可能什么也搞不出？”因为当时人们对体内是否存在EDLS仍有怀疑。然而，越是严峻的挑战越能激发我探索未知的强烈兴趣和愿望。真正的幸福是在为真理而奋斗的征途中，而不是到达终点的那一刻。我想，如果用一辈子的时间能解决一个难题或解开一个谜，哪怕得到的是阴性结果，也能使后人不再重复走弯路，应该说这辈子也值得！为此，我暗暗下了决心，把EDLS作为我毕生的研究对象。1994年我有幸被国家教委选拔赴美国，从师于在EDLS研究领域有杰出贡献，最为活跃的John M. Hamlyn教授，是他首先确定了EDLS的分子结构和生物学特性，提出EDLS即内源性哇巴因（endogenous ouabain, EO），这不仅消除了学术界对体内是否存在内源性哇巴因的怀疑，而且大大推动了内源性哇巴因领域的研究。在共同研究的日子里，他那执著追求真理的精神、求实严谨的科学态度、刻苦勤奋的工作热情和令人振奋的研究成果给了我极大的鼓舞。在Hamlyn实验室中，我和美国、英国、意大利、爱尔兰、日本、菲律宾、印度、挪威等国家的同事们一起工作，他们中有搞基础研究的，有心血管、内分泌等临床专家，我们经常在一起研讨，有时甚至是争论，大大拓宽了研究思路，形成了一个“International Ouabain Family”（国际哇巴因研究的大家庭）。Hamlyn教授要“在教科书中增添新的一页”的进取心不断鼓励我迎着困难坚定不移地向科学的高峰一步一步攀登。

15年来我和课题组的伙伴们围绕“一种新的激素——内源性哇巴因”开展了系列研究，中标了3项国家自然科学基金项目、2项国家科技部项目、1项国家卫生部科研项目和6项省级研究课题，内源性哇巴因的研究向3个方向展开：

1. 内源性哇巴因的生理学及病理生理学研究：包括内源性哇巴因的纯化、生物学特性、生物合成途径、分泌及调节特性、人体生理分泌规律、内源性哇巴因受体钠泵(亚单位的基因表达特点)等；在病理意义方面的研究包括哇巴因－高血压鼠模型的建立、哇巴因受体(亚单位的基因表达)与高血压的关系、原发性和继发性高血压与哇巴因的关系、内源性哇巴因与肾脏的关系等。研究目标是明确内源性哇巴因作为一种新激素的全貌，包括其分泌、调节、合成等；搞清内源性哇巴因异常分泌的病理生理作用和临床意义。

2. 内源性哇巴因的检测：内源性哇巴因是体内微量的生物活性物质之一，准确地检测其含量是内源性哇巴因研究的“瓶颈”。因此，检测必然成为内源性哇巴因研究领域中的热点和重点之一。我们摸索了包括放射免疫法、免疫放射法在内的多种检测方法，建立了酶联免疫吸附法检测内源性哇巴因。制备了抗哇巴因多克隆抗体、单链抗体、单克隆抗体、噬菌体抗体等，申请专利1项。但是到目前为止还未能获得满意的检测方法。理想的方法是能用一滴血直接获得准确的数据，并能制成检测试剂盒，在临床上推广应用。这有待于更深入地研究并取得突破。

3. 应用研究：理论和实验研究最终要走出实验室，应用于临床，并进入产业化，直接为人类造福。既然一系列研究结果证明过高的内源性哇巴因与高血压等病理状态有关，这就有可能通过干预，降低过高浓度内源性哇巴因所引起的不良作用，从而达到新的平衡，使患者恢复健康。我们的系列研究结果证明上述假说是正确的，而且正在一步步向临床应用的目标接近。我相信，总有一天事实将表明我们提出的“第七类抗高血压药物——钠泵抑制阻断剂”并非是幻想。

内源性哇巴因研究的意义可能还不仅仅是发现和阐明一种新的激素，至今为止内源性哇巴因和内啡肽是被发现的仅有的两种既存在于哺乳动物体内又可在植物中发现的物质。这可能具有更深层次的含义，值得深入研究。此外，随着对这种新的具有明显心血管调节功能的激素认识的深入，也使我们联想到传统的心血管病学与内分泌学之间有着许多内在的联系：内分泌功能异常可影响心血管功能，心血管疾病可并发内分泌功能的紊乱，体内还存在一系列如心房肽、脑钠素、内源性哇巴因等直接参与心血管功能调节的激素，这奠定了一门新的边缘学科——“心血管内分泌学”诞生的基础。如在有生之年，能对这门学科的建立做出一些微薄的贡献，这将是一个美妙的梦……

