REGULATION OF ENDOGENOUS OUABAIN-LIKE FACTOR PRODUCTION IN THE ADRENAL GLAND AND IN VOLUME EXPANDED PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STATES

PhD thesis

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INTRODUCTION

Cardiac glycosides (or cardiotonic steroids) are compounds with steroid structure. They are known to inhibit the membrane bound Na\(^+\)/K\(^+\)-ATPase, which is present in every cell. When the Na\(^+\) pump is inhibited, the intracellular Ca\(^{2+}\) increases, which explains the positive inotropic effect of these compounds. There was a long search after an endogenous substance that inhibits Na\(^+\)/K\(^+\)-ATPase, is natriuretic and would be involved in the pathogenesis of essential hypertension. It was shown recently that the endogenous “digitalis-like compound” (DLF) is identical to ouabain. However other compounds like bufodienolides may represent another family of the endogenous cardenolides.

The reason why we decided to study ouabain-like factor was, that OLF was previously investigated in our institute. We wanted to understand how endogenous OLF production is regulated and extend our knowledge about its role in the physiological regulation of electrolyte and fluid homeostasis.

AIMS OF THE STUDY

I. Raising ouabain antibodies to

(1) develop a sensitive and specific radioimmunoassay,

(2) identify OLF producing organs by immunohistochemistry.

II. Studying regulation of endogenous OLF secretion in vitro

(3) Adrenals are the sources of several hormones regulating ion and fluid homeostasis like even as aldosterone, atrial natriuretic peptide (ANP), angiotensin-II (A-II) to be mentioned. As the adrenal cells have been found to be immunopositive for DLF, we proposed to use human and rat adrenal tissue slices and cells in static in vitro model systems to study OLF secretion. We wished to determine which type of cells produce OLF in the rat adrenal cortex. We also wanted to identify factors regulating adrenocortical OLF secretion by measuring levels of untreated and stimulated (ACTH, A-II, ANP) rat cells’
supernatant. We also used human cells’ originating from normal adrenals, various adrenocortical tumours including accidentally diagnosed small adrenal incidentalomas.

(4) The low subnanomolar plasma level of ouabain in normal subjects opposite to the high OLF level in the adrenals suggests that it could have not only endocrine but also paracrine actions. It has been recognized long ago that the regulation of aldosterone secretion is multifunctional as to the effect of ACTH, A-II, ANP, or potassium. Among the numerous paracrine regulators now OLF has been emerged to be a new factor. Using the same model system described in (3), **paracrine effect of ouabain on in vitro aldosterone secretion and its interactions with the above-mentioned factors were investigated.**

III. Studying plasma level of OLF in volume expanded physiological and pathophysiological conditions: *in vivo* studies

(5) As levels of digitalis-like factors were shown to be increased in conditions with volume expansion, we wanted to measure **endogenous OLF from plasma and urine** samples of healthy volunteers, of patients with diabetes and/or hypertension, of pregnant individuals with hypertension and of mature and premature newborns.

(6) Using an *in vivo* model of volume expansion induced cardiac hypertrophy we performed an aortocaval shunt in rats. We intended to study correlation between the **development of cardiac hypertrophy and changes in blood OLF concentration** during a certain amount of time in these animals. As the possibility arose that one of the main sources of endogenous OLF are the adrenals we included shunted animals with adrenalectomy in our experiments.
METHODS

Immunization of rabbits

Ouabain-BSA conjugate in Freud complete adjuvant was used to immunize rabbits. Booster injections were given three times with an interval between injections of two weeks. Blood samples were taken weekly after booster injections and their anti-ouabain titers were analyzed using RIA.

Immunohistochemistry

Rats were perfused with Zamboni’s fixative (paraformaldehyde/picric acid) and adrenals, heart tissue (atrium and ventricle), and brain were paraffin embedded. Ouabain antiserum was used at 1:3000 dilution for 48 hours, and after incubation with anti-rabbit IgG colour was developed using the ABC Elite kit.

Static incubation systems

Rat adrenal capsule-glomerulosa preparations and fasciculata tissue slices, rat zona glomerulosa and zona fasciculata cells obtained by collagenase digestion of rat adrenal capsular strippings and decapsulated adrenal glands, collagenase dispersed human adrenocortical cells were incubated for 2 hrs with or without different concentrations of $K^+$, ACTH, A-II, ANP, ouabain, acetylcholine, nicotine and nicotinic receptor antagonists.

Adrenalectomy and abdominal aortocaval (AV) shunts

The adrenals were removed through the retroperitoneum and for aortocaval shunt the Garcia-Diebold method was used. Briefly, the aorta was punctured with a 18 gauge needle, then the neighbouring walls of the aorta and vena cava were perforated. The aortic puncture point was sealed with a drop of cyanoacrylate glue.

Biochemical methods

Plasma and urine samples, and adrenal supernatants were purified by SepPak C18 solid-phase extraction for adrenomedullin and ouabain radioimmunoassay.
Radioimmunoassays

Ouabain-like immunoreactivity was determined by RIA developed in our laboratory.

Radioimmunoassay was used for measurements of ANP, adrenomedullin, aldosterone and corticosterone.

