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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1. INTRODUCTION

Cardiac glycosides (or cardiotonic steroids) are compounds with steroid structure containing a 17ß unsaturated lactone ring, a 14ß -hydroxyl group and a sugar molecule in the 3ß position [**Figure 1**]. Cardenolides or butenolides are cardiac glycosides containing a five member lactone ring. Another group of glycosides, bufadienolides have a six member lactone ring. Cardiotonic steroids had been identified not only in plants, but in toad skin also [for review see 1].

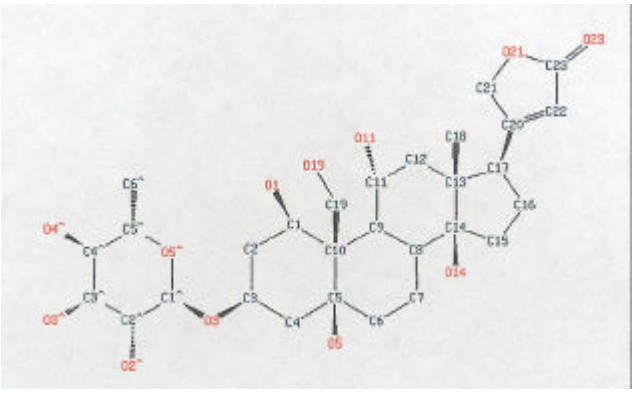

Figure 1. The steroid structure of ouabain (Strophantus gratus) contains a 17? unsaturated lactone ring and a sugar molecule in the 3? position.

Almost every cell has a transmembrane Na^+/K^+-ATPase that transports sodium out of the cell and potassium into cell in stochiometric relations. This Na/K pump is essential for maintaining the normal membrane potential of cells. It is of great importance that the cells consume 20-40% of their energy for sustaining the pump. It was demonstrated as early as during 50'ies [1, 2], that exogenous cardenolides are potent inhibitors of this membrane bound receptor/enzyme. When the Na^+/K^+-ATPase is inhibited, the intracellular Ca^{2+} increases. After the discovery of the Na^+-Ca^{2+}-exchange protein [3], the link between Na^+-pump inhibition and the rise in intracellular Ca^{2+} was elucidated. This also explained the well-known cause of the positive inotropic effect of cardenolides. The question was raised, whether these widely distributed receptors serve only for the binding of exogenous ligands.

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. Already in 1885 Ringer mentioned that some organic constituents of the blood could have an effect on the contractility of the ventricle [4]. In 1953 Szent-

Györgyi made a prophetic suggestion [5]: “We need not to be astonished if we find digitalis-like substances in any cell…. The digitalis cardiac glycosides, if I may say so, are no drugs at all: they are substitutes for a missing screw in our machinery, which has a cardinal role in one of the most basic physiological regulations.”

By the time our investigations started in the year of 1993, presence of a natriuretic and hypertensinogen substance in volume expansion had long been debated (De Wardener *et al.* [6]). The terms “endogenous digitalis-like factor” (EDLF) and “endogenous ouabain-like factor” (OLF) had appeared in the literature. By that time Hamlyn and *his coworkers* had demonstrated that EDLF is identical to ouabain [7], and evidences unfolded about the existence of other digitalis-, and bufadienolide-like substances in mammals (see details in “Background”). The fight about the nature of “the endogenous digitalis” began and for years the most important subject of the scientific investigations was to prove whether it is closely related to ouabain or it is rather a digitalis, a bufadienolide or even a peptide. Our group was among the first to suggest in 1996, and again in 1998 [8], that similarly to the mineralocorticoid/ glucocorticoid hormones there is a family of ouabain-like and digoxin-like factors that are mainly produced in the adrenal cortex.

The reason why we decided to study ouabain-like factor was, that OLF was previously investigated in our institute [9, 10]. 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.

In the first part of my thesis I present the development of an ouabain radioimmunoassay and show immunohistological data obtained with the antibody raised by our group. In the second part, I summarize the results of our *in vitro* investigations on the regulation of endogenous ouabain-like factor secretion. The third part of my work provides *in vivo* data on plasma level of endogenous OLF in volume expanded states and further validates our assay.

2. AIMS

The aims of the present study were (1) to establish the proper method to measure endogenous OLF and to identify the organ(s), which produces OLF in the body as the target of our investigations. In addition, (2) we aimed to study regulation of endogenous ouabain-like factor production *in vitro* and (3) to characterize ouabain secretion *in vivo* under physiological and pathophysiological conditions.

I. Raising ouabain antibodies to develop a radioimmunoassay and to identify OLF producing organs

(1) To measure endogenous ouabain-like factor levels in *in vivo* and *in vitro* systems first we aimed to **develop an ouabain radioimmunoassay**. As ouabain antibodies were not available commercially at the time of the beginning of our studies, we proposed to raise highly specific polyclonal ouabain antibodies in rabbits and develop a relatively sensitive radioimmunoassy, which is suitable for the serial measurements of OLF.

(2) Using our ouabain antibody we wished to **map the localization of endogenous OLF in the mammalian** body. For this purpose, we wanted to use rat tissues. As there were observations in the literature about digitalis-like factor (DLF) producing cells in the central nervous system, especially in the hypothalamus and some other tissues including the adrenals, we focused our attentions to these areas. We supposed that OLF is present in tissues, which directly or indirectly regulate ion/fluid homeostasis.

II. 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. Plasma level of endogenous 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 a possibility arose that one of the main sources of endogenous OLF are the adrenals we included shunted animals with adrenalectomy in our experiments.

3. BACKGROUND

3.1. The short history of cardiac glycosides

Plants containing compound called now cardiac glycosides were known already by the ancient people in Egypt 3000 years before, and they were utilized as poisons or as drugs, mainly erroneously. The Romans used the bulb of the "sea onion" (Scilla maritima) as a diuretic, heart tonicum, and rat poison. Chinese employed the dried skin of the common toad for centuries as a drug. Foxglove was described botanically in 1550 by Fuchsius, who named it Digitalis purpurea. In 1785, William Withering, an English physician and botanist described in his famous book, entitled *An Account of the Foxglove and Some of Its Medical Uses: with Practical Remarks on Dropsy and Other Diseases* in detail the clinical effects and indications of the extract of Digitalis purpurea. He pointed out its toxic effects, too. Besides foxglove and Scilla maritima cardiac glycosides like strophantin and ouabain are obtained also from Strophantus hispidus and Strophantus gratus, individually [**Figure 2**]. During the nineteenth century, digitalis was used for many disorders indiscriminately. In the twentieth century, it was mainly employed in the therapy of congestive heart failure and later in the treatment of atrial fibrillation [11].

Figure 2. Strophantus gratus. Photo copy-right Henriette Kress, http://www.ibiblio.org/herbmed, with permission.

3.2. Na^+/K^+-ATPase – receptor for cardiac glycosides

In 1953, before the discovery of the sodium pump Schatzmann demonstrated the inhibitory effect of cardiac glycosides on active movement of sodium and potassium across the red cell membrane [1]. Four years later Skou described the presence of the Na^+/K^+-ATPase in peripheral nerves [2]. Cardenolides were shown to

be potent inhibitors of the membrane bound Na^+/K^+-ATPase. This receptor/enzyme maintains low cytosolic Na^+ and high K^+ concentrations in all mammalian cells, using the metabolic energy of adenosine 5'-triphosphate (ATP). The pump consists of ? and ß subunits, both of which occur in multiple isoforms [for comprehensive review on Na^+/K^+-ATPase see references 12 and 13]. The isoforms are encoded in separate genes and their expression is both tissue- and cell-specific. However, the isoforms can be located in different region of the same cell. The catalytic ? subunit that is responsible for the transport properties for the enzyme contains the binding site for the cations, ATP, and the sodium pump inhibitor ouabain [13]. Low (? 1) and high (? 2, ? 3, ? 4) affinity isoforms of the subunit for ouabain were characterised [14], which also vary in their affinities for ions. However, ouabain affinity of the same isoform differs in a few species including the rat [13]. Glycosylated ß subunits (ß1-3) are essential for the normal activity of the enzyme. They are involved in the modulation of cation affinity of the sodium pump, and in vertabrates they have possible role in stabilizing the correct folding and transportation of the ? subunit to the plasma membrane [13]. A third protein (? subunit) was also described. It belongs to a family of small membrane proteins, which induce cation-selective channels when expressed in *Xenopus* oocytes. There is evidence that this subunit can modify sodium pump activity, however the exact role of the ? subunit in the Na^+/K^+-ATPase function is not clarified yet [13].

3.3. Intracellular effect of cardiotonic steroids

The general knowledge about the action of cardenolides is that these compounds exert their effect by inhibiting Na^+/K^+-ATPase, thus elevating cytosolic Na^+ concentration, which leads to increase in cytosolic [Ca^{2+}] through activating the Na^+-Ca^{2+}-exchange protein [3, 15]. The entering Ca^{2+} is then rapidly sequestered in the sarcoplasmatic and endoplasmatic reticulum allowing additional Ca^{2+} to be mobilised when the cells activated next time [11, 15]. This observation is verified using relatively high ouabain concentrations (1-1000 µM) [15, 16]. However, using therapeutic (nanomolar) concentrations of the compound, which induced hypertension in rat and augmented vasoconstriction, little or no change in intracellular [Na^+] and cytosolic resting [Ca^{2+}] was observed [16, 17]. Using immunohistochemistry, distribution of

functionally different isoforms in various cells, including myocytes, was studied and reticular occurrence of high ouabain binding isoforms in the plasma membrane that paralleled the underlying endoplasmatic or sarcoplasmatic reticulum was shown [18]. It was suggested that ? 1 isoform may regulate bulk cytosolic Na^+, whereas high ouabain binding sodium pumps may regulate reticulum Ca^{2+} content by modulating Na^+ and Ca^{2+} concentration in a restricted cytosolic space [19]. Further confirmation of this theory was accomplished by using low doses of ouabain (3-100nM), and human ouabain-like compound (see below) on primary cultured rat mesenteric arterial myocytes [20]. Augmented hormone-evoked mobilisation of stored Ca^{2+} without increasing bulk cytosolic [Na^+] was observed. The mobilisation of Ca^{2+} was inhibited by Mg^{2+}, and was dependent on extracellular [Na^+]. These results provided mechanistic details for the cardiotonic and vasotonic actions of low and evidently therapeutic doses of cardenolides [16, 21].

3.4. Novel effects of cardiac glycosides

The sodium-potassium pump (Na^+/K^+-ATPase) is the only known plasma membrane receptor for cardiac glycosides till now. However, recent studies suggest the presence of other binding site(s) for the glycosides on the cell surface. Purdy *et al.* demonstrated that ouabain is able to convert the 5-HT_{2A} receptor from low to high efficacy state and thus to increase its sensitivity to serotonin in rabbit ear artery [22]. Studying various ouabain analogs, Manunta *et al.* found negative correlation between the hypertensinogenic and Na^+ pump inhibitory ability of the compounds [23]. They suggested that ouabain may have a mechanism of action independent of the sodium pump. Using high extracellular potassium solution, which suppressed the binding of ouabain to the Na^+-pump, Ward *et al.* showed high affinity ouabain binding to a novel class of sites [24]. These were present on bovine adrenocortical cells and membranes, but were not detected in skeletal muscle or in liver membranes. Ouabain binding to these novel sites were stimulated by high extracellular potassium concentrations but was not affected by either aldosterone or cortisol. The authors speculated that these new receptors might be involved in the regulation/secretion of endogenous ouabain.

3.5. Endogenous inhibitors of the sodium pump – endogenous "digitalis-like" compounds

After the discovery of the sodium pump the question was again raised, whether this widely distributed receptor serve only for the binding of an exogenous ligand. As the binding site for cardiac glycosides is highly selective for compounds with the appropriate stereochemistry [25], it was supposed that the exogenous and endogenous ligands belong to the same chemical class; i.e. the endogenous ligand is a digitalis-like compound that inhibits Na^+/K^+-ATPase, is natriuretic and would be involved in the pathogenesis of essential hypertension. De Wardener *et al.* showed that in volume-expanded dogs a natriuretic [26] and hypertensinogenic substance is formed [6]. As the effects of this substance were nearly identical to ouabain, it was called "ouabain-like factor" (OLF) by Haddy *et al.* [27]. Later Gruber *et al.* called it "endogenous digitalis-like factor" (EDLF) because it inhibited Na^+/K^+-ATPase and could be determined by digitoxin radioimmunoassay [28]. After great efforts Hamlyn *et al.* demonstrated that there are four chromatographically distinct substances in the human circulation inhibiting the sodium pump activity of normal cells [7]. Using 85 liters of human plasma Hamlyn isolated by multiple purification procedures including HPLC, and affinity chromatography 12 µg of one of these materials, the most biologically active compound. The compound was identified by mass spectrometry as a steroid isomer of the plant glycoside ouabain. It was later shown to be produced mainly in the adrenal cortex, but it could be shown in the hypothalamus, hypophysis, heart and kidney, too [29].

In mammalian tissues till now several steroid compounds with digitalis-like activity were identified. Besides the stereoisomer of plant ouabain (OLC) found by Hamlyn *et al.* in human plasma and bovine adrenals [7, 30] another isomer of the compound was reported from bovine hypothalamus and characterized by Haupert [31]. However, these authors later reported that their hypothalamic compound was in fact ouabain, and the previous erroneous results were mainly due to the complexation of hypothalamic ouabain with borosilicates present in the glass tubes used in their purification method [32]. As another correction, Balzan and co-workers showed in their latest work [33] that the digitalis-like compound from neonatal blood they previously referred to as EDLF [34] may be identical or very similar to ouabain.

From human urine Bagrov *and his colleagues* isolated a bufadienolide-like compound (BLF), which cross-reacted with both anti-marinobufagenin (MBG) and anti-digitalis antibodies [35]. A similar material was found in bovine adrenals by Schoner and that cross-reacted with antibodies against another bufadienolide, proscillaridin A [36]. The same group showed that ouabain is also present in bovine adrenals and verified their finding in electrospray mass spectrometry and NMR [37]. Further, from peritoneal dialysate of uremic patients a highly labile digitalis-like compound was purified by Graves, which may be a sterol [38]. Further studies needed to characterize the exact structure of these compounds.

3.6. Biosynthesis of "endogenous digitalis-like compounds"

There are evidences of dietary origin of exogenous ouabain in mammalian tissues and body fluids. In rat urine Tamura found two Na^+/K^+-ATPase inhibitors that were derivatives of dietary strophanthidin [39]. Also, Kitano *et al.* studied organ accumulation of orally administered ^{3}H-ouabain, ^{3}H-digoxin, and ^{3}H-digitoxin in rats and found that they were accumulated in high levels in the adrenals [40]. However, Ferrandi *et al.* demonstrated that hypothalamic ouabain-like factor and systolic blood pressure is not influenced by dietary ouabain-like factor [41].

On the other hand some recent findings support the endogenous synthesis of cardiac glycosides. Perrin *et al.* reported that after addition of pregnenolone or progesterone there was increased secretion of ouabain-like factor from bovine adrenocortical cells. Their OLF was characterized by HPLC, mass spectrometry and radioreceptor assay [42]. Lichtstein *and his colleagues* studied the biosynthesis of digitalis-like compound both in bovine and rat adrenal homogenates and in primary rat adrenal cells, and showed production of EDLF from pregnenolone and 25-OH hydroxycholesterol [43]. Pregnenolone was also shown by Hamlyn *et al.* to stimulate aldosterone and endogenous ouabain secretion by bovine zona glomerulosa cells [44]. Interestingly, as they used an inhibitor of 3ß-hydroxysteroid dehydrogenase to reduce conversion of pregnenolone to progesterone, they were able to block aldosterone production, but the pregnenolone-augmented secretion of OLF remained increased. These results show that the biosynthetic pathways of these

steroids diverge prior to formation of progesterone. Oazzaz *et al.* described a method for simultaneous isolation of both digoxin-, and ouabain-like immunoreactive factors and their naturally existing deglycosylated congeners (DLIF-genin, DLIF-mono, DLIF-bis, and OLF-genin) from bovine adrenal cortex [45]. They also observed a compound with structural similarities to both digoxin and ouabain, which may represent a metabolic link between DLF and OLF [46]. Further, Dmitrieva *et al.* showed that endogenous mammalian bufadienolide is synthesized in the adrenal cortex from cholesterol, and the biosynthesis is independent of side-chain cleavage [47].

3.7. Regulation of endogenous ouabain secretion

After isolating endogenous ouabain from human plasma [7], immunohistological studies showed that it is present in the hypothalamus, hypophysis, heart and kidney, and also in the adrenals, in greater amounts [29]. Using primary cell culture of bovine adrenocortical cells OLF was shown to originate from zona glomerulosa [48]. Adrenocorticotropin (ACTH) and angiotensin-II (AII) stimulated both endogenous ouabain and aldosterone secretion of these cells [48, 49]. However, effect of angiotensin-II on endogenous OLF was mediated through angiotensin-II type 2 receptor, and it stimulated aldosterone and cortisol secretion via angiotensin-II type 1 receptor [50]. Further, dibutyryl cAMP and phorbol myristate acetate treatment of the cells increased aldosterone secretion without having any effect on OLF production. On the other hand 8-bromoadenosine 3',5'-cyclic monophosphate sodium salt, the membrane-permeable analog, stimulated secretion of OLF, showing that the secretion of these two adrenocortical steroids are regulated by different intracellular signaling mechanisms [51].

In vivo regulation of endogenous inhibitors of the Na^+/K^+-ATPase was studied in rats. Intramuscular ACTH treatment of animals for 8 days was found to result in increase of marinobufagenin- but not ouabain-like immunoreactivity [52]. Exposure of rats to hypoxia was observed to trigger release of endogenous ouabain (or "hypothalamic inhibitory factor") from both midbrain and adrenal tissues [53]. On the other hand treatment with Synacthen or with Dexamethasone of healthy individuals did

not lead to any significant changes in the secretion of endogenous ouabain-like factor secretion [54].

3.8. Physiological role of "endogenous digitalis-like factor"

The original hypothesis implied that endogenous digitalis-like factors would resist pressor effects of volume expansion by increasing natriuresis and diuresis. Thus, effects of acute volume expansion were studied using anesthetized dogs and general activity of endogenous cardiac glycosides was measured using an enzyme bioassay. Endogenous ouabain-like compound (OLC) was determined using ouabain antisera in plasma drawn from dogs before and 30 and 120 min after massive volume expansion with isotonic saline [55]. Urinary Na^+ excretion was significantly elevated at 30 min and remained elevated for 120 min after loading. Plasma OLF was not changed during the experiment, but in contrast, plasma endogenous digitalis-like factor (EDLF) was higher after the saline load than before in each of four dogs at 30 and 120 min after the infusion. Fedorova *et al.* also found that two-hour volume expansion in dog was associated with an increase in urinary marinobufagenin-like factor, but not ouabain-like material [56]. They also investigated changes in tissue and plasma levels of EDLF during acute volume expansion in rats, and demonstrated increase in plasma marinobufogenin-like immunoreactivity, but decrease in plasma OLF concentration [52]. At the same time, they found that plasma volume expansion increased pituitary OLF level with no significant change in pituitary MLF concentration [57]. Marinobufagenin and ouabain were shown to have their predominant effect on the two sodium pumps subunits ?-1 and ?-3, respectively [58]. These findings could explain some of the differences between ouabain and marinobufagenin, since rat vascular smooth muscle was found to contain ?-1 isoform and ?-3 was rather present in the nervous tissues [59]. Thus, elevated plasma concentration of MLF and increased brain level of OLC could have physiological significance in the inhibition of the sodium pump during volume expansion.

Effect of salt intake on endogenous digitalis/ouabain-like factor secretion was investigated, as the exact role of the compound(s) in sodium homeostasis is not known, yet. Yamada *et al.* used acute hypertonic (20 %) sodium chloride load in rats

intraperitoneally [60]. After the injection they found elevated levels of OLF in pituitary, in adrenals and in plasma, suggesting that endogenous ouabain may be involved in response to hypernatremic state. Butt *and colleagues* found significantly increased urinary excretion of OLF as the effect of increased salt intake in healthy volunteers [61]. In their recent work, they studied tissue distribution of endogenous ouabain-like factor and the effect of prolonged (7 days) high salt (1.8 % sodium chloride) intake on plasma and tissue concentration of this compound in rat [62]. They found significantly increased serum OLF concentration as a result of hypertonic load. They also showed that the adrenals contain significantly higher amount of OLF than other tissues of the animals (liver, kidney, heart and brain). Bernini *et al.* investigated the effect of acute and prolonged isotonic salt loading in both adrenalectomized and healthy subjects on EDLF and endogenous OLF immunoreactivity [63]. They found that adrenalectomized patients had similar EDLF and OLF levels to those of the controls (EDLF 310±63 versus control 297±45 fmol ouabain equivalent/ml measured by radioreceptor assay and OLF 99±19 versus control 85±19 fmol/ml measured by Du-Pont-NEN ouabain EIA, individually). Thus, they suggested that in humans not the adrenals are the main sources of these factors. Further, using the above mentioned measurements they also found that isotonic salt loading did not influence the circulating levels of these compounds either in adrenalectomized patient or in control individuals.

3.9. Exogenous and endogenous sodium pump inhibitors and hypertension

In 1976 Haddy *et al.* suggested that a circulating inhibitor of the Na^+/K^+-ATPase is involved in the mechanism of volume expanded hypertension [27]. It has been hypothesized that this compound, through inhibition of the Na^+/K^+ pump, can constrict blood vessels, enhance vasoconstriction in response to agonists, increase cardiac contractility, and raise blood pressure. It could also cause natriuresis/ diuresis that would resist the hypertensive effects of the pump inhibitors. Therefore, it was suggested to be significant in the pathophysiology of the low-renin, volume-expanded type of hypertension [64].

There were several lines of evidences supporting this hypothesis. In 1940 Solandt *et al.* reported, that cross circulating the blood from a dog with experimental low renin

hypertension to another normotensive dog rose the arterial pressure in the normotensive animal [65]. Later several groups of investigator showed similar findings among them Dahl and coworkers. They described development of hypertension in normotensive salt resistant rat that was joined by parabiotic connection with a hypertensive salt sensitive animal, after feeding salt [67]. Other investigators also showed presence of a factor with sodium pump inhibitory activity in animals with one-kidney renal hypertension [67], and "digitoxin-like activity" in monkeys with chronic Goldblatt hypertension [68].