15年来，我和课题组同事们发表了90余篇论文，不仅勾画出内源性哇巴因研究的一个轮廓，也使我国在世界该领域的研究中有了一席之地，在世界学术讲台上能听到我们中国人的声音。近年来我们不断接到国内外对内源性哇巴因研究感兴趣的学者们的来信，索取论文或进行讨论，为此我们萌发出

版课题组在内源性哇巴因方面的研究论文集，这不仅是课题组成员们15年来孜孜不倦、刻苦钻研、勇于探索的成果和结晶，也是一大批年轻的硕士、博士们成长历程的真实记载。如今其中的大部分仍活跃在国内外科学研究的第一线，并取得了出色的成绩。对于长期以来各级组织，包括国家科技部、国家自然科学基金会、卫生部、陕西省科技厅、卫生厅和西安交通大学的领导、国内许多心血管界和内分泌界老前辈的热情关怀和帮助，Hamlyn教授、Hamilton教授、Boschi博士等专家的潜心指导和鼓励，以及家人亲友们的理解和全力支持，文集也是奉献给他们的一份礼物，并借此文集出版之际向他们致以真挚的谢意。葛蘅博士负责全部论文的收集、整理和校对工作，文集的出版凝集了她的无私奉献和辛勤劳动。作为一个系列研究比较完整的论文集，反映了课题组的研究思路和步骤，以供同道们交流、批评和指正。我们期待这一文集能对我国心血管内分泌领域的研究有所推进。如能这样，也就圆了我的梦。

西安交通大学医学院

吕卓人

2002年11月22日

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第一部分

论　文

——内源性哇巴因的生物学特性研究——

The Partial Purification and Detection of Endogenous Sodium Pump Inhibitor (s) from Human Plasma

Lü Zhuoren(吕卓人), Boschi Stefano*, Borghi Claudio*, Munarini Alessandra*
(Institute of Cardiology, Xi'an Medical University, Xian 710061, China)

Abstract In the present paper, the methodology of purification and detection of endogenous sodium pump inhibitor(s) (ESPI) from human plasma was introduced. Furthermore, some biological properties of the plasma ESPI were found: ① inhibition of Na^+, K^+ – ATPase; ② inhibition of sodium pump activity; ③ competive inhibition of ^3H – ouabain binding to RBC receptors or ATPase; ④ "dose – response" inhibition; ⑤ reversible inhibition; ⑥ stabilizing the enzyme E_2 conformation.

Key words endogenous sodium pump inhibitor; purification; Na^+, K^+ – ATPase; high – performance liquid chromatography(HPLC)

The accumulation of experimental evidence suggests that an endogenous sodium pump inhibitor(s) (ESPI) which has natriuretic, pressor, vascular sensitizing and digitalislike activities may be involved in regulating cation transport, sodium excretion by kidney and vascular reactivity in arterioles, and may participate in the development of hypertension[1, 2]. But some contradictory results obtained with laborotary-specific preparation and assay methods were reported. Unfortunately, the digoxin radioimmunoassay(RIA) which is employed widespreadly to detect ESPI is the least reliable among the methods for the detection of ESPI, since some compounds may bind non – specifically to the antibody[3]. In this paper, the methodology of the partial purification and detection of ESPI from human plasma will be introduced, which was developed by Boschi in the Institute of Clinical Pharmacology of Bologna University, Italy. Also, some biological properties of ESPI will be discussed.

METHODS

Sample preparation and extraction

Plasma was applied to a C18 disposable column (SPE 7020 J T Baker USA) and eluted with the mixture of acetonitrile (CH_3CN) and trifluoroacetic acid (TFA) 0.15% (40/60).

Purification with high – performance liquid chromatography (HPLC)

HPLC on a reverse – phase column (LiChrosorb Merck RP18) was carried out using increasing linear concentration gradient of 80% CH_3CN at a flow rate of 1ml/min. The absorbance wavelength was monitored at 225 nm. Fraction collector (Gilson Model 201) was applied and 20 fractions were collected within 60 min. For further purification, Fraction 1 ~ 4 were subjected to HPLC on Column DIOL (LiChrosorb) with a decreasing linear gradient of 5% to 99.9% (1 ml/min). Also, 20 fractions were collected (UV absorbance at 225 nm).

The $\gamma - ^{32}P$ ATP method

HEPES-Mg^{2+} buffer was prepared with $MgCl_2$ 5mmol/L, EGTA 1mmol/L, BSA 2mg/ml, HEPES 80 mmol/L HEPES – Tris buffer contained $MgCl_2$ 6 mmol/L, HEPES 160 mmol/L, NaCl 300 mmol/L, KCl 20mmol/L, EGTA 6 mmol/L, ATP 4 mmol/L and $\gamma - ^{32}P$ ATP were put in HEPES – Tris

* Institute of Clinical Pharmacology, Bologna University, Italy

buffer to be $\gamma-^{32}P$ ATP buffer. The mixture of ATPase or EDTA, Norepinephrine (NE) and fractions or HEPES – Mg^{2+} buffer was preincubated at 37℃ for 30 min. Then, $\gamma-^{32}P$ ATP buffer was added for incubation 20 min. It was stopped by addition of cold perchloric acid(20%). Labeled inorganic phosphate was separated from ATP by absorption of ATP on charcoal. Free phosphate inos were counted in a Liquid Scintillation counter.