High performance liquid chromatography (HPLC)

Plasma and urine samples, adrenal cortex cells’ supernatant, incubation media, commercial ouabain, and water extracted adrenal parts were chromatographed after SepPak extraction, and eluted with increasing concentration of acetonitrile.

RESULTS

I. Raising ouabain antibodies

(1) We developed a highly sensitive and specific ouabain-radioimmunoassay using $^3$H-ouabain tracer, which we further improved by using iodinated ouabain. This assay made it possible to measure low (physiological) OLF levels.

(2) Using our antibody we showed presence of ouabain-immunopositive cells in the adrenal cortex.

II. Regulation of endogenous OLF secretion in vitro

(3) Using dispersed rat adrenocortical cells we provided evidence that not only the glomerulosa but also the fasciculata/reticularis cells produce OLF. Using reverse phase HPLC we proved that endogenous OLF co-elutes with authentic ouabain. We demonstrated that in the human adrenals and in the rat zona glomerulosa besides ACTH and angiotensin-II the extracellular [K+] modulates endogenous OLF secretion. In the rat zona fasciculata only ACTH and angiotensin-II had stimulatory effect. As “the major regulator” of endogenous ouabain we showed the striking stimulatory effect of nicotine on rat adrenocortical cells. Acetylcholine and eserine modulated OLF production in zona glomerulosa biphasically. In zona
fasciculata Ach had no effect, but eserine stimulated OLF secretion. ACTH strongly potentiated the OLF stimulatory effect of nicotine in the zona glomerulosa, however we were not able to show significant interactions between nicotine and ACTH or A-II on the OLF production of faciculata/reticularis cells.
To provide pharmacological evidence that nicotinic acetylcholine receptors may be involved in the regulation of OLF, we used the ganglionic blocking hexamethonium and mecamylamine, and the ?7-receptor antagonist methyllycaconitine (MLA). The ganglionic blocking compounds further potentiated the effect of nicotine. MLA dose-dependently inhibited the effect of nicotine in the glomerulosa, however in the faciculata/reticularis cells it potentiated the nicotine-induced OLF secretion.
We showed OLF production in human adrenocortical incidentalomas, which was modulated by the extracellular potassium concentrations.
We also found that nicotine and eserine dose-dependently inhibited adrenocortical aldosterone secretion, but acetylcholine had no effect. The ganglionic blocking compounds and MLA (in lesser extent) antagonized the inhibitory effect of nicotine.
(4) Studying the paracrine effect of OLF we found that ouabain at 10^{-4} M increases aldosterone production however, higher and lower concentrations of the cardenolide did not have any significant effect on the aldosterone secretion. We also found that ouabain interacts with ANP and angiotensin-II at different extracellular [K^+] on the aldosterone secretion.

III. Studying plasma level of OLF in volume expanded conditions: \textit{in vivo} studies

(5) Analyzing OLF in volume expanded states we found elevated plasma OLF levels in patients with moderate form of congestive heart failure, which may contribute to the compensated state of this disease. We also showed that during pregnancy diabetes further augments plasma and urinary OLF concentrations. As the plasma OLF level was more elevated in gestational diabetes than
in pregnant women with IDDM, our data may explain why gestational diabetes predisposes more often to pre-eclampsia. As another novel result we found correlation between gestational age and plasma immunoreactive OLF levels in newborns and showed that mature infants have lower OLF level at birth than premature newborns, which can provide important new information for newborn physiology.

(6) In our in vivo experiments we found that in volume overloaded rats during the development of cardiac hypertrophy endogenous OLF and adrenomedullin are substituted after adrenalectomy from other organs to provide positive inotropic substances to the failing heart. This observation further supports the role of these hormones in the compensated state of heart failure.

CONCLUSIONS

In conclusion, we provided new evidence on localization, intra-adrenal regulation of endogenous ouabain-like factor, and its interaction with endogenous vasoactive substances. Also, we identified nicotinic regulation as the major modulator of OLF, and provided data for its role in diabetes, during development of cardiac hypertrophy, and in newborns.

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PUBLICATIONS

List of publications related to the thesis

Original academic contributions:


modulated by nicotinic mechanisms in rat adrenocortical cells. (Submitted for publication: Life Sciences).

Abstracts

International congresses (31 total)


Hungarian congresses (12):


Other publications

Original academic contributions:


Gööz, Monika, Maria Shaker, Pal Gööz, Adam J. Smolka: Role of Cytokines in Helicobacter pylori-Induced Gastric Epithelial Cell Matrix Metalloproteinase Secretion and Activation. (Under revision: Gut).

**Abstracts**


Gööz M, CH Hammond, KA Larsen, AJ Smolka. Host-specific sensitivity of H,K-ATPase \(^{-}\)-subunit gene 5'-flanking sequence to


**M. Gööz, JR. Raymond, MN. Garnovskaya.** Cross talk between serotonin (5HT2A) and epidermal growth factor receptors (EGFR) involves heparin-binding EGF-like growth factor (HB-EGF) and activation of metalloproteinase-like enzyme(s) in rat mesangial cells. International Congress of Nephrology, June 8-12, 2003 Berlin, Germany.


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