In plasma of spontaneously hypertensive rats elevated levels of material able to inhibit 86Rb uptake, an indicator of sodium pump activity, compared with Wistar-Kyoto rats (WKY) were found [69]. It was shown however, that the lowered ^{86}Rb uptake could not be attributed to authentic ouabain, as ouabain-like levels were down-regulated in SHR rats as showed by radioimmunoassay (0.62 ng/ml versus 1.9 ng/ml in WKY).

The possible hypertensive effects of endogenous Na^+/K^+ pump inhibitors bufalin, ouabain, and its aglycone ouabagenin were investigated in normotensive rats [70]. Bufalin given during a 30 minutes time produced significant dose-dependent increases in blood pressure, heart rate, and excretion of urinary volume and sodium; however ouabain had no significant effect. In contrast, given chronically during a 6-7-week period, ouabain proved to be as effective as bufalin in increasing blood pressure both in normal rats and 70 % reduced renal mass rats on a salt-free diet [70].

Manunta *et al.* investigated the effects of ouabain, its aglycone ouabagenin, digoxin and digitoxin on blood pressure. They found that ouabain- and ouabagenin-infused rats became hypertensive after four weeks, but digoxin/digitoxin treatment did not induce chronic hypertension. In contrast, secondary infusion of digoxin or digitoxin in rats with ouabain-dependent hypertension normalized their blood pressure. [71].

Both digoxin and ouabain are potent inhibitors of the sodium pump, but only ouabain appears to induce hypertension [72]. The structure-activity relationship for the hypertensinogenic activity of ouabain was recently studied and a hypothesis was put forward that different structural elements are responsible for the sodium pump inhibitory and the hypertensinogenic effect of ouabain and related compounds [23]. The hypertensinogenic activity was found to be high when the lactone ring of ouabain was

saturated (dihydro-and iso-ouabain), or opened. However, these compounds proved to be weak inhibitors of the Na^+/K^+-ATPase. This finding again, as detailed in 3.4., suggests that ouabain may have a mechanism of action independent of the sodium pump.

3.10. Animal models for hypertension studies

A novel hypertensive animal model was developed when the mouse Ren-2d renin gene was integrated into the rat's genome [73]. Besides hypertonia resting cytosolic Na^+ concentration was significantly higher in the lymphocytes of these transgenic animals than in the wild ones. Ouabain failed to inhibit Na^+/K^+-ATPase and to further increase cytosolic sodium in these cells compared to cells from normotensive Sprague-Dawley rats. Further, Na^+/K^+-ATPase activity of transgenic mice erythrocytes was significantly reduced compared to normotensive animals. It was concluded that reduced Na^+/K^+-ATPase activity leads to elevated cytosolic sodium in this model of genetic hypertension. However, endogenous ouabain level of these animals was not characterized.

Another model was used in studying the development of hypertension in insulin- dependent diabetes mellitus and the possible role of endogenous Na^+/K^+ pump inhibitors [74]. Rats receiving streptozotocin followed by 25 % reduction of renal mass developed insulin-dependent diabetes mellitus and low-renin volume-expanded hypertension. Plasma level of digoxin-like immunoreactive factor (DIF), determined with a digoxin radioimmunoassay was significantly higher in these hypertensive rats than in normotensive controls. DIF level correlated inversely with myocardial Na^+/K^+-ATPase activity and positively with systolic blood pressure.

3.11. Natriuretic effect of EDLF

Several early investigations supported the concept that volume expansion leads to inhibition of the sodium pump and to natriuresis. Buckalew *et al.* [75] showed inhibitory effects of plasma filtrates of saline loaded dogs on toad bladder sodium

transport. Kramer *and colleagues* studied decrease in renal Na^+/K^+ATPase activity by volume expansion [76]. Hillyard *et al.* investigated the inhibitory effect of a natriuretic factor from volume loaded rats on short circuit current and sodium pump; and concluded that the factor acts by reducing active sodium transport via inhibition of Na^+/K^+ATPase [77].

In 1984 Haupert *et al.* described a hypothalamic inhibitory factor: HIF [78]. It proved to be a high-affinity, Mg^{2+}-dependent reversible inhibitor of Na^+/K^+ATPase. It was as potent as ouabain, but its ligand requirement for optimal activity differed from that of ouabain. Later these authors however demonstrated that their HIF is ouabain [79]. An ouabain-like factor in Milan hypertensive rats was identified [80] by following the ouabain-sensitive ^{86}Rb uptake into human erythrocytes, displacement of [^{3}H]ouabain binding, and inhibition of purified dog kidney Na^+/K^+ATPase.

Takada *and colleagues* investigated the urinary excretion of endogenous immunoreactive ouabain-like and digitalis-like factors in 5/6 reduced renal mass rats, a model of volume-expanded hypertension [81]. Systolic blood pressure and urinary Na^+ excretion were also measured during 4 weeks of 1 % saline load and proved to be higher in reduced renal mass animals than in controls. They also found that both OLF and DLF coexist in rat urine and that urinary level of DLF increased and reached a peak in the first week while OLF increased continuously during the time of investigation. Thus, they suggested contribution of DLF in renal sodium excretion and role of OLF in development and maintenance of hypertension.

In their recent article, Fedorova *and colleagues* studied the role of the endogenous ?-1 sodium pump ligand marinobufagenin in hypertension in Dahl salt sensitive rats. These animals have a mutation in the ?-1 subunit of Na^+/K^+ATPase, and on high salt intake they retain Na^+ and demonstrate increased blood pressure. Fedorova found that within two weeks of high salt (8 %) diet in these rats, besides elevation in systolic blood pressure, renal excretion of marinobufagenin increased compared to Dahl salt resistant animals. As marinobufagenin inhibits the ?-1 isoform of the sodium pump, the major Na^+/K^+ATPase present in the kidney, this compound can promote natriuresis in hypertensive Dahl sensitive rats [82]. Previously, the same authors also measured correlation between endogenous ouabain level and natriuresis [83]. However, as using

their ELISA they measured as high as 200 pg/ml resting OLC level, their conclusion about endogenous ouabain secretion may not be valid.

Other forms of EDLF were also investigated for their natriuretic effects. Kramer and co-workers identified a group of Na^+/K^+ATPase inhibitors with unknown origin as vanadium diascorbates, which are vasoconstrictor but more potent natriuretic agents than ouabain [84]. Pregnanes, a class of synthetic progesterone-like steroids with sodium pump inhibitory action were investigated by Smyth *et al.*. and proved to be both inotropic and natriuretic, too [85]. However, none of these compounds showed any kaliuretic property [84, 85].

Novel natriuretic factors were also described lately. Murray *et al.*. reported a factor, which is synthesized endogenously from ?-tocopherol, and inhibits the apical potassium channel in the ascending loop of Henle [86]. Garay *et al.* described two inhibitors of Na/K/2Cl transport, one of which was characterized as a phytoestrogen [87].

3.12. Vasoconstrictor effect of EDLF

Many of the candidate endogenous sodium pump inhibitors (EDLF) have been tested for either direct vasocontrictor activity or the ability to enhance vascular responses to norepinephrine and other agonists. In 1989 Weber *et al.* tested a substrate obtained from urine of hypertensive humans by adding it to the incubation media of rabbit femoral artery rings. They detected direct, concentration-dependent vasoconstrictor activity [88]. The urinary factor was able to increase the sensitivity of the artery ring to the vasoconstrictor effect of norepinephrine, clonidine and angiotensin-II. However, in contrast to ouabain, the factor did not have any inotropic effect on beating rabbit atria.

A Na^+/K^+-ATPase inhibitor from peritoneal dialysate of volume-expanded hypertensive patient with chronic renal failure was isolated by Krep and *co-workers* [89]. This compound, similar to ouabain, proved to be vasoconstrictor on rabbit aorta strips, and its effect was antagonized by digoxin antibody Fab fragments. However, because of its labile nature it was unlikely to be identical with ouabain.

The hypothalamic sodium pump inhibitor originally purified by Haupert *et al.* [78] was also tested for its vasoconstrictor ability [80]. It was shown that the factor was able to cause contraction on different arteries [90]. However, the hypothalamic pump inhibitor had no direct effect on vascular smooth muscle, but, rather through inhibition of sodium pump activity of adrenergic nerve terminals, it triggered release of catecholamines from intramural sympathetic nerves [91].

Effect of ouabain and marinobufagenin on vascular tone was studied by Bagrov *and colleagues* [58]. They showed that both compounds elicited vasoconstriction in mesenteric artery rings and the same concentrations of the agents inhibited sodium pump activity also. As presence of two Na^+/K^+-ATPase isoforms was shown: ?-1 in sarcolemmal membrane, and ?-3 mainly in nerve endings, differential regulation of the pump(s) by the inhibitors was suggested. The same group found that bufalin, another constituent of Bufo marinus venom, can cause rapidly developing vasoconstriction which was blocked by alpha adrenoceptor antagonist in contrast to marinobufagenin which effect is phentolamine–insensitive [92]. They suggested that marinobufagenin exert its effect by acting directly on vascular smooth muscle cells. Vasoconstrictor effect of ouabain independent of Na^+/K^+-ATPase inhibition was investigated on rabbit ear artery (see above). Xu et al. found that ouabain is able to enhance sensitivity of this vessel to serotonin. The effect was not mediated through ? adrenerg but rather 5-HT_{2A} receptors [93].

3.13. Sympathoexcitatory effect of EDLF

In 1957, Smith first proposed secretion of an endogenous cardiac glycoside from human hypothalamus [94]. Since then, Thymiak described an ouabain isomer isolated from bovine hypothalamus [95]. Later Zhao *et al.* compared this substrate with the OLF isolated by Hamlyn from human plasma and found that the hypothalamic compound was ouabain [31]. In 1997 Huang and his coworkers characterized a non-ouabain Na^+/K^+-ATPase inhibitor from bovine hypothalamus [96], originally isolated by Illescas *et al.* [97], the cardiovascular effects of which could not be blocked by Fab fragments of an antibody raised against digoxin (Fab fragments).

EDLF containing neurons were shown in the hypothalamus by immunohistochemistry using Fab fragments binding either digoxin or ouabain. The immunoreactivity was shown to be present mainly in parvocellular and magnocellular neurons of the paraventricular nucleus and supraoptic nucleus [98, 99]. Also, activation of the sympathetic nervous system by brain “ouabain” (through brain renin-angiotensin system) was shown to be mediated in part by the media preoptic nucleus and the ventral part of the anteroventral third ventricle (AV3V) region [100].

Huang and colleagues suggested that ouabain-induced reversible form of hypertension was developed due to increased activity of the central nervous system [101]. They showed that increased brain "ouabain" may desensitize the arterial baroreflex and thereby facilitate the development of hypertension in Dahl salt-sensitive rats on high sodium [102]. It was also proved that blockade of brain "ouabain" prevents sympathoexcitatory and pressor responses to high sodium in spontaneously hypertensive rats [103]. Further, their results indicated that chronic administration of ouabain activates the brain renin-angiotensin system, resulting in decreased sympathoinhibition and increased sympathoexcitation, impairment of baroreflex function, and hypertension [104]. Later they showed that chronic blockade of brain 'ouabain' by i.c.v. Fab fragments prevented the high salt diet-induced increases in blood pressure, in the expression and activity of angiotensin converting enzyme (ACE) in the hypothalamus and pontine medulla in Dahl S rats. Therefore, effects of high salt intake on ACE mRNA and activity appeared to be secondary to activation of brain 'ouabain' [105]. Using transgenic rats, deficient in brain angiotensinogen, they found attenuated sympathoexcitatory and pressor responses to ouabain and Na^+-rich artificial cerebrospinal fluid. These findings provide further evidence for the importance of local brain renin-angiotensin system in the sympathoexcitatory effects of ouabain and sodium [106].

3.14. Clinical studies: endogenous DLF/ OLF in physiological and pathophysiological volume expansion

Data were increasing during the past 30 years that sodium pump inhibitors are involved in volume expanded hypertension [27]. Facts included pressor activity of low

renin hypertensive blood, natriuretic and sodium pump inhibiting activities of volume expanded blood and potassium vasoactivity, which was blocked by ouabain.

Rossi *et al.* found that a large portion of Caucasian patients with essential hypertension have elevated circulating levels of endogenous ouabain (3.39±0.57 nmol/L versus healthy controls 0.53±0.1 nmol/L), possibly caused by an inherited or acquired renal defect in clearance of this steroid [107]. As ouabain proved to be hypertrophic on isolated cardiac myocytes [108], Manunta and *co-workers* studied relationships among plasma OLF, left ventricular mass and stroke volume in essential hypertensives. They found that 50% of the patients have elevated-high plasma OLF concentration (377±19 pmol/L versus normotensive (253±53 pmol/L), higher diastolic blood pressure, greater left ventricular mass and stroke volume and increased heart rate [109]. Pierdomenico *et al.* investigated correlation between plasma level of endogenous ouabain and sytemic hemodynamics and left ventricular geometry in patients with recently diagnosed essential hypertension [110]. They showed positive correlation between OLF and mean blood pressure and relative wall thickness and found that left ventricular mass and endogenous OLF was significantly higher in patients with concentric remodeling (489±211 pmol/L), than in those with either normal geometry, or eccentric nondilated or concentric hypertrophy (285±114, 313±134 and 348±180 pmol/L, respectively).

Borghi *et al.* [111] investigated the effect of saline (0.9 %) infusion in essential hypertensives and found short-term plasma renin activity suppression (PRA -1.32±0.5 ng/mL/h versus control -1.72±0.2 ng/mL/h) and release of a plasma endogenous Na^+/K^+-ATPase inhibitor, which was measured as % of control (without plasma) Na^+/K^+-ATPase activity (after saline loading 38±2.8 % versus before loading 45.4±1.7 % activity). In their recent work, Manunta and coworkers (Salt Sensitive Study Group of the Italian Society of Hypertension) aimed to clarify the role of endogenous ouabain-like factor, as natriuretic agent in salt sensitive hypertension in a randomized clinical trial [112]. They measured the plasma endogenous OLF levels in salt resistant and salt sensitive hypertensives during acute sodium intake (325.8±32.4 versus 340.9±35.3 pmol/L) and during acute and chronic sodium depletion (421.7±37.1 versus 383.6±26.6 pmol/L, 475±59.3 versus 485.9±75.5 pmol/L, respectively) and showed that circulating

OLF is raised specifically by procedures that lead to sodium depletion. They confirmed that isotonic saline infusion is not a stimulus for plasma ouabain-like factor. Further, they could not show any difference between basal plasma OLF levels of patient with salt-resistant or salt-sensitive hypertension (340.1±25.9 versus 337.4±23.6 pmol/L). As a conclusion, they suggested that endogenous OLF is involved in the adaptation of humans to sodium depletion however it is not a natriuretic hormone.

Lopatin *and colleagues* measured circulating marinobufagenin-like and ouabain-like compounds in preeclampsia [113]. They found that in third semester of noncomplicated pregnancy MBG level was elevated (0.625+/-0.067 versus non-pregnant control 0.190+/-0.04 nmol/L), but OLC level remained steady (0.32+/-0.07 versus 0.297+/-0.037 nmol/L). In patients with preeclampsia however, both MBG and OLC were dramatically increased (2.63+/-0.10 nmol/L and 0.697+/-0.16 nmol/L, respectively) suggesting that OLC has pathogenic role in preeclamptic hypertension. Gonick *et al.* analyzed plasma levels of marinobufagenin-like factor and endogenous ouabain from patients with various diseases with volume expansion [114]. They found elevated level of MBG in idiopathic hyperaldosteronism, acute congestive heart failure, chronic renal failure, and untreated essential hypertension. However, OLF level was elevated only in idiopathic hyperaldosteronism.

Ebara *et al.* showed previously presence of a natriuretic factor measured by ^{125}I-digoxin RIA in cord blood [115]. They also investigated occurrence of digoxin-like immunoreactive substance (DLIS) in the serum and urine in children with nephrotic syndrome [116]. They found positive correlation between disease state and urinary DLIS level. Balzan *et al.* measured high level of endogenous digitalis-like factor(s) in human neonate plasma [117]. After characterization of this factor using rat Na^+/K^+-ATPase isoforms, they claimed that it is similar but not identical to ouabain. It needed preincubation with the membranes in order to fully inhibit the sodium pump, and had a much steeper dose- response curve for rat ? 1 and ? 2 isoform than either ouabain or digoxin [118]. They suggested that its role would be to maintain high Na^+ excretion by reducing tubular sodium reabsorption, showed by Shimabukuro [116], in spite of the high and continuous ingestion of sodium from the amnionic fluid.

Chimori *et al.* studied cation transport in erythrocytes in essential hypertension and in normotensive and hypertensive diabetes mellitus [119]. They found elevated sodium influx into ouabain-treated erythrocytes both in patients with essential hypertension and in hypertensive diabetics. Diabetes itself also increased Na^+ influx. They suggested that abnormalities in the sodium transport may be related to the elevated blood pressure. Data are accumulating that both in diabetic and in hypertensive patient the clinical evidences of correlation of DLIF to blood pressure, insulin levels and the degree of insulin resistance, together with the experimental findings of decreased Na^+/K^+-ATPase activity, increased $Ca^{2+}{}_i$ and decreased $Mg^{2+}{}_i$ support the hypothesis on DLIF being a causative agent in these diseases [119, 120].

These studies were carried out from 1988-2001 in different laboratories. As one of the biggest challenges in endogenous DLF/ OLF research is the lack of standardized measurements, the results can be compared with each other only with a certain reservation. Antibodies raised and used by distinct groups are often poorly characterized and high basal DLF/ OLF levels measured [107, 108, 113, 117] can originate from cross-reactivity with other steroid compounds. Thus in these cases conclusions can not be drawn.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals are listed at the methods they were used.

4.1.2. Human adrenal samples

Two patients with incidentalomas (one aldosteronoma and one incidentaloma with normal serum steroid secretion pattern) were studied. As control, adjacent normal tissue of the aldosteronoma was used. The patients were operated at the Department of Surgery, Haynal Postgraduate Medical School, Budapest, Hungary. After unilateral adrenalectomy the tissues were put and stored in ice-cold M199 medium (Cambrex, Belgium) for transport to the laboratory.

4.1.3. Human serum and urine samples

Plasma and urine samples of healthy volunteers (n=16), of hypertensive patients (n=20) with congestive heart failure (CHF, New York Heart Association class II and III), of diabetic pregnant women (n=22), of non-pregnant diabetic patients (n=43), and of newborns (n=21) were studied. Blood samples were collected into EDTA tubes and were immediately centrifuged. Plasma was taken at 7 am during bed rest and urine was collected over 24 hours at the same day. The samples were stored at –20^{o}C. The adult samples were provided by Dr. Miklós Tóth from the 1st Department of Medicine, Faculty of Medicine, Semmelweis University, Budapest, Hungary. Newborn plasma and urine samples were provided by Dr. Barna Vásárhelyi from the Heim Children's Hospital, Budapest, Hungary.

4.1.4. Rat adrenal samples

Male Sprague-Dawley rats (n=30 per experiment) weighing 220-250 g were used. The animals were housed in plastic cages in a room with controlled 40 % humidity and temperature of 22^{o}C. A 12-hour light/dark cycle was maintained. Food and water was

given ad libitum. The animals were killed and decapitated. The adrenals were removed and freed from adipose tissue. The glands were decapsulated resulting in “capsula-glomerulosa“ preparation and the remainder fasciculata-reticularis, and after collagenase digestion were used in a static incubation system.

4.2. Experimental protocols

4.2.1. Immunization of rabbits

All chemicals were obtained from Sigma Co., (St. Louis, MO, USA). Hapten-carrier conjugates: Ouabain-dialdehyde was prepared by mixing 670 mg ouabain with 6.7 ml distilled water and 4.5 ml acetone. The solution was heated to 70^{o}C, and then cooled to room temperature. 500 mg sodium (meta)periodate ($NaIO_4$), 500 mg potassium phosphate dibasic (K_2HPO_4) and 56 mg meso-erythritol was added and mixed overnight. The solution was then lyophilized. BSA (260 mg) was previously activated by 1.75g hexane diamine and 500 mg N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDAC) in 10 ml water. The BSA solution was then dialyzed against 30 mM K_2HPO_4 and coupled to ouabain-dialdehyde using 240 mg sodium cyanoborohydrate ($NaBH_3CN$) overnight. The conjugate was dialyzed against 25 mM Tris buffer (pH 7.9), lyophilized, resuspended in Tris buffer, purified on Sephadex G25 column and dialyzed against Tris buffer again.

Anti-ouabain antiserum was then prepared. A solution comprised of 24 mg/ml ouabain-BSA lyophilisate in water was dissolved in 1 ml of Freud's complete adjuvant and injected subcutaneously into rabbit. 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.

4.*2.2. Immunohistochemistry*

Tissue immunostaining was performed with the anti-ouabain antibody developed in our laboratory (see details in 3.1.5.). Rats were perfuse with Zamboni’s fixative (paraformaldehyde/ picric acid) and adrenals, heart tissue (atrium and ventricle), and brain were paraffin embedded. Paraffin embedded tissues (10? m slides) were fixed to

gelatine pre-treated glass slides. After deparaffination and rehydration with graded alcohols, the slides were incubated in 0.3% H_2O_2 in methanol, then rinsed with phosphate–buffered solution (PBS) and incubated with 10% non-immune goat serum for 30 minutes at room temperature. Excess serum was removed and the tissues were incubated with 1:3000 dilution of anti-ouabain antiserum for 48 hours at 4°C in a humidified chamber. After three washes with PBS for 10 minutes, biotinylated anti-rabbit IgG as secondary antibody was applied for 1 hour at room temperature. Colour was developed using the ABC Elite kit (Vector Laboratories, USA) according to the manufacturer's recommendations with 3'-3'-diaminobenzidine as a substrate, and with a peroxidase concentration of 0.3% (w/v). The tissues were dehydrated, mounted and examined using light microscope. Negative control stainings were obtained by substitution of the primary antibody with either non-immune rabbit serum or antibody saturated by ouabain.