Coupled enzyme assay

Tris – Mg^{2+} buffer contained Tris 40 mmol/L, $MgCl_2$ 10 mmol/L. Solution of kinetics consisted of NaCl 104.2 mmol/L, KCl 20.83 mmol/L, $MgCl_2$ 5.2mmol/L, HEPES 20.83 mmol/L, DTT 1.042mmol/L. Reaction mixture had ATP 2.6 mmol/L, NADH 0.26 mmol/L, PEP 1.4 mmol/L, LDH 10 U and PK 10 U in the solution for kinetics. BSA and EGTA were put in an aliquot, and dried in a lyophilizator for measurement. The reaction was initiated by rapid addition of NE, Tris – Mg^{2+} buffer or fractions and ATPase in the aliquots. After the preincubation, it was mixed with the reaction mixture. The oxidation of NADH was monitored by recording the decrease in absorbance at 340 nm with a spectrophotometer (BECKMAN DU-5).

Inhibition of ^{86}Rb uptake by erythrocytes

Fresh human red cells were washed and suspended with Ringer's buffer (NaCl 140 mmol/L, MgSO4 1mmol/L, Na_2HPO_4 5 mmol/L, Glucose 5.6 mmol/L, KCl 5 mmol/L and $CaCl_2$ 1 mmol/L). The numbers of cells were measured with a Coulter Counter. The cells were preincubated with or without fraction and Ringer's buffer or unlabeled ouabain(2×10^{-3}mol/L) for measurement of non-specific uptake. Transport was initiated by addition of ^{86}Rb, and they were incubated for 1h, The reaction was stopped by addition of cold buffer. Cells were separated by alternate wash with Ringer's buffer and determined for ^{86}Rb radioactivity in a Liquid Scintillation Counter.

Inhibition of ^{3}H – ouabain binding to erythrocytes

Fresh red cells were washed and suspended with Tris buffer (NaCl 130 mmol/L, Tris 10 mmol/L, Glucose 10 mmol/L, Sucrose 20 mmol/L), Paralled incubation of red cells and ^{3}H-ouabain was performed with addition of an excess of unlabeled ouabain (2×10^{-3}mol/L), for measuring nonsaturable binding, and with or without fractions, After 5h-incubation, the binding was stopped by rapid cooling. Bound and free radioactivites were separated by filtration on glass filter (Whatman GF/F). The filters were washed with cold Tris buffer and counted in a Liquid Scintillation Counter.

Inhibition of ^{3}H – ouabain binding to ATPase

The assay was similar to that of ^{3}H – ouabain binding to erythrocytes. The differances only were the buffer (NaCl 100 mmol/L, Tris 50 mmol/L, $MgCl_2$ 3mmol/L) and the incubation time (1h).

Inhibition of p-nitrophenylphosphatase (pNPPase) activity

The preparation and the preincubation were the same as described for the coupled enzyme assay. After preincubation, pNPP buffer (Tris 51.02 mmol/L, $MgCl_2$ 3.06 mmol/L, KCl 10.2 mmol/L and pNPP 3.06 mmol/L) was added and the mixture was incubated. The reaction was stopped by addition of 20% cold trichloroacetic acid (TCA) and a portion of the supernatant fluid was added with Tris 1 mol/L. The hydrolysis of pNPP was monitored spectrophotometrically at 420nm.

"Dose – response" for inhibition

Fractions were added into aliquots in increased concentrations. The "dose-response" for inhibition was measured by means of the $\gamma-^{32}P$ ATP assay.

Reversibility

The reversibility of the inhibition to ATPase was studied using the coupled enzyme assay. The control or sample was mixed with the reaction mixure and then measured for enzyme activity at 340 nm using the kinetic assay with a spectrophotometer. After addition of NaCl(120 mmol/L) and EDTA (15mmol/L), the same kinetic assay was performed with each interval of 30 min.

RESULTS

Purification with HPLC and inhibition of Na^+, K^+ – ATPase activity of fractions with the $\gamma-^{32}P$ ATP assay

HPLC of CH_3CN/TFA extract of plasma on RP18 column showed several peaks. The first peak of absorbance eluting with a retention time from 3 to 6 min was found to have high inhibition of ATPase activities. The Fractions 1 ~ 4 pool were further chromatographed on DIOL coulmn and the second Fractions 4 ~ 7 pool were detected to have high inhibition of Na^+, K^+ – ATPase activity.