4.2.3. Static incubation systems

We used the following experimental models: 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. Rat adrenal glands, after decapsulated, were minced and digested by 0.1% collagenase (Wothington Biochem. Corp. (Freehol, NJ) in 0.2% BSA (Sigma Co., St. Louis, MO, U.S.A.) for 2x25 min followed by mechanical agitation to yield zona glomerulosa and fasciculata cells. The zona fasciculata cell contamination in the zona glomerulosa cell suspension was less than 5%.

Human and rat adrenocortical cells were prepared in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 2 g/L glucose (KRBG) and 40 g/L human serum albumin. Cells were incubated at 37 °C at a density of approximately $2x10^5$ cells/ 2mL (rat) or 10^5 cells/ 2mL (human) in a shaking bath under an atmosphere of 95% O_2 and 5% CO_2 for 2 hours in KRBG containing 0.5 % human serum albumin (KRBGA) alone or with the following compounds: ACTH (Sigma Chemical Co., St. Louis, MO, U.S.A.), ouabain (BHD Chemicals Ltd. Poole, England), A-II (Sigma Chemical Co., St. Louis,

MO, U.S.A.) and ANP (Peninsula Laboratories, Belmont, CA) were added to the samples in final concentrations: 10^{-5}-10^{-3}, 10^{-8}, 10^{-11}-10^{-7} M, respectively. The potassium concentration of the medium was changed by adding different amounts of KCl solution to the potassium free KRBGA to final concentrations of 3.6 mM, 5.4 mM, and 8.3 mM. Acetylcholine, nicotine, eserine, hexamethonium, methyllycaconitine, and mecamylamine (Sigma Co., St. Louis, MO, USA) were used at concentrations of 10^{-7}-10^{-3}, $1.6x10^{-6}$-10^{-3}, 10^{-6}-10^{-3}, 10^{-7}-10^{-4}, 10^{-8}-10^{-7}, 10^{-5}-10^{-4}M. Experiments were performed in a randomised block format to eliminate bias due to systematic error. The experiments were carried out with 3 or 4 incubations in duplicate or triplicate at each dose.

4.2.4. Adrenalectomy and abdominal aortocaval (AV) shunts in the rat: Garcia-Diebold method [121].

Male Sprague-Dawley rats weighing ~200g were used. Both surgical procedures were carried out in pentobarbital anaesthesia, and 20 animals per condition were used.

Adrenalectomy was performed by small retroabdominal laparotomy below the lowest rib. The adrenals were lifted from the retroperitoneum to the skin level by a sterile forceps and the glands were excised by sterile scissors. The laparotomy then was closed.

Abdominal laparotomy was performed to expose the inferior vena cava and abdominal aorta. The aorta was punctured caudal to the renal arteries with an 18 gauge disposable needle, which was moved forward into the vessel. Then neighbouring walls of the aorta and vena cava were perforated, penetrating the needle into the latter. A vascular clamp was placed across the aorta cephalic to the puncture to avoid bleeding. The needle was carefully withdrawn, and the aortic puncture point was sealed with a drop of cyanoacrylate glue. The clamp was removed 30 s later. Successful shunts were immediately verified visually by admixture of arterial and venous blood and by swelling of the vena cava. Then the laparotomy was closed.

4.3. Biochemical methods

Aldosterone and corticosterone radioimmunoassays (RIAs) and extraction procedures for ouabain RIA were performed in the Institute of Experimental Medicine, Hungarian Academy of Sciences. Ouabain RIAs used were developed in the Hungarian Academy of Sciences and in the Department of Physiology, University of Oulu, Finland. High performance liquid chromatography (HPLC) was carried out in the Department of Physiology, University of Oulu, Finland.

4.3.1. SepPak solid-phase extraction of samples for adrenomedullin and ouabain radioimmunoassays

Adrenal gland supernatants (1ml), rat plasma, human plasma and urine (2ml) were extracted using SepPak C18 cartridges (250 mg/3 ml, Baker, Budapest). The columns were preconditioned with 2-propanol and 0.1 % trifluoroacetic acid (TFA, Merck, NJ, USA). The samples were precipitated with 1 % TFA, centrifuged at 3000 rpm, 10 ^{o}C, for 10 minutes, and passed through the cartridges. The columns were washed with 0.1 % TFA and OLF was eluted with 3ml of 40% acetonitrile (Merck, NJ, USA) in 0.1 % TFA. After evaporation the extracts were reconstituted in RIA buffer. Duplicates from the samples were assayed.

4.3.2. Radioimmunoassays

4.3.2.1. Determination of ouabain-like immunoreactivity

Ouabain content of incubation media, rat plasma, and human plasma and urine samples was determined after C18 column extraction by ouabain RIA developed in our laboratory [122] and in the Department of Physiology University of Oulu, Finland [123]. See detailed description in RESULTS.

4.3.2.2. Determination of ANP-like immunoreactivity

Plasma ANP levels were measured by radioimmunoassay developed and described in detail by Vuoltenenaho *et al.* [124]. Briefly, rabbit antisera was generated against a

human ANP[99-126]-tyroglobulin conjugate. Dilutions of synthetic human ANP[96-126] (Sigma Chemical Co., St. Louis, MO, USA) was used as calibrator. As tracer human [^{125}I]-ANP[96-126] from Amersham (Buckinghamshire, UK). The assay sensitivity and EC50 were 0.8 pg and 20 pg per tube, respectively. Intraassay and interassay coefficients were less than 10% and 15%, respectively.

4.3.2.3. Adrenomedullin radioimmunoassay

Rat plasma adrenomedullin levels were measured by radioimmunoassay after C18 (SepPak) extraction as described earlier [123]. Briefly, tissue extract, reconstituted in RIA buffer after evaporation, and synthetic rat ADM[1-50] standards (Phoenix Pharmaceuticals) were incubated with rabbit anti-rat ADM serum (Phoenix Pharmaceuticals) at 4^{o}C for 24 hrs. Then ^{125}I-rat ADM[1-50] was incubated with the antiserum and the sample for 24 hrs. The free and bound fractions were separated by double antibody precipitation. The sensitivity of the assay was 1 fmol/ tube; the intra- and interassay coefficients were <10 % and 15 %, respectively.

*4.3.2.4. Aldosterone and **corticosterone** radioimmunoassay*

The corticosterone contents of the incubation media (both zona glomerulosa and fasciculata) were determined by radioimmunoassay (RIA) after chloroform extraction. Aliquots of the chloroform extracts of the zona glomerulosa were assayed for aldosterone content by radioimmunoassay (RIA) without chromatographic separation [125].

4.3.2.5. Measurement of human plasma ACTH, PRA, aldosterone and cortisol levels

Plasma hormone levels were measured in a lege artis medication-free period: ACTH by RIA (CIS, Gif-Sur-Yvette, France), PRA by RIA (DuPont, Boston, MA), serum aldosterone by RIA (Serono, Milan, Italy), cortisol diurnal rhythm by [^{3}H] competitive protein binding method (Haynal University of Health Sciences, Budapest, Hungary).

4.3.3. High performance liquid chromatography (HPLC)

Plasma and urine samples (1-2 ml), adrenal cortex cells' supernatant (1-2 ml), KRBGA (1-2 ml), commercial ouabain, and water extracted adrenal parts were acidified with 0.1-0.2 ml 1.8 % HCl-glycine solution passed through C-18 columns, evaporated and reconstituted in H_2O containing 0.1% TFA and chromatographed on a reverse phase column (Sperisorb OD2, 10 mm x 15 cm , 3m particle size) in a Waters Automated gradient controlled 680 HPLC system. The column was washed for 20 minutes under pre-equilibration conditions and eluted with acetonitrile containing 0.1% trifluoracetic acid. The flow rate was 1 ml/ min throughout, and 1 minute fractions were collected from 0 to 25 minutes into the gradient: from 0% to 20% acetonitrile. After evaporation the fractions were reconstituted in RIA buffer and measured in ouabain-RIA.

4.4. Statistical analysis

Data were expressed as % of untreated control in each experiment and used in statistical comparisons. Statistical significance was set at $p<0.05$ and comparisons between control and different drug effects were made by Student t-test and ANOVA, followed by Newmann-Keul post hoc comparison using Statistica Software (StatSoft Inc., Tulsa, OK). For visualization of results the non-linear regression analysis by Prism Software (GraphPad Software Inc., San Diego, CA) was used.

5. RESULTS

5.1. Histological study on ouabain immunoreactivity in the mammalian adrenal glands

Our first aim was to characterize which organs produce OLF in the mammalian body. Ouabain antibody was successfully raised in rabbit using a purified immunogen, ouabain-dialdehyde conjugated to BSA, as decribed in METHODS 3.2.1. The antiserum containing the highest titer of anti-ouabain antibodies (see details below) was used for immunostaining to characterize localization of OLF in different mammalian tissues.

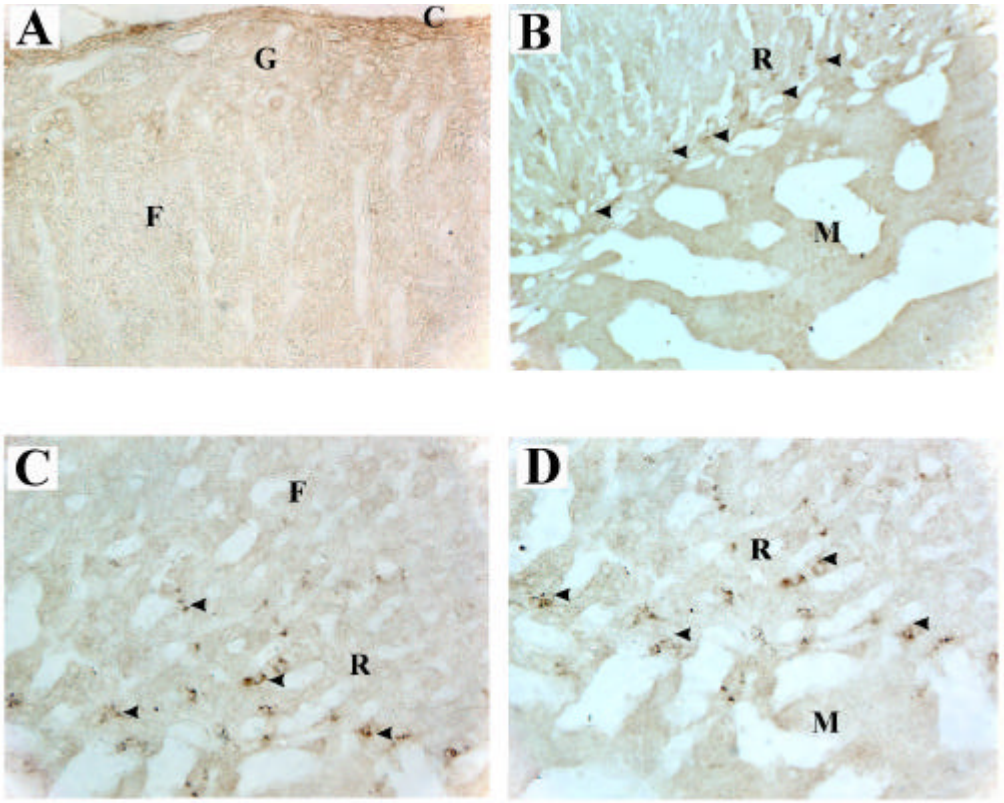

Figure 3. Light micrograph of paraffin sections processed using polyclonal antibody against ouabain diluted 1:3000 and the biotin-streptavidin peroxidase complex revealed by ABC (Vector Lab). A: Adrenal cortex showing the capsule [C], zona glomerulosa [G] and fasciculata [F] (400x). B: Zona reticularis [R] and adjacent medulla [M] (400x). C: Zona faciculata [F] and reticularis [R] at magnification 1000x. D: Zona reticularis [R] and medulla [M] (1000x).

Ouabain immunoreactivities were observed as form of dark-brown deposits present in the adrenal cortex. The staining was intracytoplasmic. **Figure 3A** shows the capsule of the adrenal gland [C], the zona glomerulosa [G] and fasciculata layers [F] of the adrenal cortex at 400x magnification. Very weak immunopositivity was present in the zona glomerulosa. Staining of the capsule was non-specific. However, prominent immunoreactivity was present in the zona reticularis cells [R] as seen in **Figure 3B** at 400x magnification, in **3C** and **D** at 1000x magnification in marked contrast with the adjacent cells of both the medulla [M] and the zona faciculata [F]. Arrowheads highlight some of the immunopositive cells.

We studied several other tissues, too. Weak staining was present in the atrial myocardium and in the hypothalamus: mainly in the nucleus supraopticus (data not shown). No activity was observed in any of the negative controls used, i.e. neither when pre-immune serum was used nor when the antiserum was preincubated with commercial ouabain.

5.2. Development of ouabain radioimmunoassay

As our immunohistological data demonstrated that the adrenal gland contains the most immunopositive cells among the tissues investigated, we wanted to measure tissue and circulating level of OLF. For this purpose, we developed an ouabain radioimmunoassay.

5.2.1 Antibody production and characterization

The antiserum containing the highest titer of anti-ouabain antibodies was harvested at 12 weeks after initial injections (bleed #8, see details in METHODS 3.2.1.). This antiserum was subsequently used in the assay.

Specificity. A comprehensive characterization of antibody specificity is shown in **Table 1**. Cross-reactivity was determined as 50 % of displacement of ^{3}H-labeled ouabain on weight basis. Only compounds containing an intact bufodienolide ring showed significant cross-reactivity with the ouabain antiserum. Ouabagenin showed the highest cross-reactivity (67 %), followed by digitoxin (15 %), and strophantidin (10 %). Digoxin showed only low (2 %) cross-reactivity.

Compound	Cross-reaction (%)
digitoxin	15
digoxin	2
ouabagenin	67
strophantidin	10
aldosterone	<0.001
progesterone	<0.001
deoxycorticosterone	<0.001
cortisone	<0.001
corticosterone	<0.0001
testosterone	<0.0001
cholesterol	<0.001
taurocholic acid	<0.001
hydrocortisone	<0.001

Table 1. Cross-reactivity of various analogs of ouabain and of other related compounds with ouabain antiserum. Compounds containing an intact bufodienolide ring showed significant cross-reactivity, however, none of the other steroids tested showed any appreciable cross-reactivity (<0.001) with the ouabain antiserum.

Binding capacity and optimal assay range. Binding capacity analyses showed that the optimal dilution of the antiserum was 1:20,000 with the tritiated tracer. The antiserum bleed selected bound 25 % of the ^{3}H-labeled ouabain tracer used (see details below). The optimal assay range was 0.2-6.4 pmol/ml (defined as linear assay range). Typical standard curve of ouabain radioimmunoassay is shown in **Figure 4**.

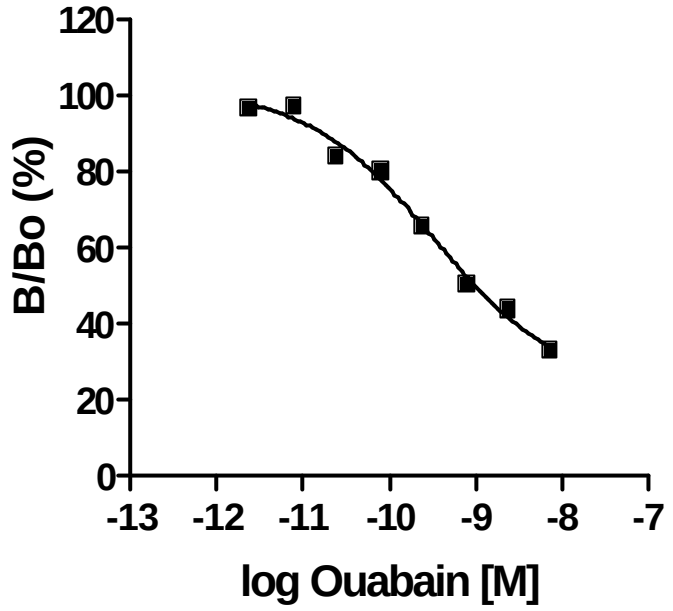

Figure 4. Typical standard curve of ouabain radioimmuno-assay using ^{3}H-ouabain tracer and 1:20,000 dilution of our antibody (bleed N^{o} 8).

Assay variability. Assay variability was assessed by estimating the coefficient of variation of the calculated ouabain concentration. Intra-assay variation was determined by assaying 10 samples with 2 nmol/l ouabain concentrations in the same assay. Inter-assay variability at 2 nmol/l dose level was calculated from 10 separate assays. The intraassay and interassay coefficients were less than 2.2 % and 10.3 %, respectively.

Analytical recovery. Known amount of ouabain (2 nmol/l) were given to a pooled plasma sample and extracted as described in 4.3.1. before assay. The recovery value of the sample was 74±6 %.

5.2.2. Treatment of plasma and urinary samples and adrenal gland supernatants

As ouabain concentration was very low in our samples, we could not measure endogenous OLF level by direct assay. Using C18 columns as described in METHODS 4.3.1., we were able to purify and concentrate OLF by washing and elution with 40 %

acetonitrile.

5.2.3. Ouabain radioimmunoassay

Several dilution of the ouabain antibody was tested in the assay. Also antibody and tracer were given either together or sequentially for various lengths of time. The most sensitive assay condition was the following: Serial dilutions of commercially available ouabain (BHD Chemicals) ranging from 2.4 pmol/L to 8000 pmol/L were prepared from stock and used as calibrators. Unlabeled ouabain and extracted samples (100 ?l) in duplicates were measured. Reaction mixture (500 ?l) consisted of ouabain antibody (at a final dilution of 1:20,000) in phosphate buffered saline (pH 7.4) containing 0.1 % sodium azide, and of sample or unlabeled ouabain, which were incubated together for two hrs at room temperature.

	Ab final dilution	**Incubation time (hrs)**	**B_{max}/B_0 (%)**
A	1: 98,000	O/N	40
B	1:200,000	O/N	14
C	1: 98,000	16 + 6	25
D	1: 60,000	2 + 1	24
E	1: 98,000	2 + 1	26
F	1:98,000	5 + 2	17
G	1:200,000	5 + 2	28
H	1:100,000	2 + 1	15
I	1: 24,000	2 + 1	42

Table 2. Assay conditions and ^{125}I-labeled tyrosyl-ouabain binding maximum of various dilutions of ouabain antibody. Overnight (O/N) and 16 hrs incubations were carried out at 4^{o}C. All other incubations were done at room temperature.

Tritiated ouabain (10,000 cpm/tube from Amersham) was sequentially added to the samples and incubated for 1 hr at room temperature. The bound and free fractions was separated by adding 500 ?l of charcoal suspension (containing 9 g of Wako SX, 0.62 g of dextran T-40 and 1g of sodium azide in 1 liter of 0.1 M Tris-HCl buffer, pH 7.8). The suspension was centrifuged at 3,000 rpm for 10 min. The supernatants were assayed by liquid scintigraphy.

Using ^{125}I-labeled tyrosyl-ouabain tracer in RIA

With our radioimmunoassay using ^{3}H-ouabain as a tracer, we were able to measure ouabain from rat adrenal supernatants and from some human plasma samples, however the assay sensitivity did not make it possible to measure the low plasma OLF content of healthy subjects. Later we used ^{125}I-labeled tyrosyl-ouabain, as a label for increased sensitivity [224]. **Table 2** shows details of the different assay conditions and **Figure 5** shows typical standard curves under the conditions investigated.

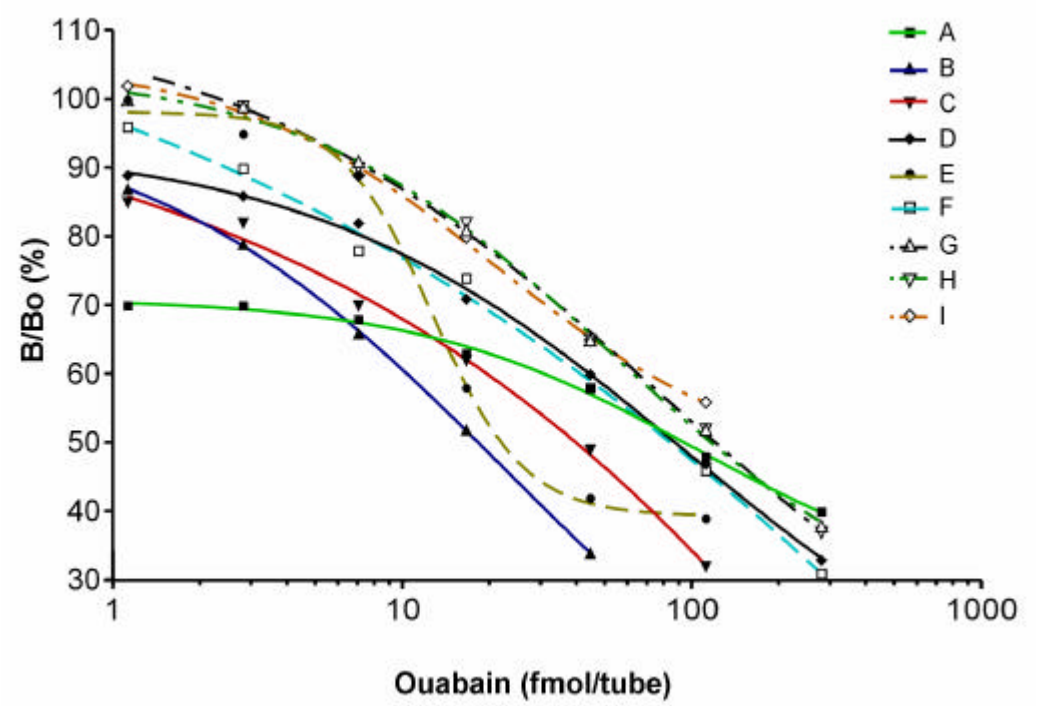

Figure 5. Typical standard curves of ^{125}I-RIA using various dilutions of the antiserum under different assay conditions. Colors refer to respective curves and A-I letters refer to conditions listed in **Table 2.**

We used our antibody at dilutions of 1:24,000-1:200,000. The tracer was either incubated together with the antibody overnight at 4^{o}C (A, B) or given subsequently for 6 hrs at room temperature after incubating the samples with the antiserum for 16 hrs (at 4^{o}C). Other conditions included in preincubation of the samples with the antiserum at room temperature for 2 hrs or 5 hrs, and subsequent addition of the tracer for 1 or 2 hrs at room temperature. The maximum binding of the ^{125}I-labeled tyrosyl-ouabain ranged from 14-42 %. The most sensitive assay conditions with a good tracer binding (B_{max}/B_0=25 %) proved to be incubating the standards with our ouabain antibody 16 hrs subsequently with the iodinated tracer for 6 hrs (C). We were able to increase the optimal antibody dilution from 1:20,000 (^{3}H-ouabain) to 1:98,000 (^{125}I-labeled tyrosyl-ouabain). The assay sensitivity also increased to 15 fmol/tube ouabain compared to the 200 fmol/tube ouabain concentration achieved by the ^{3}H-ouabain assay.

We also tried to adopt a different separation technique. Instead of using mixture charcoal and dextrane, we used polyethylene glycol (PEG) 6000 in a final concentration of 57 g/L. However, this did not significantly influence the assay quality.

5.3. Regulation of endogenous ouabain-like factor secretion in the adrenal gland

Our next aim was to study OLF production and its regulation in the mammalian adrenal gland. As human adrenals are hard to obtain, first we studied OLF secretion in rat adrenals.

5.3.1. Tissue OLF content of rat zona glomerulosa and zona faciculata

To test whether adrenals contain endogenous OLF recognizable by our assay, we measured OLF content of zona glomerulosa (ZG) and fasciculata/reticularis/medullary (ZFRM) tissues extracted as described in METHODS. Tissue OLF concentration of ZG was non-significantly lower than OLF content of ZFRM: 176.02±8.34 pg/g versus 208.62±16.8 pg/g (mean±SEM, n=5, p=NS).

5.3.2. Production of OLF by zona glomerulosa and fasciculata cells: modulatory role of ACTH, angiotensin-II (A-II) and extracellular potassium

As ACTH, A-II and extracellular potassium concentration has a main role in regulation of adrenocortical aldosterone secretion, we first investigated their effect in modulation of endogenous OLF secretion. ACTH (10^{-9} and 10^{-8} M) induced three times increase in OLF secretion by the glomerulosa cells (**Figure 6A**). Angiotensin-II 10^{-8} and 10^{-7} M induced only a 30 % and 50 % increase, respectively.

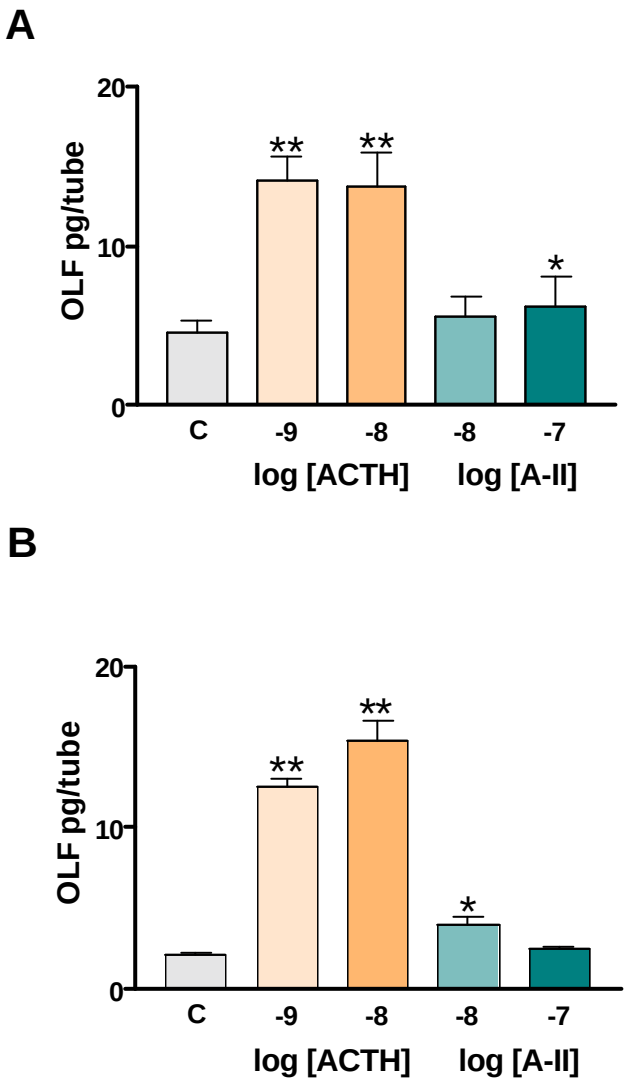

Figure 5. Effect of ACTH and angiotensin-II (A-II) on the immunoreactive ouabain concentrations of zona glomerulosa [A] and

zona fasciculata [B] cell supernatants. Values are mean?SEM, n=16, *p<0.05, ***p<0.001 versus control).

In zona fasciculata cells ACTH (10^{-9} and 10^{-8}M) induced dose-dependent 6- and 7-fold increases in OLF secretion, respectively (**Figure 6B**). Angiotensin-II at 10^{-8}M induced a two-fold increase but at 10^{-7}M it had no effect on OLF production.

Using different potassium concentrations in the incubation media (**Figure 7**), we found that the zona glomerulosa cells' OLF secretion increased by 15 % at 6 mM [K^+], and decreased both at 1.2 and 9 mM extracellular potassium concentrations (by 40 % and 20 %, respectively). There was a ~2.6- fold increase in aldosterone secretion at 6 and 9 mM potassium, but no significant difference was observed at 1.2 mM [K^+]. In contrast to zona glomerulosa, changes in extracellular potassium concentration did not modulate fasciculata/reticularis cells' OLF secretion (data not shown).

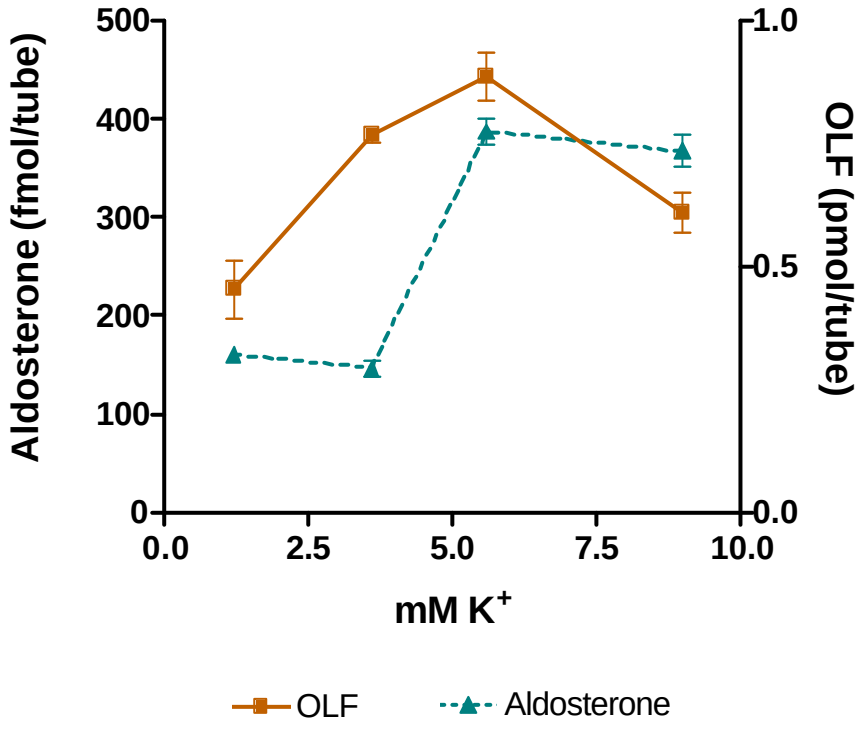

Figure 7. Effect of extracellular K^+ concentration on OLF and aldosterone production of rat adrenocortical capsule-glomerulosa preparation (mean±SEM, n=12, *p<0.05 versus 3.6 mM K^+).

5.3.3. Cholinergic influence on endogenous OLF secretion

Since there was experimental evidence that the adrenal cortex contains cholinergic nerve fibers [126], we also wanted to characterize the cholinergic influence on the endogenous OLF secretion in collagenase-dispersed adrenocortical cells.

5.3.3.1. Effect of ACh, eserine and nicotine on in vitro OLF secretion of ZG

Data are expressed as % of untreated control in each experiment. Absolute OLF concentrations in ZG and in ZFR cells' supernatant ranged from 6-142 pg/ml and 6-137 pg/ml, respectively.

OLF production of the glomerulosa cells was modulated by acetylcholine biphasically (**Figure 8A**). ACh stimulated OLF secretion at concentrations of 10^{-7}-10^{-6} M but at higher concentrations (10^{-4}-10^{-3} M) its effect was mainly inhibitory. Interestingly, employing the well-known cholinesterase inhibitor eserine to avoid possible degradation of exogenous ACh by tissue enzymes, we found modulation in OLF secretion by eserine alone. Similarly to ACh eserine at lower concentrations (10^{-6}-10^{-5} M) stimulated whereas at higher concentrations (10^{-4}-10^{-3} M) inhibited the OLF output (**Figure 8B**). As this compound is known for its ability to act as a noncompetitive agonist on nAChRs [127], the possibility raised that there may be nicotinic receptor(s) present on adrenocortical cells. In contrast to these results, nicotine ($1.6x10^{-6}$-10^{-3} M) had a prominent dose-dependent stimulatory effect on OLF secretion (**Figure 8C**). The OLF concentration in the incubation medium increased as highly as 50-100 times by $4x10^{-5}$-10^{-3} M nicotine ($p<0.001$). Note that OLF concentrations are expressed in log scale in **Figure 8C**.

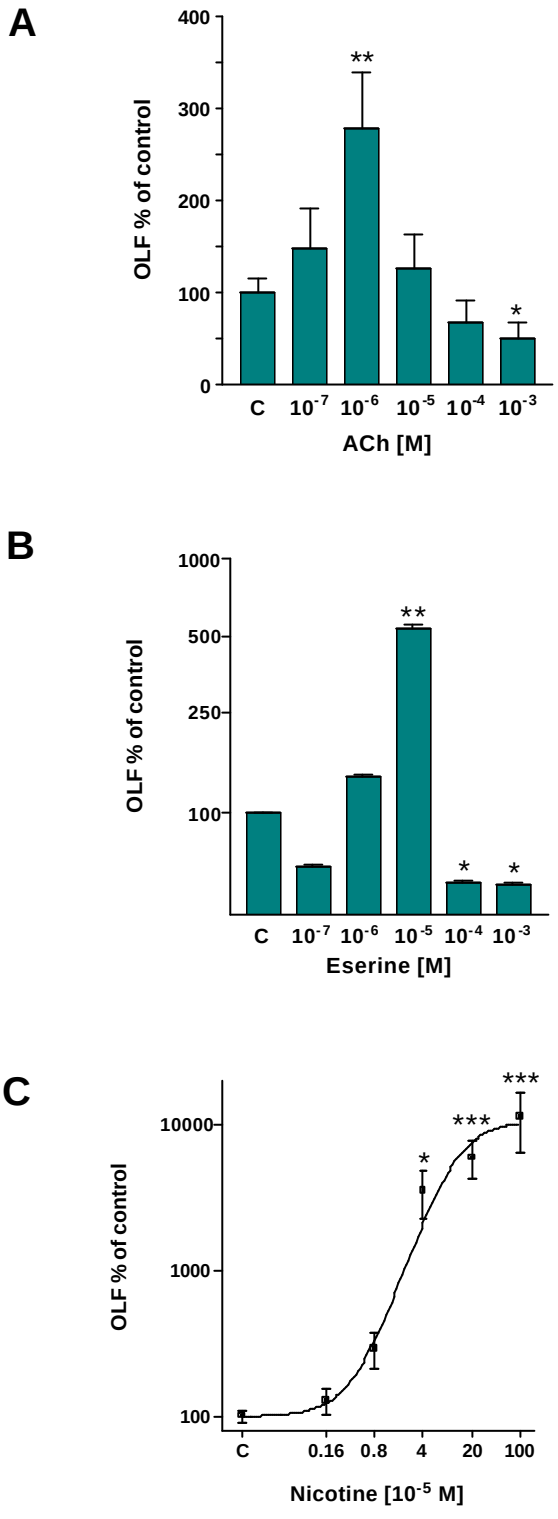

Figure 8. Effect of acetylcholine (Ach) [A], eserine [B] and nicotine [C] on rat zona glomerulosa OLF production (mean±SEM, n=8, *p<0.05, **p<0.01, ***p<0.001 versus control).

5.3.3.2. Effect of ACh, eserine and nicotine on in vitro OLF secretion of ZFR

As we showed presence of endogenous OLF not only in the zona glomerulosa but also in the zona fasciculata tissue, we studied cholinergic and nicotinic regulation of these cells, too. Despite ACh (10^{-7}-10^{-3} M) stimulated endogenous OLF secretion of ZG cells, it had no effect on OLF production of ZFR cells (**Figure 9A**).

Eserine however, induced a remarkable elevation in OLF output of ZFR cells. As shown in **Figure 9B** maximal stimulation was achieved by the same concentration as in glomerulosa cells (10^{-5} M, $p<0.001$). However, the ZFR cells seemed to be more sensitive, because a 4-fold stimulation was already obtained by 10^{-6} M eserine ($p<0.001$). Eserine at 10^{-5} M increased OLF secretion 10 times above control value ($p<0.001$). In contrary to zona glomerulosa, inhibitory effect of eserine was not observed at higher concentrations (10^{-4} M, 10^{-3} M).

Nicotine ($1.6x10^{-6}$-10^{-3}M), similarly to its effect in zona glomerulosa, elicited a large (2-100-fold) increase in OLF secretion (**Figure 9C**). It was less effective at $8x10^{-6}$ M, $4x10^{-5}$ M and $2x10^{-4}$ M concentrations compared to that on the ZG, though at a concentration of 10^{-3} M nicotine exerted the same rate of stimulation on ZFR as on ZG cells. Note that OLF concentrations on **Figure 9C** are expressed in log terms.

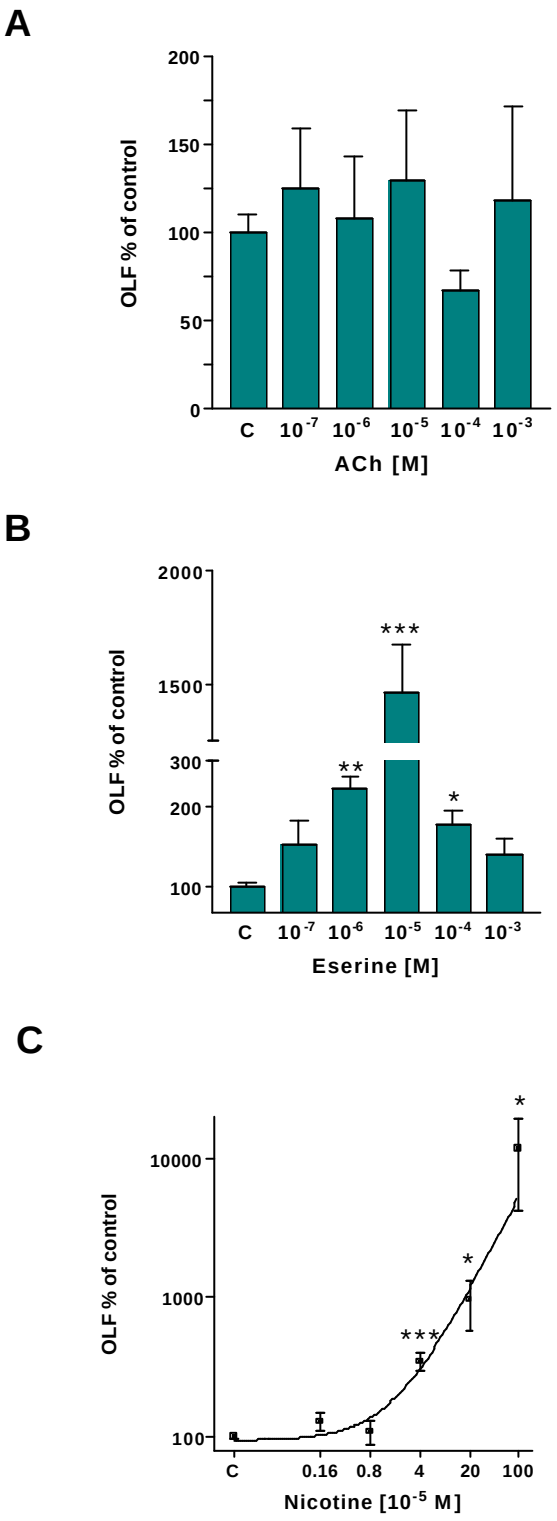

Figure 9. Effect of acetylcholine (Ach) [A], eserine [B] and nicotine [C] on rat zona fasciculata OLF production (mean±SEM, n=8, *p<0.05, **p<0.01, ***p<0.001 versus control).

5.3.3.3. Reverse phase high performance liquid chromatography of endogenous OLF

In order to identify the nature of OLF immunoreactivity we had measured in our studies with adrenal cells we applied nicotine-stimulated glomerulosa cells supernatants to reverse phase high performance liquid chromatography (**Figure 10**). As a control we used authentic ouabain and nicotine, both dissolved in KRBGA, and unconditioned KRBGA extracted by the same way as the glomerulosa samples. The ouabain-like immunoreactivity was eluted mainly at 14 min of our gradient and minor activity was observed sometimes also at 15 min. The retention time for plant-derived ouabain was about 14 min (fraction 14, n=3) indicating that OLF immunoreactivity of adrenal cells is ouabain. There was no immunoreactivity in the unconditioned culture medium or in nicotine-containing KRBGA (data not shown).

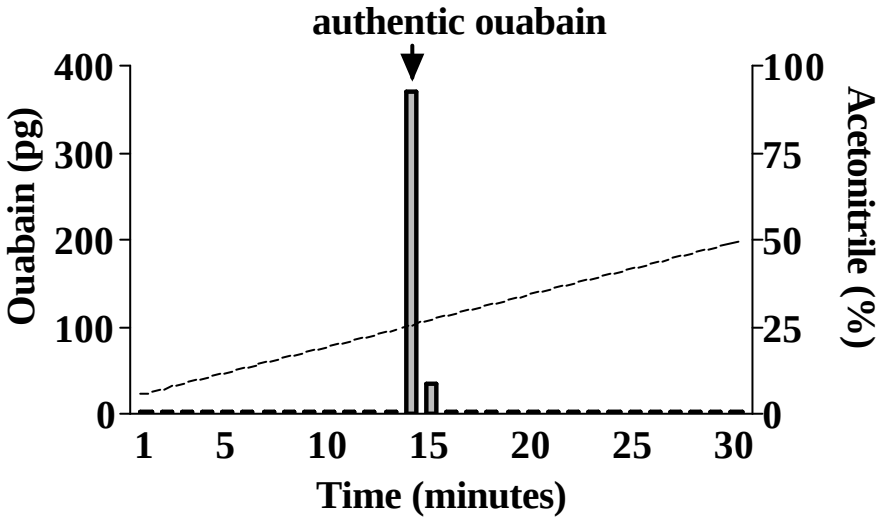

Figure 10. High performance liquid chromatography of nicotine-stimulated glomerulosa cell cells' supernatant. Bars represent endogenous ouabain-like immunoreactivity. Arrow shows retention time for authentic ouabain. Dashed line displays increasing concentration of acetonitrile in the elution buffer. One representative measurement out of 3 is shown.

5.3.3.4. Interaction of ACTH, A-II and nicotine on endogenous OLF secretion

Till now ACTH and A-II were known as the only stimulatory factors of endogenous OLF secretion. As the possibility raised that nicotine may also have a role as a potent stimulator, we wanted to study its interaction with ACTH and A-II on OLF secretion. In ZG cells ACTH alone increased OLF production by 67 % (from 90.8?6.4 pg/ml to 152?20.4 pg/ml, $p<0.05$). In combination with different concentrations of nicotine ($8x10^{-6}$ M, $4x10^{-5}$ M and $2x10^{-4}$ M) the ACTH-induced OLF level of the conditioned media raised 5.3, 17.2 and even 126 times higher than the control (ACTH only) levels (**Figure 11A**).

A-II (10^{-8}M) made a moderate, non-significant (19 %) increase in OLF production (from 90.8?6.4 pg/ml to 107.8?12.3 pg/ml). In combination with nicotine $8x10^{-6}$ M, $4x10^{-5}$ M and $2x10^{-4}$ M the OLF concentration in the culture buffer increased 4.6, 22.9 and 71.7 times, respectively (**Figure 11B**). Nicotine alone at the same concentrations had a 4.4, 29.8 and 99.4-fold stimulatory effect, respectively compared to untreated control (**Figure 11A** and **B**). ACTH strongly potentiated the OLF stimulatory effect of nicotine ($p<0.0001$), whereas A-II (10^{-8} M) did not show any interaction with nicotine on OLF production.

In the zona fasciculata-reticularis cells ACTH (10^{-8} M) stimulated OLF production by 82 % (from 86?10.3 pg/ml to 155.3?33 pg/ml, $p<0.05$). The combination of ACTH and different concentrations of nicotine ($8x10^{-6}$-$2x10^{-4}$ M) revealed 2.8, 24.2 and 102-fold increases in OLF secretion compared to ACTH alone (**Figure 12A**). A-II (10^{-8} M) alone had no stimulatory effect on OLF secretion (from 86?10.3 pg/ml to 81.3?7.5 pg/ml). The same nicotine concentrations used above in combination with A-II increased the OLF level 4.8, 35 and 182 times above control (**Figure 12B**). Nicotine alone at $8x10^{-6}$ M, $4x10^{-5}$ M and $2x10^{-4}$ M stimulated OLF secretion dose-dependently causing a 3.2, 37.7 and 158-fold increase, respectively. Although ACTH seemed to potentiate the effect of nicotine on the OLF secretion, we were not able to show significant interactions between nicotine and ACTH or A-II on the OLF production of ZFR cells.