"Dose – response" for inhibition to ATPase

The results showed a"S" curve following the increase

of the concentrations both of Fractions 1 ~ 4 pool from HPLC on RP18 column and Fractions 4 ~ 7 pool from the further purification.

Reversibility of the inhibition to ATPase by ESPI

The inhibition of ESPI was measured dynamically. It showed the decline of the inhibition after addition of Na^+ and EDTA.

Inhibition of ^{86}Rb uptake by erythrocytes

The results showed that the inhibition was increased as the concentrations of the second Fractions 4 ~ 7 pool increased.

Inhibition of ^{3}H – ouabain binding to erythrocytes and ATPase

Specific binding of ^{3}H – ouabain was inhibited by ESPI and the inhibition of binding was paralleled to the amounts of ESPI.

Inhibition of pNPPase activity

The inhibitions were increased with the increase of the amounts both of Fraction 1 ~ 4 pool (RP18 column) and Fraction 4 ~ 7 pool (DIOL column).

DISCUSSION

Like all enzymes, Na^+, K^+ – ATPase is readily inhibited by numorous substances in human plasma, for example, free fatty acids (FFA), peptides, lipids, proteins, endogenous compounds, waste products and cations[1]. For this reason, four steps of purification were employed in our experiment: treatment of plasma by a disposable C18 column, elution with CH_3CN/TFA, HPLC on RP18 column (lowsurface-energy, aliphatic hydrocarbon, bonded-phase packing with relatively nonpolar suspending liquid) and DIOL column (hydrophilic organic-modified silicas with relatively polar liquid)[4] with CH_3CN/H_2O. Furthermore, in our Na^+, K^+ → ATP ase inhibition assay, some substances were served for reducing inferences. Albumin can bind FFA and NE can block the vanadate inhibition by chelating. Additionally, EGTA shows a great capacity to chelate heavy metals and Ca^{2+} without significantly chelate Mg^{2+} at physiological pH[5].

Na^+, K^+ – ATPase activity can be measured by either the γ-^{32}P ATP assay or the coupled enzyme assay. In the former the activity was assayed with measuring the radioactivity of ^{32}P liberated from γ-^{32}P ATP hydrolyzed by ouabin-sensitive Na^+, K^+ – ATPase[6]. It is a direct, simpler and more sensitive assay, and many samples can be measured in the same time with this assay. But it can not be applied in the kinetic study of the ATPase activity because the concentrations of ADP increases gradually during ATP hydrolysis, which is an ATPase inhibitor. On the contrary, the coupled enzyme assay is appropriate in the continuous observation for Na^+, K^+ – ATPase inhibition because constant ATP concentration is maintained and the accumulation of ADP is prevented[7, 8]. However, the coupled enzyme assay is less sensitive and only one sample can be measured each time with this assay.

^{86}Rb is commonly used to investigate sodium pump activity as it substitutes for potassium at potassium binding sites on the ATPase[3]. Also, the human erythrocyte is usually served as a model for ouabin-sensitive ^{86}Rb uptake and ^{3}H-ouabain binding. Ouabain is well-known to bind RBC receptors (Na^+, K^+ – ATPase or sodium pump). The reduction of ^{3}H-ouabain binding on RBC or ATPase as parallel incubation with fractions indicates that the ESPI was competively bound to the specific ouabain binding sites.

Na^+, K^+ – ATPase is thought to exist in two major conformational states, E_1 and E_2. The E_1 conformation binds and is stabilized by sodium and ATP, but the E_2 is stabilized by potassium or phosphate[9]. Hydrolysis of the pseudosubstrate pNPP is a Na^+ – independent, K^+ – dependent reaction of the ATPase, which apparently requires only the E_2 conformation [10, 11]. Therefore, the inhibition of pNPPase activity by ESPI suggests that the Na^+, K^+ – ATPase inhibition results from the ESPI stabilized in E_2 conformation. Moreover, in the reversibility study, the inhibition of ATPase activity was abolished by addition of sodium and EDTA which change the enzyme to the E_1 conformation[5]. The results of the both studies support that the E_2 conformation is probably involved in the binding of ESPI.

In conclusion, some bioloigical properties of the plasma ESPI were found in the present study and consistent with those characterized by some investigators[7, 12, 14], including: ① inhibiton of Na^+, K^+ – ATPase activity; ② inhibition of sodiun pump activity; ③ specific competive inhibition of ^{3}H – ouabain binding to RBC receptors or Na^+, K^+ – ATPase binding sites; ④ "dose – response" inhibition; ⑤ reversible inhibition; ⑥ stabilizing the enzyme in E_2 conformation. Although a great deal of efforts in many laboratories have been exerted, a purified ESPI of known structure is not in hand. Further study is necessary to identify the ESPI, which requires complete purification and determina-