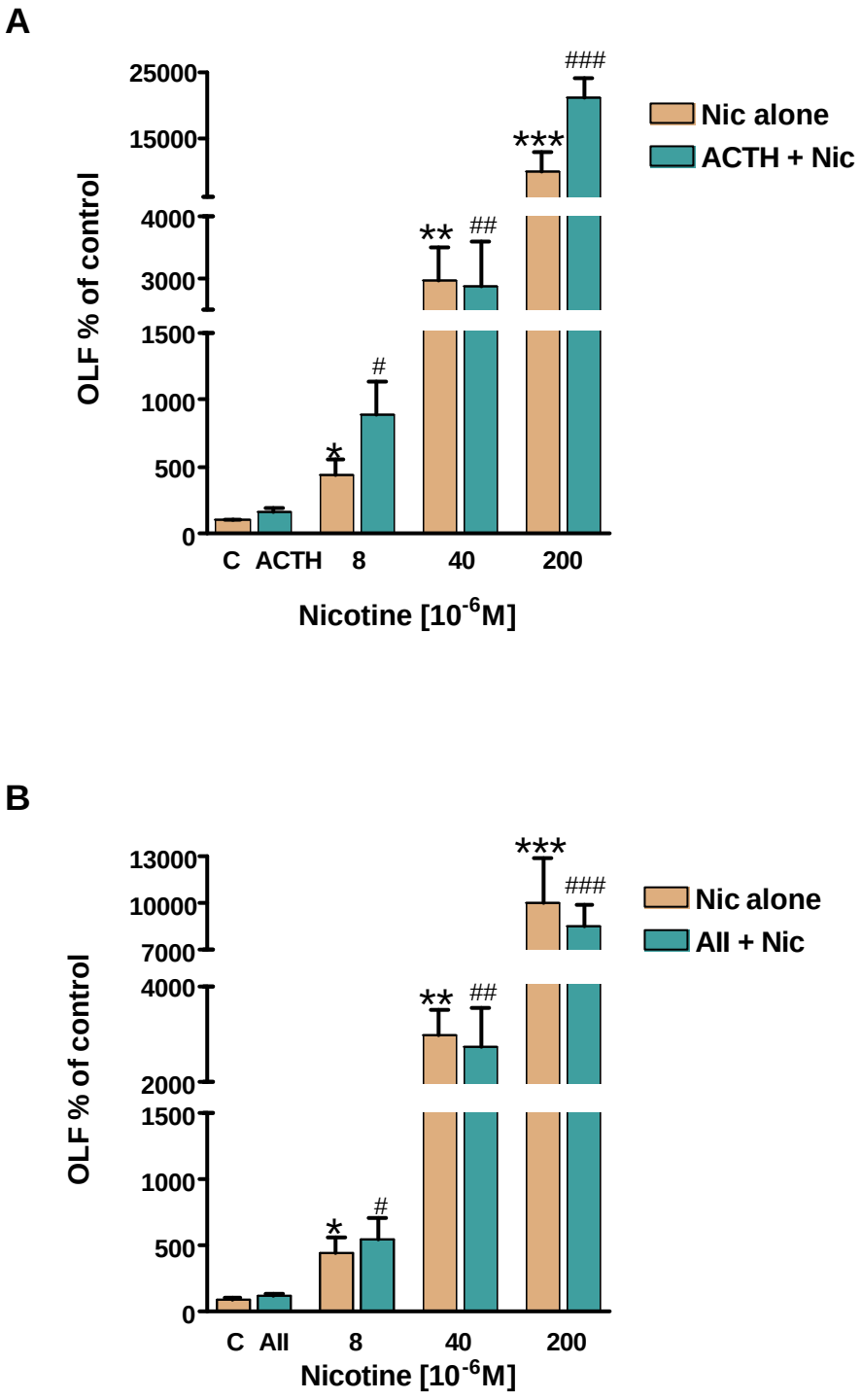

Figure 11. Interaction of ACTH and nicotine [A], and angiotensin-II (AII) and nicotine [B] on zona glomerulosa OLF secretion. Values are mean±SEM, n=4, *p<0.05, **p<0.01, ***p<0.001 versus control, #p<0.05, ##p<0.01, ###p<0.001 versus ACTH or A-II alone.

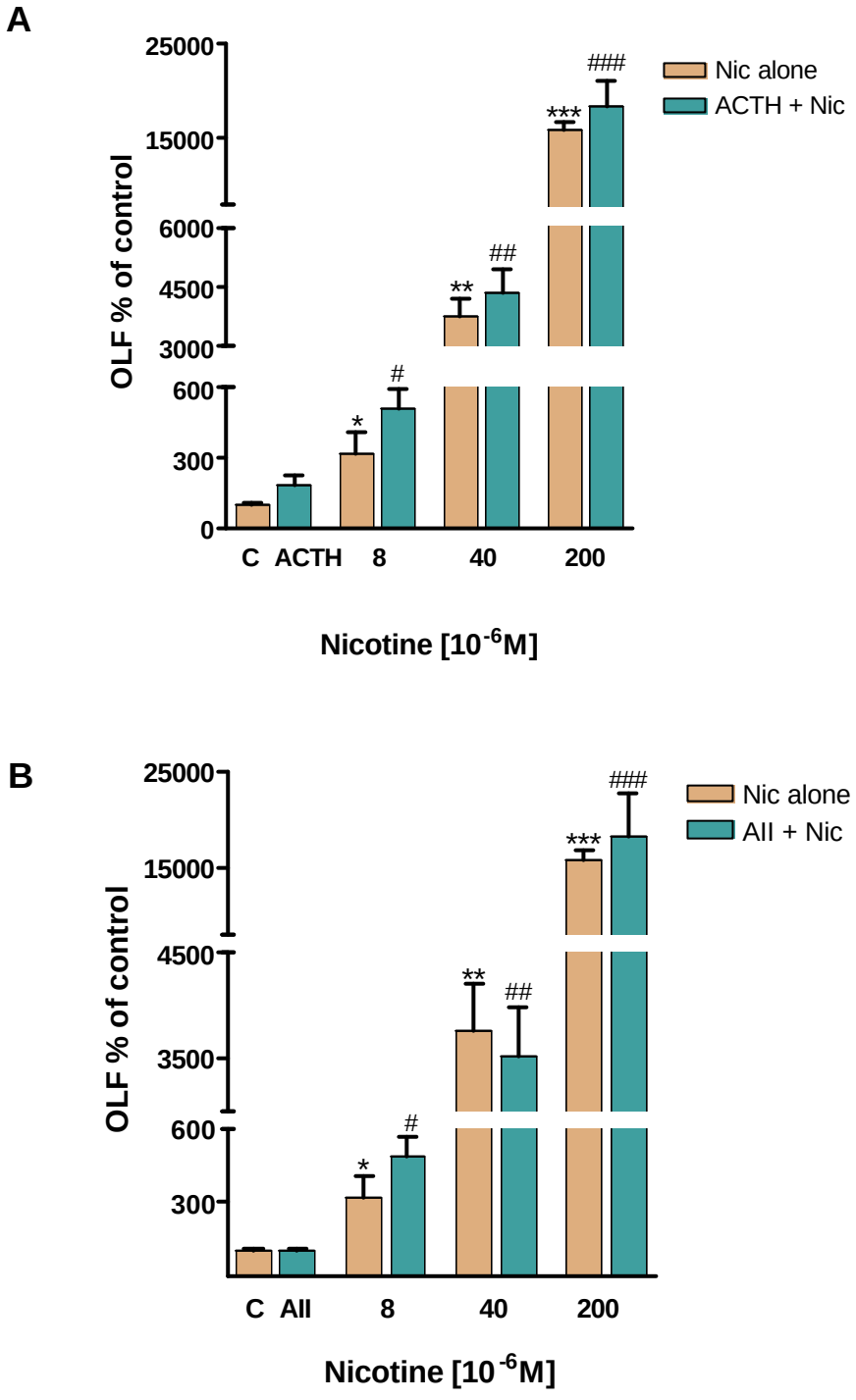

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Figure 12. Interaction of ACTH and nicotine [A], and angiotensin-II (AII) and nicotine [B] on zona fasciculata OLF secretion. Values are. mean±SEM, n=4, *p<0.05, **p<0.01, ***p<0.001 versus control, #p<0.05, ##p<0.01, ###p<0.001 versus ACTH or A-II alone.

5.3.3.5. Effect of the ganglion blockers hexamethonium and mecamylamine and the ? 7 nicotinic receptor antagonist methyllycaconitine on adrenocortical OLF secretion

Our experiments using nicotine and eserine, the allosteric non-competitive agonist of nicotinic acetylcholine receptor (nAChR) suggested that there might be functional nicotinic receptors present in the rat adrenal cortex.

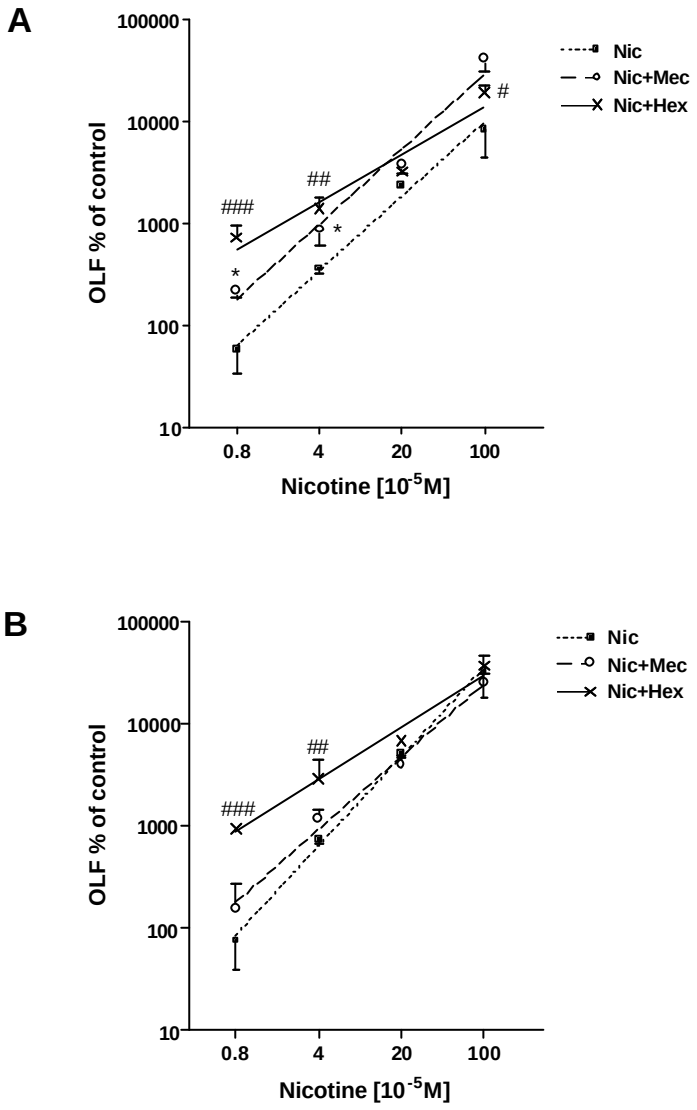

Figure 13. Effects of the ganglionic blockers hexamethonium (Hex 10^{-4}M) and mecamylamine (Mec 10^{-4}M) on the nicotine-induced (Nic) OLF secretion in zona glomerulosa [A] and in zona fasciculata [B]. Values are mean±SEM, n=4, *p<0.05, **p<0.01, ***p<0.001 Nic+Mec versus control, #p<0.05, ##p<0.01, ###p<0.001 Nic+Hex versus control).

To provide pharmacological evidence and to test the hypothesis, first we used the ganglionic blocking hexamethonium at different concentrations (10^{-7} -10^{-4} M) in combinations with various concentrations of nicotine. In zona glomerulosa cells 10^{-4} M hexamethonium potentiated the $8x10^{-6}$-10^{-3} M nicotine-stimulated endogenous OLF production by 10, 4, 1.4 and 1.7 times ($p<0.001$, $p<0.01$, NS, $p<0.05$), respectively (**Figure 13A**).

Mecamylamine, another ganglionic blocking compound, (10^{-4} M) in combination with nicotine potentiated the nicotine-induced OLF secretion by 3, 2.8, 1.7 and 4 times ($p<0.05$, $p<0.05$, NS, $p<0.05$), individually. The effect of hexamethonium was more prominent in combination with lower ($8x10^{-6}$-$4x10^{-5}$ M) concentrations of nicotine. However, mecamylamine showed a steady stimulatory effect at all nicotine concentrations used.

In zona fasciculata-reticularis the ganglionic blocking drugs had similar effect on nicotine-stimulated OLF secretion as in zona glomerulosa. However, effects of mecamylamine used with higher nicotine concentrations ($2x10^{-4}$ M, 10^{-3} M) proved to be rather inhibitory (**Figure 13B**). Mecamylamine lowered the nicotine evoked OLF secretion by 17 %, 31 % (NS), respectively.

To study possible presence of another type of nicotinic acetylcholine receptor we used the ? 7-receptor antagonist methyllycaconitine in our experiments. As shown in **Figure 14A** in zona glomerulosa cells methyllycaconitine at lower concentration (10^{-8} M) potentiated the effect of $1.25x10^{-5}$ M and $2.5x10^{-5}$ M nicotine, but it inhibited the stimulatory effect of higher nicotine concentrations ($5x10^{-5}$-$2x10^{-4}$ M) used. It decreased the secreted OLF concentration by 27 %, 7 % and 14 % (NS, NS $p<0.05$), respectively. At 10^{-7} M it inhibited the effect of nicotine ($2.5x10^{-5}$ M, $5x10^{-5}$ M, 10^{-4} M and $2x10^{-4}$ M) by 44 %, 27 %, 24 % and 31 % (NS, NS, NS, $p<0.05$), respectively. In ZFR cells MLA influenced OLF production differently from that in ZG (**Figure 14B**).Both concentrations of the compound (10^{-7} M and 10^{-8} M) potentiated the stimulatory effect of all the nicotine concentrations used ($1.25x10^{-5}$-$2x10^{-4}$ M). MLA at 10^{-8} M had no effect at the lowest nicotine concentration but increased OLF production by 3.5, 2.2, 1.5 and 1.5 times at higher nicotine concentrations ($p<0.01$, $p<0.05$, $p<0.05$, NS), individually. At 10^{-7} M it had no effect at $2x10^{-4}$ M nicotine

concentration, but significantly ($p<0.05$) potentiated the effects of the other nicotine concentrations used. Its effect resulted in a 1.5, 1.7, and 2-fold increase ($p<0.05$) in OLF production in combination with $1.25x10^{-5}$ M, $5x10^{-5}$ M, and $2x10^{-4}$ M nicotine, respectively.

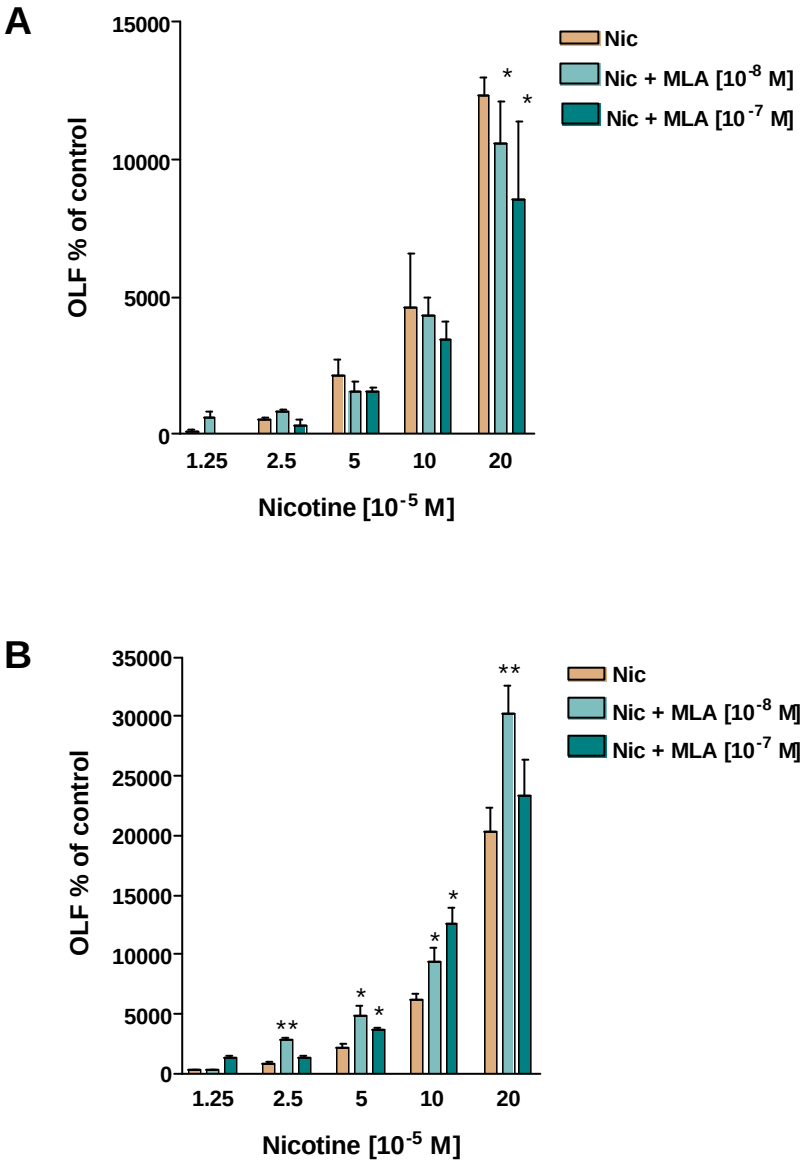

Figure 14. Effect of the a7 nicotinic receptor antagonist methyllycaconitine on nicotine induced OLF secretion in zona glomerulosa [A] and in zona fasciculata [B]. Values are mean±SEM, n=4, *p<0.05, **p<0.01 versus same concentration of nicotine alone.

5.3.4. Cholinergic influence on zona glomerulosa aldosterone secretion

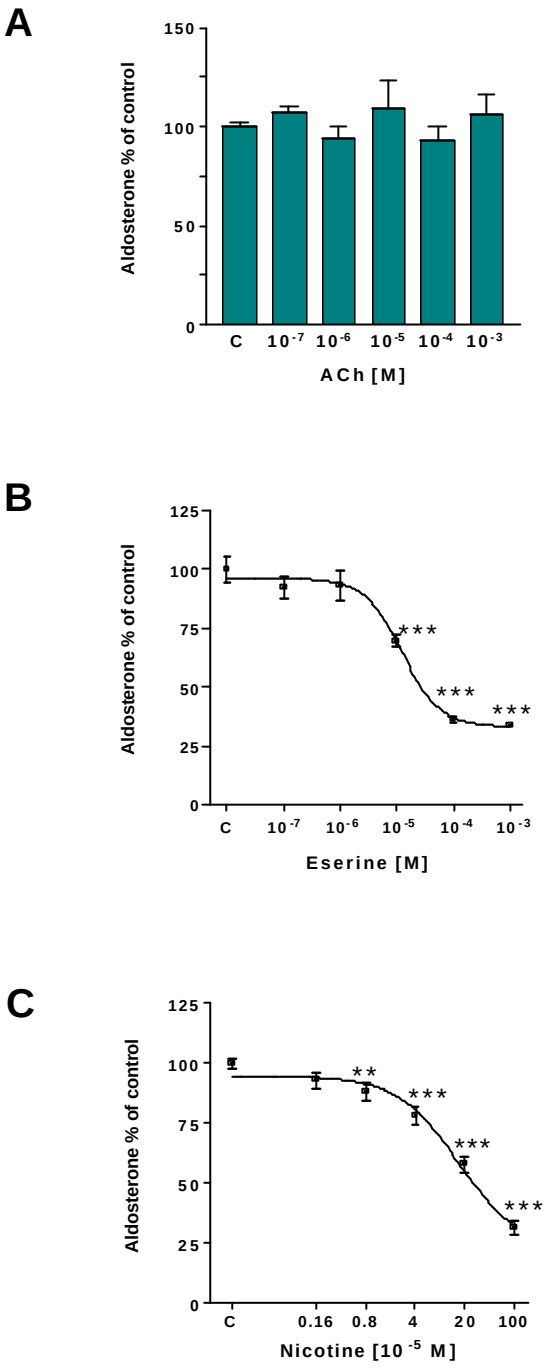

Figure 15. Effect of acetylcholine (Ach) [A], eserine [B] and nicotine [C] on adrenocortical aldosterone secretion. Values are mean±SEM, n=8, *p<0.05, **p<0.01, ***p<0.001 versus control.

Based on our result on cholinergic regulation of endogenous OLF secretion, we also proposed to characterize modulation of adrenocortical aldosterone secretion by acetylcholine, eserine and nicotine.

5.3.4.1. Effect of acetylcholine, eserine and nicotine on in vitro aldosterone secretion by zona glomerulosa cells

ACh (10^{-7}-10^{-3} M) had no effect on aldosterone secretion in 5 out of 6 experiments (**Figure 15A**), however in one experiment it stimulated the aldosterone production (data not shown). This correlates with our earlier finding using perifused capsule-glomerulosa tissue [126]. On the other hand, we observed strongly significant ($p<0.001$) dose dependent inhibition of aldosterone secretion at eserine concentrations of 10^{-5}-10^{-3} M (**Figure 15B**), which decreased the aldosterone secretion by 30%, 65%, and 70%, respectively. Employing nicotine itself we found dose-dependent inhibitory effect on aldosterone secretion (**Figure 15C**), which proved to be significant $p<0.01$ at $8x10^{-6}$ M and $p<0.001$ or less at higher nicotine concentrations ($4x10^{-5}$-10^{-3} M) used. Nicotine ($8x10^{-6}$-10^{-3}M) decreased the aldosterone secretion by 20%, 25%, 40% and 70%, respectively.

5.3.4.2. Interaction of ACTH, angiotensin-II and nicotine on aldosterone secretion

As A-II and ACTH are known to stimulate aldosterone secretion, we wondered whether nicotine interacts with them. ACTH (10^{-8} M) stimulated aldosterone secretion by about 18 times (from 1541?130 fmol/tube basal concentration to 29410?5169 fmol/tube). This effect was inhibited by nicotine ($8x10^{-6}$-$2x10^{-4}$ M) dose dependently employed in combination with ACTH by 24 %, 37 % and 57 % ($p<0.05$), respectively compared to ACTH alone (**Figure 16A).**

A-II (10^{-8} M) alone stimulated aldosterone secretion about 2.5 times (from 1541?130 fmol/tube basal concentration to 4111?494 fmol/tube). In contrast to its effect on ACTH stimulated aldosterone production, $8x10^{-6}$ M nicotine did not influence the effect of A-II. Only higher concentrations of nicotine ($4x10^{-5}$ M and $2x10^{-4}$ M) reduced the angiotensin-II evoked aldosterone secretion by 11 % and 49 % (NS,

$p<0.05$), respectively (**Figure 16B**). Nicotine alone used at the same concentrations reduced aldosterone production by 0 %, 24 % and 30 % compared to untreated control (**Figure 16A and B).** No significant interactions were shown with ACTH and nicotine, however ANOVA analysis and post hoc comparison showed an interaction between angiotensin-II and nicotine at a significance level of p=0.0507.

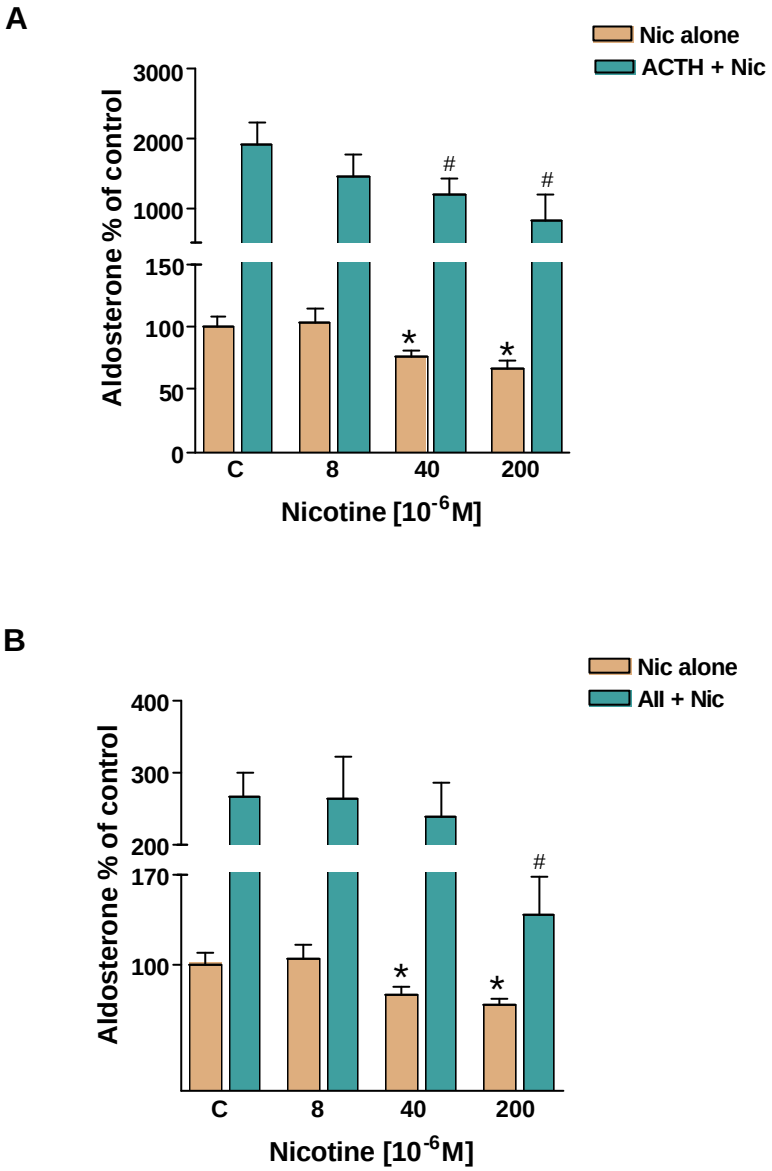

Figure 16. Interaction of ACTH [A] and nicotine, and angiotensin-II (AII) and nicotine [B] on zona glomerulosa aldosterone secretion (mean±SEM, n=8, *p<0.05, **p<0.01 versus nicotine control, #p<0.05 versus ACTH or A-II alone.

5.3.4.3. Effect of the ganglion blockers hexamethonium and mecamylamine and the ? 7 nicotinic receptor antagonist methyllycaconitine on adrenocortical aldosterone secretion

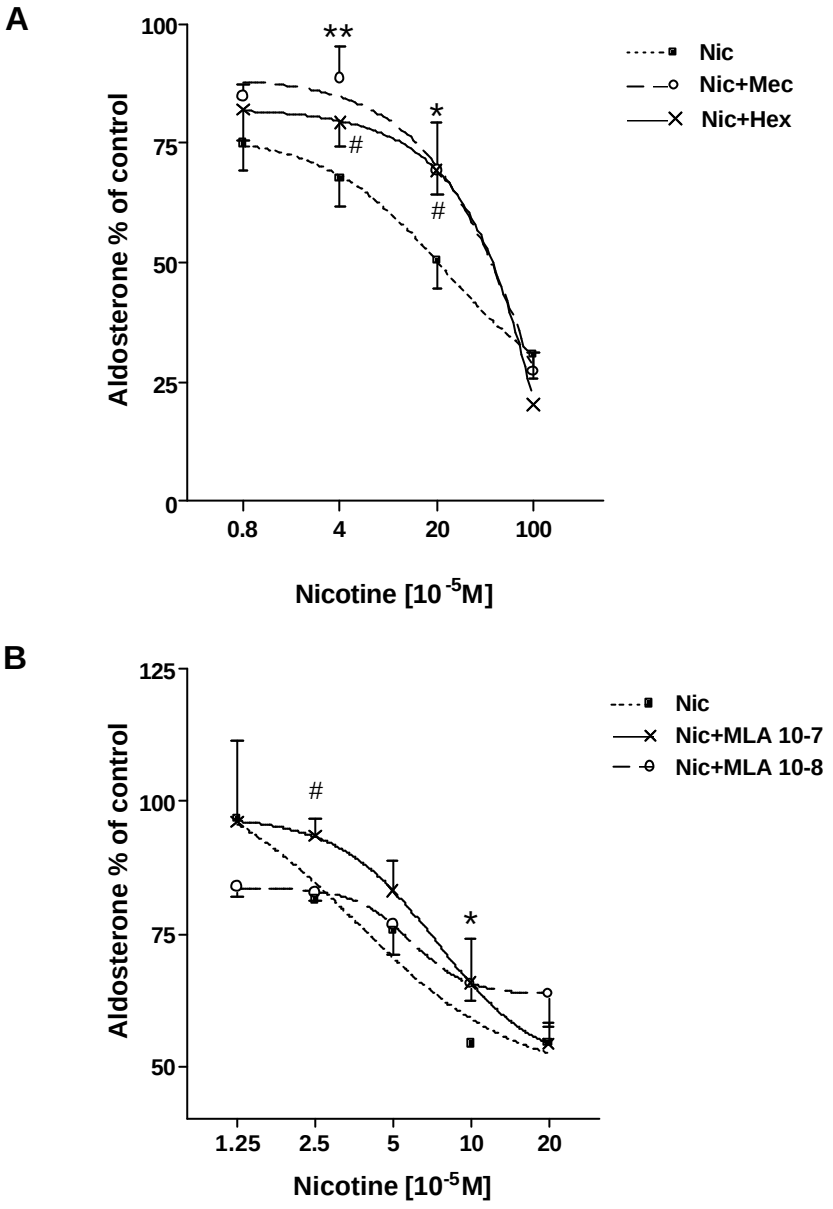

Figure 17. Effects of the ganglionic blocker hexamethonium (Hex 10^{-4}M) and mecamylamine (Mec 10^{-4}M) [A] and the a7 nicotinic receptor antagonist methyllycaconitine [B] on adrenocortical aldosterone secretion. Values are mean±SEM, n=4, *p<0.05, **p<0.01 Nic+Mec versus control, #p<0.05 Nic+Hex versus control).

As shown in **Figure 17A** hexamethonium (Hex) at 10^{-4} M partially antagonized the effect of $8x10^{-6}$ M, $4x10^{-5}$ M and $2x10^{-4}$ M nicotine by 28 %, 31 % ($p<0.05$) and 60 % ($p<0.05$) respectively, but had no influence on the inhibition by 10^{-3} M nicotine. Lower concentrations of hexamethonium (10^{-7}-10^{-5} M) had no impact on the inhibitory effect of nicotine and higher concentration of the compound (10^{-3} M) inhibited the aldosterone secretion (data not shown). The other ganglionic blocking drug: mecamylamine (Mec) at 10^{-4} M concentration was able to antagonize partially or almost completely (by 20 % NS, 69 % $p<0.01$ and 40 %, $p<0.05$) the inhibitory effect of $8x10^{-6}$-$2x10^{-4}$ M nicotine (**Figure 17A**). However, similar to hexamethonium, it was not able to influence the inhibitory effect of 10^{-3} M nicotine.

The ? 7-receptor antagonist methyllycaconitine (MLA) at 10^{-8} M partially antagonized the inhibitory effect of 10^{-4} M nicotine on aldosterone secretion (by 27 %, $p<0.05$) but showed no significant effect at lower nicotine concentrations (**Figure 17B).** As shown in **Figure 17B** 10^{-7} M methyllycaconitine had a more prominent effect. It antagonized the effect of $2.5x10^{-5}$ M, $5x10^{-5}$ M and 10^{-4} M nicotine by 65 % ($p<0.05$), 32 % (NS), and 27 % (NS), respectively.

5.4. Role of endogenous ouabain-like factor secretion in the adrenal gland: Interaction between ouabain, atrial natriuretic peptide (ANP), angiotensin-II and potassium: effects on rat zona glomerulosa aldosterone production

5.4.1. Interaction of ouabain and ANP at 3.6 mmol potassium

In order to study the effect of ouabain in the adrenal gland we treated our dispersed rat glomerulosa cells with different concentrations of the compound and measured aldosterone secretion of the cells. Ouabain at 10^{-4} M increased aldosterone production by 120 % ($p<0.001$), however, higher (10^{-3}M) and lower (10^{-5}M) concentrations of ouabain did not have any significant effect on the aldosterone secretion (**Figure 18**).

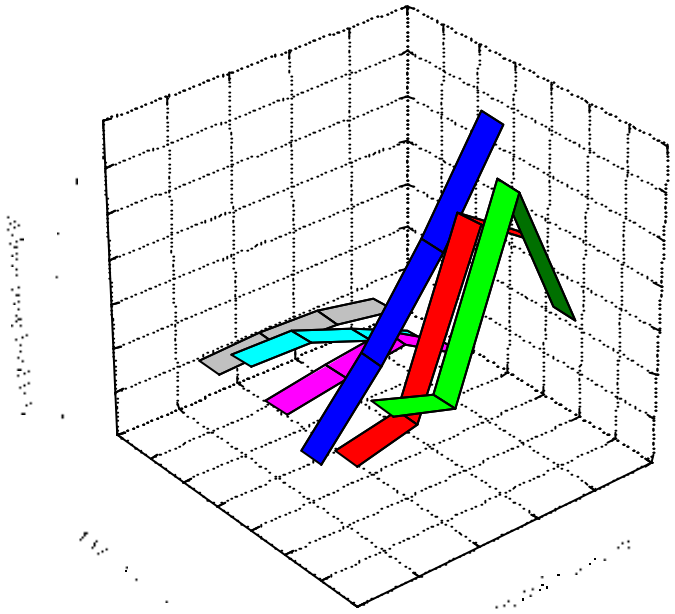

Figure 18. Interactions of different doses of ouabain and ANP on aldosterone secretion by zona glomerulosa cells at 3.6 mM [K^+]. Values shown are means from 4 separate experiments. Significant interactions ($p<0.05$) were found at 10^{-9}-10^{-7}M ANP and 10^{-4}M ouabain versus 10^{-4}M ouabain, and at 10^{-11}-10^{-10}M ANP and 10^{-3}M ouabain versus 10^{-3}M ouabain.

ANP (10^{-11}-10^{-7}M) alone inhibited basal aldosterone production by 60-24 % ($p<0.001$-$p<0.05$). Interestingly, lower doses caused higher degree of inhibition. Further, ANP at 10^{-11}M and 10^{-10}M did not influence effect of 10^{-4}M ouabain on the aldosterone production. However, 10^{-9}-10^{-5}M ANP completely inhibited the ouabain evoked increase in aldosterone secretion ($p<0.05$). An unexpected interaction ($p<0.05$) could be observed between the effect of 10^{-11}-10^{-10}M ANP and 10^{-3}M ouabain: together they increased aldosterone production by 1.5 and 2-fold, while 10^{-3}M ouabain alone was ineffective.

5.4.2. Interactions of ouabain, ANP and A-II at different potassium concentrations

As angiotensin-II and changes in extracellular potassium concentration are the major regulators of adrenocortical aldosterone secretion we aimed to study their interaction with ouabain and atrial natriuretic peptide. Ouabain at 10^{-4}M, ANP (10^{-9}M) and A-II (10^{-8}M) were used at 3.6, 5.4 and 8.3 mM potassium concentrations (**Figure 19A, B** and **C**).Rising the medium potassium concentration to 5.4 mM and 8.3 mM caused 3.5 ($p<0.01$) and 10.5-fold ($p<0.001$) increases in aldosterone production, respectively. Ouabain (10^{-4}M) increased aldosterone production at 3.6 and 5.4 mM [K^+] by 2.2 and 2 times, individually ($p<0.01$), but had no effect at 8.3 mM extracellular potassium concentration. ANP (10^{-9}M) decreased aldosterone production at every (3.6-8.3 mM) potassium concentrations by 40 % ($p<0.05$), 62 % and 79 % ($p<0.01$), respectively. Angiotensin-II (10^{-9}M) increased aldosterone synthesis at 3.6 and 5.4 mM [K^+] by 4.6 and 1.7-fold ($p<0.001$ and $p<0.05$), but had no significant effect at 8.3 mM K^+ concentration.

ANP (10^{-9}M) inhibited the effect of ouabain at 3.6 mM and 5.4 mM potassium concentrations by 66% and 75%. At 8.3 mM potassium there was no difference between the effect of ANP alone and the combined effect of ANP and ouabain. ANP inhibited angiotensin-II induced increase in aldosterone production at all potassium concentrations. Ouabain (10^{-4}M) significantly inhibited the aldosterone stimulating effect of angiotensin-II at the lowest potassium concentrations used by 66% (at 3.6 mM); however the degree of the inhibition was smaller at higher potassium levels: 29%

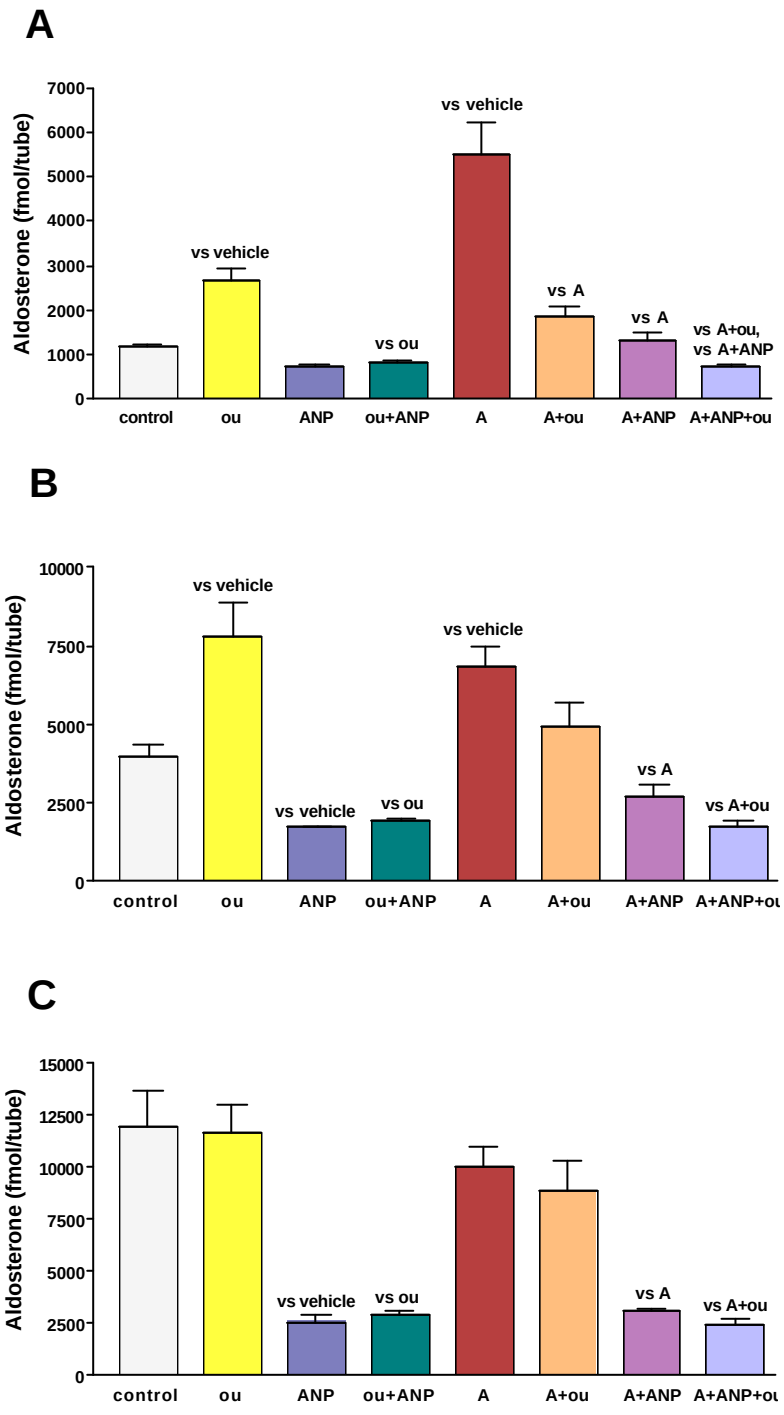

Figure 19. Effects of 10^{-9}M ANP (ANP), 10^{-8}M angiotensin-II (A) and 10^{-4}M ouabain (ou) on aldosterone secretion of zona glomerulosa cells at [A] 3.6 mM, [B] 5.6 mM and [C] 8.3 mM K^+ concentrations. Values are mean±SEM from 4 separate experiments. Text above columns shows significant differences between treatments ($p<0.05$).

at 5.4 mM, and 10% at 8.3 mM, respectively. Ouabain enhanced the inhibitory effect of ANP on angiotensin-II induced aldosterone synthesis at all potassium concentrations.

The interactions calculated between the effect of ouabain, A-II and ANP at different potassium concentrations are shown in **Table 3.**

[K+]	ANP-ou	A-II-ou	ANP-A-II	ANP-A-II-ou
3.6 mM	0.37	0.000***	0.07	0.000***
5.4 mM	0.045*	0.000***	0.63	0.085
8.3 mM	0.54	0.01*	0.23	0.49

Table 3. Significance level of interactions among 10^{-9}M ANP, 10^{-8}M A-II and 10^{-4}M ouabain (ou) at different (3.6 mM, 5.6 mM and 8.3 mM) potassium concentrations. Results shown are from 3-4 separete incubations. Neuman-Keul post-hoc comparison with log aldosterone levels was used.

5.5. Production of adrenocortical steroid hormones by human adenoma cells

Two patients with incidentalomas: one aldosteronoma and the adjacent normal tissue, and one incidentaloma with normal steroid secretion pattern (incidentaloma) were studied. As shown in **Table 4** aldosterone level in Patient A returned to normal right after operation (<160 pg/ml). Other hormone levels remained normal after the operation as ACTH was < 70 pg/ml, plasma renin activity (PRA) was 1-9 ng/ml/h, cortisol levels were 140-690 nmol/ml at 8 a.m. and 80-330 nmol/ml at 4 p.m., and free (urinary) cortisol was 55-248 nmol/day. Control measurements were taken two years after operations. Aldosterone level remained normal: 79 pg/ml, and ACTH increased to 83 pg/ml. Plasma renin activity (PRA) also became normalized: 1.1 ng/ml/h. Cortisol levels (8 a.m., 4 p.m. and free cortisol) were in normal range. In Patient B (**Table 5**) there was an increase in the 8 a.m. and 4 p.m. cortisol levels immediately after operation, but aldosterone and ACTH levels were in the normal range. Two years later

both ACTH and plasma glucose became normal, and blood pressure decreased also to normal level (data not shown).

	Before operation	After operation
Aldosterone (pg/ml)	624	75
PRA (ng/ml/h)	0.4	0.45
Cortisol (nmol/ml) 8h 16h	470 436	625 441
free urinary	116	236
ACTH (pg/ml)	<25	<25

Table 4. Patient A: Cortical adrenal adenoma (aldosteronoma). Hypertension.

	Before operation	After operation
Aldosterone (pg/ml)	99	75
PRA (ng/ml/h)	0.8	0.45
Cortisol (nmol/ml) 8h 16h	267 321	625 855
free urinary	82	48
ACTH (pg/ml)	<25	61

Table 5. Patient B: Incidentaloma: Adrenocortical adenoma. Hypertension. NIDDM.

5.5.1. Ouabain-like factor concentrations of adrenal supernatants is modulated by extracellular potassium

Basal OLF production of dispersed incidentaloma and aldosteronoma cells was 20-fold and 40-fold higher, respectively than that of the adjacent normal tissue ($p<0.001$, **Table 6**). To modulate steroid secretion we used different concentrations of K^+ in the incubation media. We found that OLF production was increased at 5.4 and 8.3 mM potassium concentrations in the normal adjacent tissue about 20-fold ($p<0.001$, **Figure 20C**) and in the incidentaloma about 2.5 and 1.5 times, respectively ($p<0.05$, **Figure 20A**), compared to the secretions at 3.6 mM potassium. However, OLF concentration was decreased in the aldosteronoma by ~50 % ($p<0.05$) (**Figure 20B**) at both 5.4 and 8.3 mM extracellular potassium concentrations.

	OLF (pmol/ml)	**Aldosterone (pmol/ml)**
Normal (adjacent)	0.01±0.001	2.0±0.36
Incidentaloma	0.21±0.04***	1.2±0.004*
Aldosteronoma	0.42±0.02***	35.5±2.6***

Table 6. Basal OLF and aldosterone production of incidentally detected human adrenal adenomas; *p<0.05, ***p<0.001 versus normal.

5.5.2. Aldosterone and Cortisol

*Basal aldosterone secretion of dispersed incidentaloma cells were 50% lower ($p<0.05$) and of aldosteronoma cells 17-fold higher ($p<0.001$) compared to normal (adjacent) tissue. In the adjacent "normal" adrenal tissue, high [K^+] did not increase aldosterone secretion (**Figure 20C**). We found a significant ~50 % decrease (*p<0.05) in aldosterone secretion by aldosteronoma cells both at 5.4 and 8.3 mM potassium*

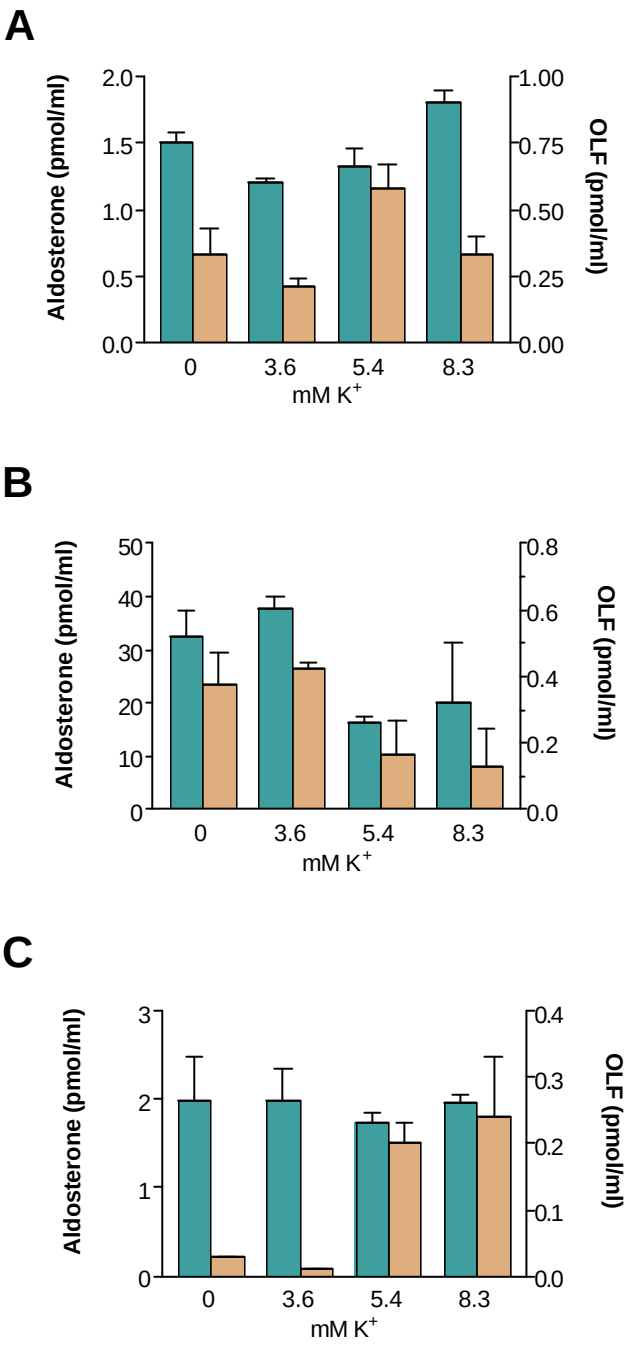

Figure 20. *OLF (orange bars) and aldosterone (turquoise bars) production of incidentally detected human adrenal adenomas. Effect of changes in extracellular [K^+] on incidentaloma with normal steroid level [A], on aldosteronoma [B], and on the adjacent normal part [C] of the tumour. Values are mean±SEM, n=3, *p<0.05, ***p<0.001.*

*(**Figure 20B**). In the incidentaloma sample changes of the extracellular [K^+] increased aldosterone secretion close to the well-known pattern (**Figure 20A**).*

Cortisol levels of incubation media of adjacent tissue (960±75 pmol/ml), aldosteronoma (587±35 pmol/ml) and of the incidentaloma (552±33 pmol/ml) did not change significantly using higher (5.4 mM, 8.3 mM) or lower (0 mM) potassium concentrations (data not shown on **Figure 20**).

5.6. Plasma levels of endogenous ouabain-like compound (OLF) and adrenomedullin (ADM) in experimental cardiac hypertrophy in rats

To test the hypothesis that circulating endogenous positive inotropic substances may enhance contractility of the hypertrophied myocardium to improve heart failure, we characterized the plasma levels of OLF and adrenomedullin during development of left ventricular hypertrophy in shunted animals. As these hormones were previously shown to be secreted mainly by the adrenals [48-51], our model system using shunted and/ or adrenalectomized animals seemed to be ideal to study development of cardiac hypertrophy in the presence or absence of these substrates.

I. 5.6.1. Induced left ventricular hypertrophy; atrial natriuretic peptide (ANP) as marker of cardiac hypertrophy

Changes in body weight and left ventricular weight of the animals were measured at 1, 2 and 4 week after the operations. As shown in **Table 7** volume overload was accompanied by a significant left ventricular hypertrophy both two and four weeks after the interventions (938±83 mg versus 828±52 mg, $p<0.05$ and 1213±199 mg versus 1032±62 mg, $p<0.05$ shunt versus sham, respectively).

Since shunted rats gained less weight as sham operated, atrial natriuretic peptide was used as a biochemical marker to control cardiac hypertrophy. Plasma levels of ANP were 0.06-fold, 4.2-fold ($p<0.05$) and 5.6-fold ($p<0.05$) of control 1, 2 and 4 weeks after shunting, respectively.

Treatments	n	Time (weeks)	Body weight (g) (mean ±SEM)	Left ventricular weight (mg) (mean ±SEM)
Control	7	1	298±15	811±58
Shunt	12	1	285±25	878±107
Control	10	2	312±21	828±53
Shunt	12	2	306±20	938±83*
Control	8	4	388±20	1032±62
Shunt	16	4	372±27	1213±199*

Table 7. Changes in body weight and left ventricular weight of shunted and control operated animals 1, 2 or 4 week after surgery; *p<0.05 versus control.

II. *5.6.2. Plasma concentrations of OLF and ADM*

One week after operation there was a nonsignificant increase in OLF plasma concentration of shunted animals and a nonsignificant decrease in adrenalectomized (ADX) rats (**Figure 21A**). Significant decrease (42 %, $p<0.05$) was observed in adrenalectomized and shunted (ADX+Shunt) rats compared to sham operated ones. At two weeks OLF plasma concentration in shunted animals was increased significantly (1.9-fold increase, $p<0.05$) compared to sham operated controls (**Figure 21B**). Unexpectedly, the increase was more expressed in "ADX+Shunt" rats (2.97-fold, $p<0.01$). There was no significant change (any decrease) in adrenalectomized animals. Four weeks after surgery however, ouabain levels decreased in all groups compared to sham (**Figure 21C**). The decrease was significant in adrenalectomized (ADX), and in adrenalectomized and shunted (ADX+Shunt) animals (49 %, $p<0.05$ and 40 %, $p<0.05$ versus control, respectively). However, in the shunted group the change (20 % decrease) was not significant.

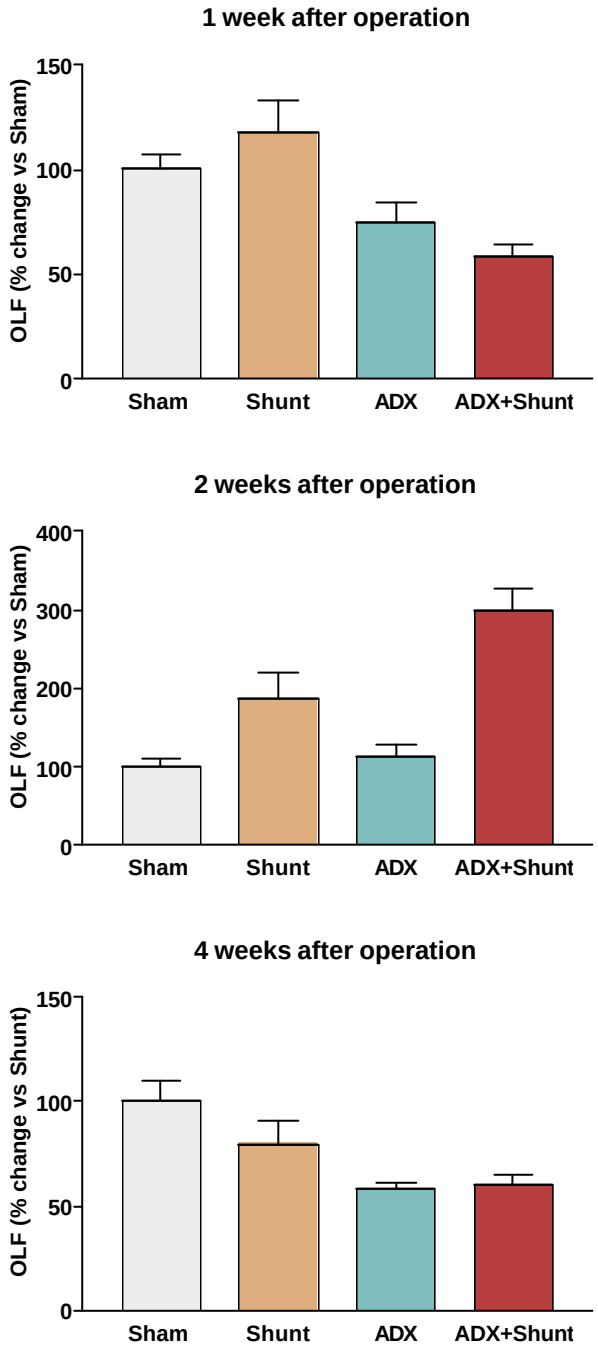

Figure 21. Plasma levels of ouabain (OLF) in shunted, adrenalectomized (ADX), and adrenalectomized and shunted (ADX+Shunt) animals 1, 2, and 4 weeks after operation (A, B and C, respectively). Values are expressed as % change versus Sham operated; *p<0.01 versus Sham, #p<0.05 versus ADX, $p<0.05 versus Shunt.

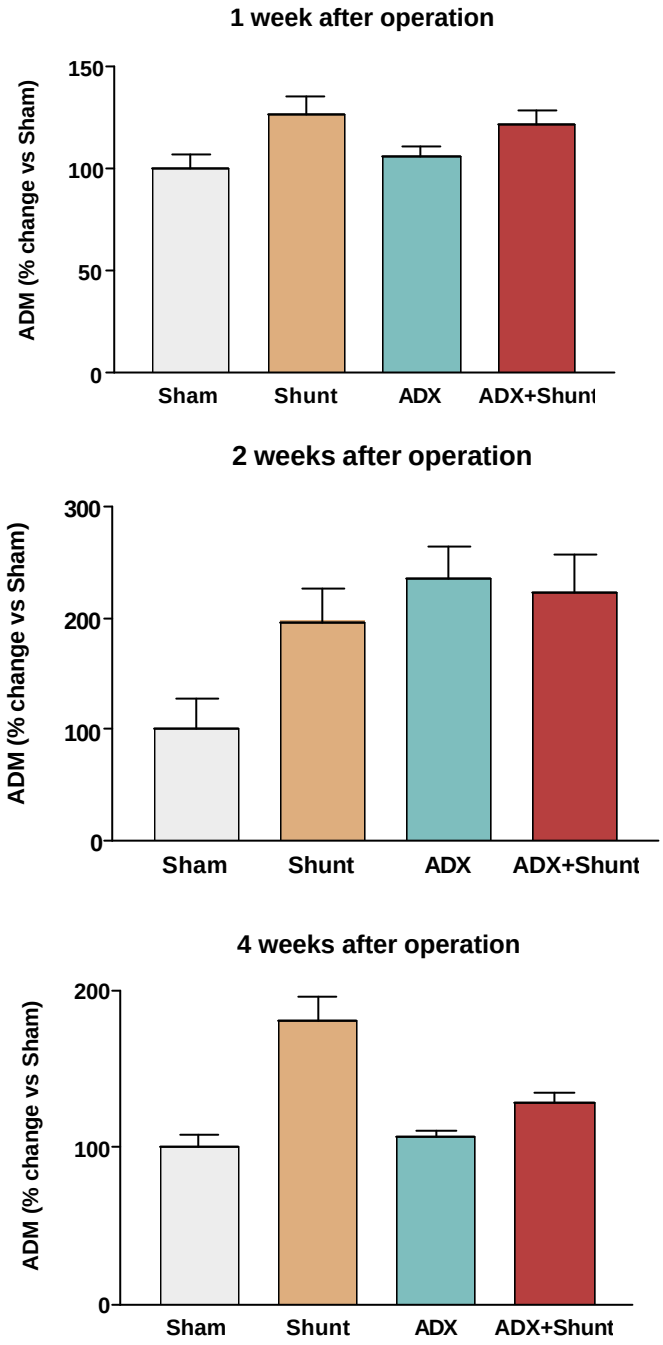

Figure 22. Plasma levels of adrenomedullin (ADM) in shunted, adrenalectomized (ADX), and adrenalectomized and shunted (ADX+Shunt) animals 1, 2, and 4 weeks after operation (A, B and C, respectively). Values are expressed as % change versus Sham operated; *p<0.01 versus Sham, **p<0.01 versus Sham, #p<0.05 versus ADX.

Plasma levels of adrenomedullin were significantly increased at week one in shunted (1.3-fold $p<0.05$), and adrenalectomized and shunted (ADX+Shunt: 1.2-fold $p<0.05$) rats (**Figure 22A**). Similar to OLF, plasma adrenomedullin of shunted (2-fold, $p<0.05$) and ADX+shunted animals (2.2-fold, $p<0.05$) showed significant increase at two weeks compared to controls (**Figure 22B**). Unexpectedly, plasma level of adrenomedullin in ADX rats also increased (2.3-fold, $p<0.01$) raising the question whether the adrenal gland is really the main source of ADM. At week four adrenomedullin level raised in shunted rats 1.8-fold ($p<0.05$) compared to controls (**Figure 22C**). Further, there was a significant increase in ADM plasma level of ADX+shunted animals compared to both sham operated (1.27-fold, $p<0.05$) and adrenalectomized rats (1.2-fold, $p<0.05$). However, there was no significant difference between the plasma ADM levels of control and adrenalectomized animals at this time point.

5.7. Plasma Levels and Urinary Excretion of Endogenous Ouabain-like Compound in Volume Expanded Conditions in Humans

As increased production of endogenous substances with positive inotropic and natriuretic effect were described in physiological and pathophysiological states with volume overloading [128], we aimed to study the circulating level of OLF in these cases. Our main goal was to validate our assay and compare our results with the previous findings of other investigators on endogenous ouabain- (digitalis)-like factors.

5.7.1. Plasma levels and urinary excretion of OLF in patient with congestive heart failure

As shown in **Figure 23**, extracted plasma OLF levels and urinary OLF concentrations were 1.7 and 1.85 times higher respectively in patients with congestive heart failure (CHF) than in healthy controls (5.3±0.9 versus control 3.1±1.1 pg/ml, $p<0.05$ and 54.1±10.0 versus control 29.2±10.3 pg/ml, $p<0.05$, individually). Twenty-four hours urinary OLF excretion was more than two times higher in patients with CHF compared to control individuals (72.4±13.4 versus 31.9±11.3 ng/24 hours, $p<0.05$). OLF plasma clearance (13.8±2.6 versus control 13.4±4.7 L/ 24 hours, p=NS) and

urinary/plasma concentration ratios (11±2 versus control 14±5, p=NS) were similar in both groups.

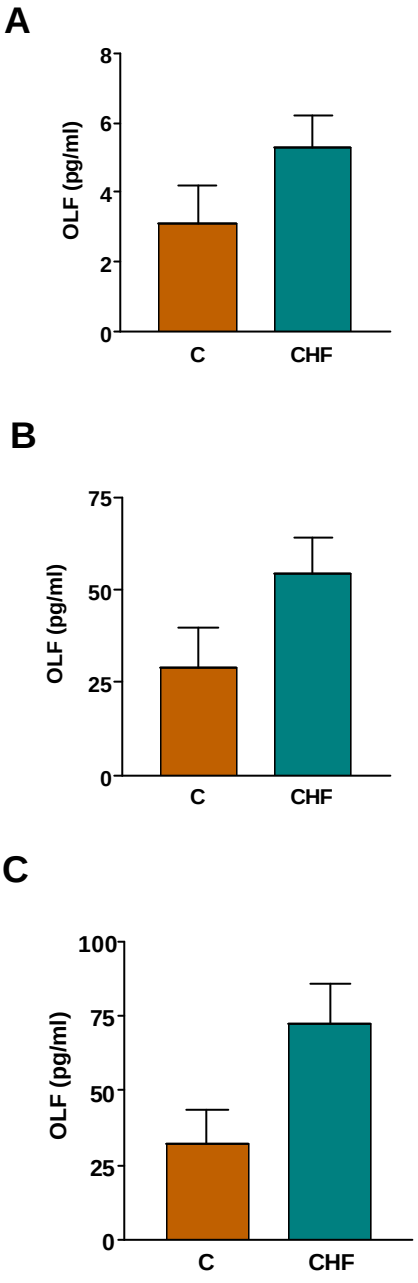

Figure 23. Plasma [A] and urinary [B] levels and twenty-four hours urinary OLF excretion [C] of patients with congestive heart failure (CHF, n=20) and of healthy controls (C, n=16); *p<0.05 versus control.

5.7.2. Plasma and urinary levels of OLF in diabetic patients

We extracted plasma samples of 43 diabetic patients. OLF levels of 72 % of the samples were out of assay range and only in twelve samples were we able to measure plasma OLF concentration. Mean±SEM level were 9.91±2.51 pg/ml (n=12). This proved to be significantly higher than the OLF levels measured in healthy subjects (3.1±1.1 pg/ml, $p<0.05$). Correlating gender with plasma OLF levels (**Figure 24A**) we found that plasma level of ouabain-like factor in males is about two times higher than in females (15.04±5.37 versus 6.25±0.87 pg/ml, $p<0.001$). Urinary OLF of 15 patients was measured and levels were detectable in all samples. OLF concentration ranged 27±4.04 pg/ml, which was not significantly different from healthy controls (29.2±10.3 pg/ml).

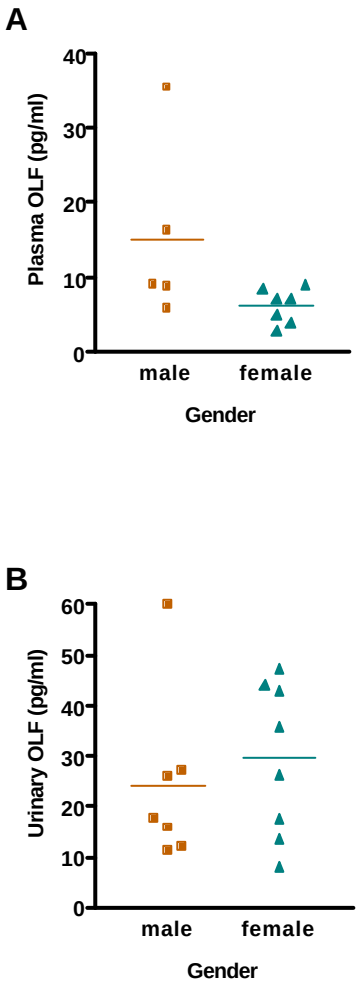

Figure 24. Plasma [A] and urinary [B] OLF concentrations in diabetic patients. Mean values are expressed as solid lines for each group.

As shown in **Figure 24B**, there was no significant difference in urinary OLF of males compared to females (24.24±6.4 versus 29.43±5.36 pg/ml, p=NS), and we could not find correlation between plasma and urinary OLF concentrations.

As we did not have much plasma OLF data, we did analysis on urinary OLF levels. Grouping our data by diagnosis (**Figure 25A**): non-insulin dependent diabetes (NIDDM) versus insulin dependent disease (IDDM), we did not find any statistically significant differences (24.53±7.3 versus 27.92±5.01 pg/ml, p=NS). We also compared urinary OLF levels of normotensive and hypertensive diabetic subjects (**Figure 25B**). Again, there was no difference in the urinary OLF concentrations (31.24±9.4 versus 26.78±4.1 pg/ml, p=NS).

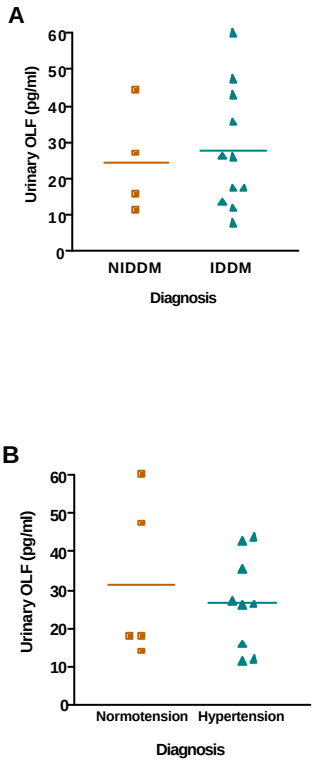

Figure 25. Comparison of urinary OLF levels in NIDDM and IDDM [A] and in normotensive and hypertensive diabetic patients [B]. Mean values are expressed as solid lines for each group.

5.7.3. Plasma and urinary level of OLF in diabetic pregnant women

Plasma OLF was measured in first, second and third trimester of pregnancy in 25 diabetic patients (**Figure 26A**).

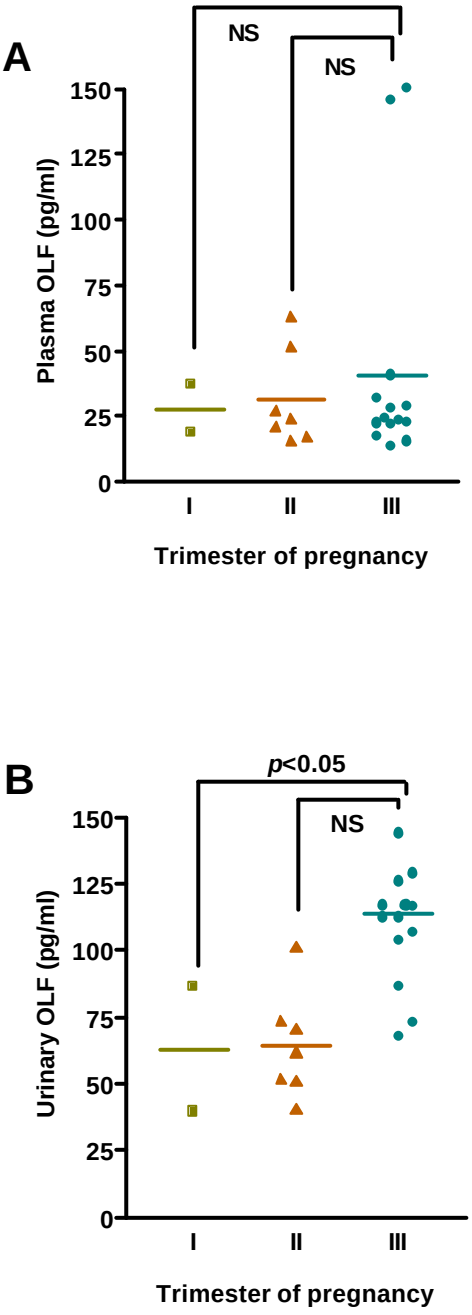

Figure 26. Plasma [A] and urinary [B] OLF levels in first, second and third trimesters of pregnancy in diabetic patients. (NS=nonsignificant difference)

Levels of two plasma samples were below assay sensitivity, which means 8 % of the data, compared to the 72 % found in non-pregnant diabetic patients. Mean values of samples showed a non-significant increase in the third trimester (40.47±11.4 pg/ml) compared to levels of the first (27.73±9.3 pg/ml) and second trimesters (31.39±6.97 pg/ml). (OLF concentration of "first trimester" group is rather symbolic as we had only two useful data.) Plasma concentrations in every subgroup were significantly higher compared to OLF level in healthy subjects (versus 3.1±1.1 pg/ml, $p<0.01$).

A more expressed increase was visualized using urinary OLF for comparison (**Figure 26B**). Concentration of ouabain-like factor was increased significantly in the third trimester (113.7± 7.56 pg/ml) compared to the first (63±23.4 pg/ml, $p<0.05$) and the second (64.59±7.5 pg/ml, $p<0.001$) ones. Urinary concentrations in "second trimester" and third trimester" groups were significantly higher compared to OLF level in healthy subjects (versus 29.2±10.3 pg/ml, $p<0.05$, $p<0.01$ respectively).

OLF data of patients were subdivided into three groups according to their diagnosis (**Figure 27A**). Only one patient had NIDDM. Thirteen patients had insulin dependent disease (IDDM) and 10 had gestational diabetes induced either during their present or developed already at the time of their previous pregnancy. There was significant difference between plasma levels of OLF in IDDM and gestational diabetes (23.6±2.28 pg/ml versus 54.74±16.24 pg/ml, $p<0.05$). However, as shown in **Figure 27B,** urinary levels of OLF were in the same range (90.98±10.23 versus 96.73±11.95 pg/ml, p=NS). Association between plasma/ urinary OLF and HbA1C levels in these two groups of patients (i.e. IDDM and gestational DM) was also analyzed. No significant correlation was found in any of the groups. **Figure 28A** and **28B** show HbA1C versus plasma and urine OLF in IDDM patients and in gestational DM, respectively. Comparing plasma OLF levels of pregnant groups with non-pregnant diabetic females (31.39±6.97 and 40.47±11.4 pg/ml versus 6.25±0.87 pg/ml, $p<0.05$), we found about 4-5 times higher plasma OLF level in pregnant diabetic patients.

Urinary OLF level (64.59±7.5 and 113.59±7.56 pg/ml versus 29.43±5.36 pg/ml, $p<0.01$) proved to be three times higher in pregnant patients with DM.

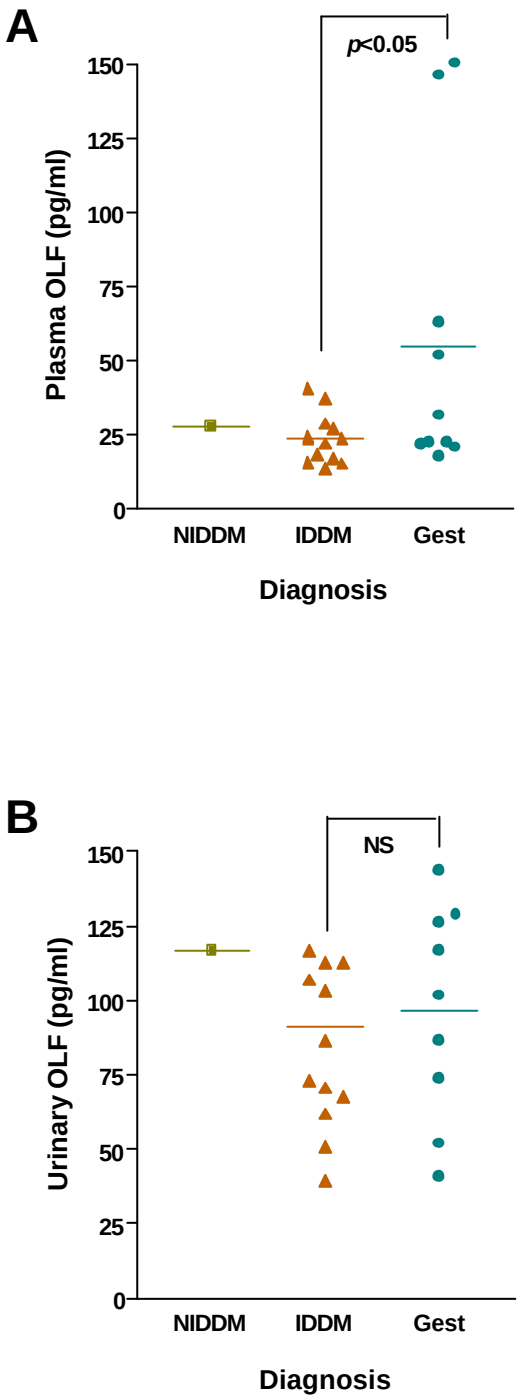

Figure 27. Plasma [A] and urinary [B] OLF levels of pregnant NIDDM, IDDM and gestational diabetic patients. (NS=nonsignificant difference)

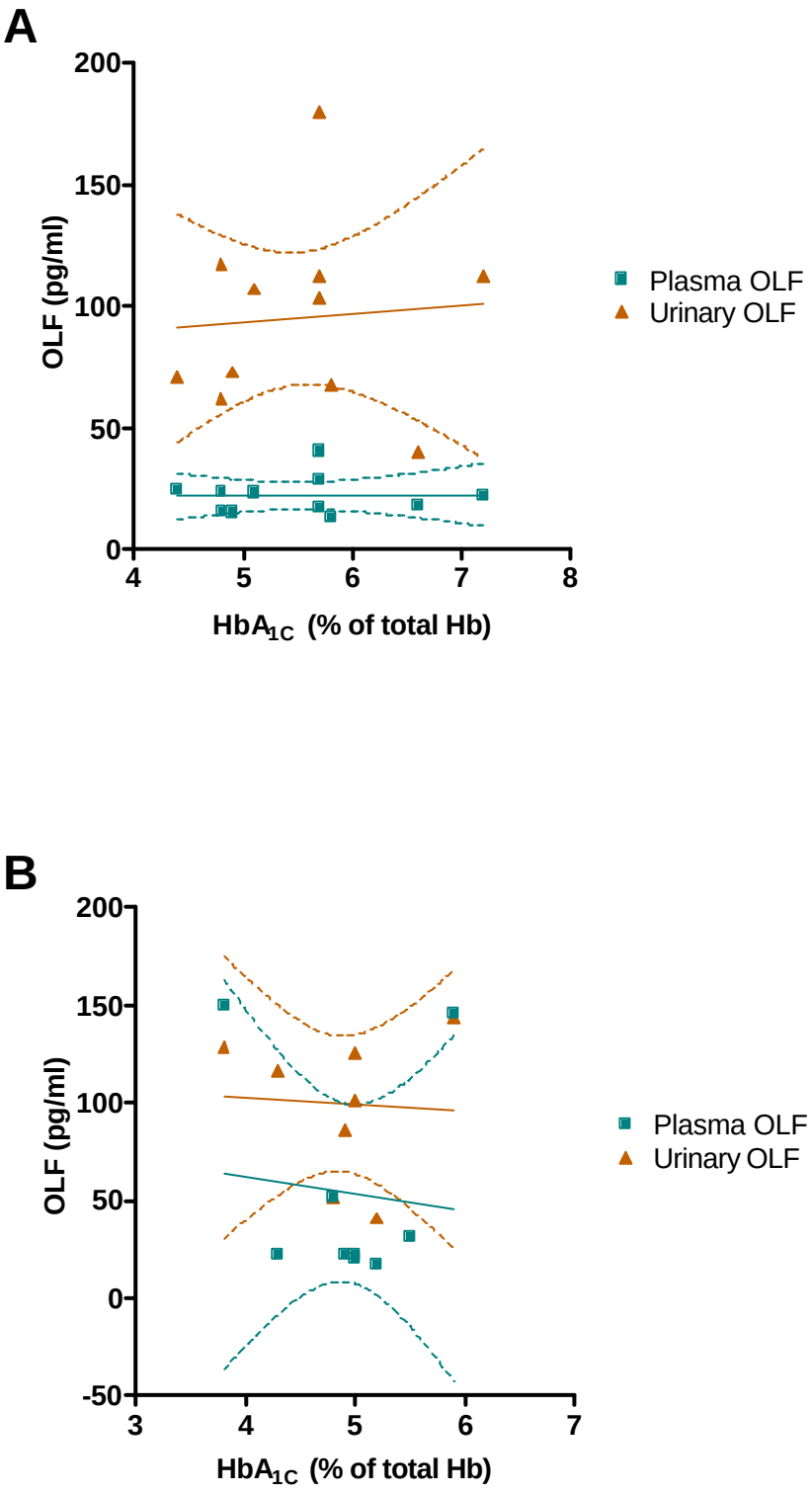

Figure 28. Correlation between plasma/ urinary OLF and HbA_{1C} in pregnant IDDM [A] and gestational diabetic patients [B].

5.7.4. OLF level in premature and mature newborns

As newborns have relative high fluid volume compared to their body mass we aimed to measure plasma level of OLF in babies. Compared to healthy adults newborns (28-41 weeks, n=25) had about twelve times higher OLF levels (3.1±1.1 versus 41.96±4.64 pg/ml, $p<0.001$). We proposed to analyze relationship between the age of the newborns (gestational weeks) and their umbilical cord plasma OLF level. There was a highly significant correlation between maturity and OLF concentration ($p<0.004$). As shown in **Figure 29**, ouabain-like factor level increased with maturity, however there was a rapid drop in its concentration at week 39 (43.26±7 versus 35.47±1.84 pg/ml, p=NS).

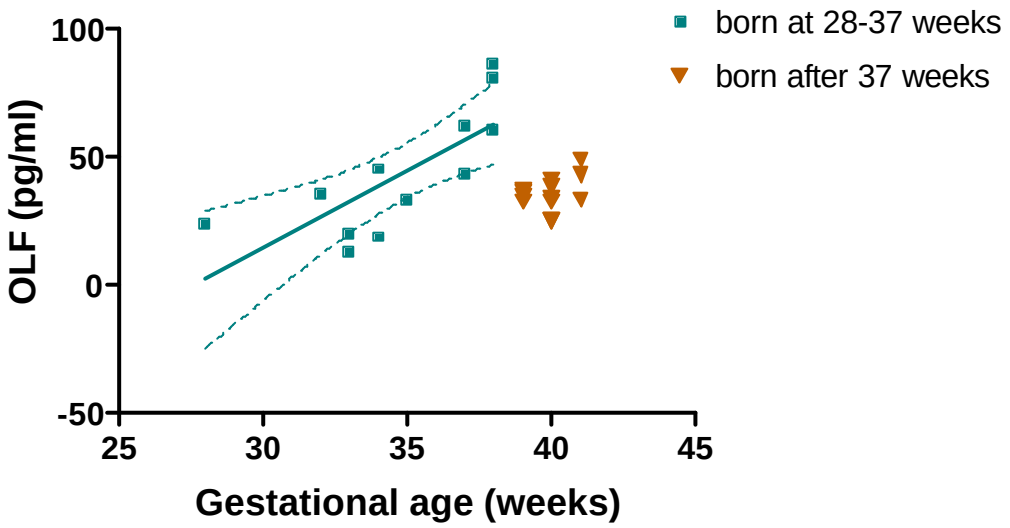

Figure 29. Relationship between plasma OLF and gestational age in newborns; p<0.004 premature versus mature group. ?Cord blood?????l) was extracted and analyzed in ouabain RIA.

6. DISCUSSION

6.1. Presence of ouabain-like immunopositive cells in the mammalian adrenal cortex

Previous studies using digoxin or digitoxin antiserum showed presence of immunopositive cells in the rat paraventricular and supraoptical nuclei. In some cases, digitalis-like (DL) immunopositivity was co-localized with vasopressin and/or oxytocin and because of its localization EDLF was suggested to be a neurohormone or neurotransmitter [126]. Ouabain-like immunoreactivity was also shown to be present in the same areas [127]. However, there is only one study published up to now reporting strong reactivity in the adrenal cortex by using anti-digoxin serum [128]. Our data now provide evidence that there is also OLF-like immunoreactivity in the mammalian adrenal cortex. Interestingly, similar to the data on digitalis- and ouabain-like activity in the central nervous system, we found the strongest ouabain-like immunoreactivity at the same zona (zona fasciculata) where presence of digitalis-like reactivity was shown previously [128]. An explanation for these observations could be that the antibodies cross-react with other cardenolides, thus presenting similar results. Though, careful analysis of the antibodies provided by the authors show that the investigators (including us) worked with highly digitalis- or ouabain-specific antisera. Another explanation could be that both digitalis and ouabain-like factors are produced by the same type of cell in both the central nervous system and in the adrenals. However, this hypothesis could be confirmed or rejected after pathway of synthesis for endogenous cardenolides is solved.

6.2. Ouabain radioimmunoassay

By the time our RIA using ^{3}H-ouabain tracer data was published in 1996, several other groups managed to develop enzyme linked immunosorbent assay (ELISA) or RIA for measuring endogenous ouabain-like factors in body fluids [129-132], and the number of various assays are still growing [123, 133-134]. We improved our assay sensitivity by using iodinated tracer [123], which made us possible to measure low OLF levels. Further, using HPLC, we proved that there is a rather homogenous endogenous material present in our samples (both in plasma/ urine and in adrenal supernatant), and that the majority of ouabain immunoreactivity of our samples eluted identically with authentic ouabain. These results validate our assay for measurement of endogenous ouabain-like factor. Interestingly, however, for measuring OLF from healthy subjects we still needed to use an even more sensitive assay with different ouabain antibodies [135].

This fact immediately highlights the main problem of endogenous OLF quantification. Namely, previous studies disclose large variations in endogenous OLF levels. Different groups with similar assay sensitivity report highly divergent plasma ouabain concentrations. The plasma concentrations in solid-phase-extracted samples measured vary from <5 pmol/L (15) to 100–300 pmol/L [130,133-134]. Some authors described even higher (>1000 pmol/L) OLF levels in human plasma [129]. In addition, ouabain concentrations of extracted rat plasma ranged from <50 pmol/L [136] to >200 pmol/L [134]. As a main reason for these discrepancies Vakkuri *et al.* discussed in their recent paper presence of nonspecific interferences during the measurement of low concentration of biologically active substances by RIA and ELISA [123]. An additional source of inconsistency could be the use of tritiated or enzyme-labeled tracers, which can lead to low sensitivity and thus resulting greatly variable plasma ouabain concentrations [123]. Differences in the degree of specificity of ouabain antibodies developed in the individual laboratories, heterogeneity of human samples and contamination by exogenous ouabain can possibly play some role [134]. In some cases lack of comparison of immunological and chemical properties of the endogenous ouabain-like substance with authentic ouabain by HPLC makes the results also questionable [132-133].

Further work needed to develop a rather uniform extraction and quantification methodology, which would make it possible not only to compare data of different study groups but also to measure clinical samples routinely.

6.3. Regulation of endogenous ouabain-like factor secretion in the adrenal gland

6.3.1. Rat adrenal zona glomerulosa and fasciculata has similar tissue OLF concentrations

Ouabain tissue content of the rat adrenals was measured previously by Ferrandi *et al.*, however they did not investigated the ouabain content of the zona glomerulosa and fasciculata separately. They used methanol extraction and HPLC fractionation and found 1.32-1.6 ng/g wet tissue weight OLF in the adrenals [134]. Our data showed 3-4 times lower tissue OLF content, which could be due to the different extraction method and RIA used. We also found that zona glomerulosa and fasciculata/reticularis OLF concentrations are similar (176.02± 8.34 versus 208.62±16 pg/g wet tissue weight). There are no other reports on rat adrenals till now. Laredo *et al.* [137] investigated bovine adrenals and found that the zona glomerulosa tissue content was 5.7-fold higher than the zona fasciculata OLF concentration, which can be explained due to difference between species. These data show that in various species the contribution of the adrenal layers to the OLF production can be very different.

6.3.2. *Regulation of endogenous ouabain-like factor secretion in the adrenal gland: effect of AII, ACTH, ANF, and extracellular potassium*

There are not many data on regulation of endogenous OLF in the adrenals. Laredo and his coworkers [48] previously showed that in *bovine* adrenal zona glomerulosa cells 10^{-8}M ACTH or angiotensin-II induced significant increase in OLF secretion. However, they claimed that ACTH or A-II had no effect on zona fasciculata cells. The basal OLF production in their system is 4 times less than the cells' of the glomerulosa layer [48].

To our knowledge our report is the only one on regulation of rat adrenal OLF till now. In our studies we found that the rat zona glomerulosa and fasciculata/reticularis layers not only have the same tissue concentration of ouabain, but their basal OLF productions are about the same: 6-10 pg/ml medium. ACTH (even at 10^{-9} M) induced prominent increase in OLF production of both the fasciculata and glomerulosa layers. However, angiotensin-II had more prominent effect on fasciculata cells then in zona glomerulosa. These results again confirm that in rat both layers are important contributors to endogenous OLF production.

There are no previous studies reporting on effect of extracellular potassium concentration on adrenal OLF secretion either, however role of K^+ in adrenocortical aldosterone secretion is well investigated [138-140]. We found that unlike aldosterone, endogenous OLF secretion is decreased both at very high (9 mM) and low (1.2 mM) K^+ concentrations, but similar to aldosterone its level is increased at 6 mM extracellular potassium concentration. The difference can be due to the different role of aldosterone and endogenous OLF in the electrolyte homeostasis. The fact, that extracellular potassium concentration influenced OLF production only in zona glomerulosa but not in faciculate/ reticularis, suggest that potassium may not have a direct effect on endogenous ouabain secretion. Rather, other factors modulated by the changes in extracellular [K^+] –like aldosterone – can act as indirect regulators of OLF production.

6.3.3.-6.3.4. Cholinergic influence on endogenous OLF and aldosterone secretion: effect of acetylcholin, nicotine and eserine

In the control of endogenous OLF secretion the role of ACTH and A-II has been discussed till now (see above). These compounds, however, have only a moderate stimulatory effect on OLF secretion, especially A-II, which effect seems to be more prominent in bovine [50] than in rat adrenocortical cells [122]. Our data now provide evidence for more potent activators of endogenous OLF production; namely nicotine and acetylcholine, which can be “the key modulators” of OLF secretion. The huge OLF

secretory response induced by these compounds was confirmed by HPLC also proving that the endogenous OLF we measured is similar to authentic ouabain.

In our previous work we showed presence of extended peripheral cholinergic network in the zona glomerulosa [141]. Cholinergic nerve fibres terminating in this area can form the physiological source of OLF secretory stimulator, and acetylcholine released from the nerve endings can exert direct neuronal modulation on the steroidogenesis in zona glomerulosa cells. Although, muscarinic regulation of cortisol and aldosterone in bovine adrenocortical cells [142-144] and in perifusion of rat adrenal capsule-glomerulosa preparation [141] was already shown, we have some data for nicotinic modulation of steroidogenesis in cat [145] and rat [146] adrenocortical cells also. Formerly, nicotine and/or related constituents were already shown to repress glucocorticoid and sex steroidogenesis by inhibiting rat adrenal 21-hydroxylase, 11?-hydroxylase [147] and conversion of corticosterone to 18-hydroxycorticosterone [146]. Skowronski and Feldman confirmed inhibition of ACTH and A-II stimulated aldosterone secretion in rat adrenal cells using nicotine and cotinine, however they did not investigate modulation of basal aldosterone secretion [146]. Influence of nicotine on secretion of other steroids was also investigated. It elicited a dose-dependent increase on steroidogenesis in cat adrenocortical cells [145], inhibited androgen biosynthesis by cultured rat testicular cells [147] and steroidogenesis in mouse Leydig cells [148]. In this study we confirmed, that nicotine inhibits ACTH and A-II stimulated aldosterone secretion. Further, we showed a dose dependent inhibitory effect on the basal aldosterone secretion also, using one order of magnitude lower concentration of nicotine than published previously [146].

Eserine or physostigmine, a well-established acetylcholinesterase inhibitor, was shown to interact directly with nicotinic acetylcholine receptor [149]. Modulation of the receptor is noncompetitive (allosteric); the nicotinic acetylcholine receptors were shown to contain loci different from the acetylcholine binding site through which eserine can modulate the receptor function (for review see ref 150). Although nicotinic receptors were not proved to be present on adrenocortical cells, the fact that not only nicotine but also eserine inhibited aldosterone secretion dose dependently and

stimulated endogenous OLF production suggests the existence of functional nicotinic acetylcholine receptor(s) on cells in the rat adrenal cortex.

Studies revealing presence of neuronal nicotinic acetylcholine receptors on non-excitatory cells were published already (for review see ref 151). Thus, skin keratinocytes [152], bronchial epithelial cells [153] and vascular endothelial cells were proved to possess functional nicotinic receptors, similar to those expressed in sympathetic ganglia, based on their ion-gating properties [154]. Further, in human nasal epithelial cells nicotine (3mM) and its derivate nicotine di-D-tartrate caused a methyllycaconitine-sensitive increase in intracellular [Ca^{2+}], indicating direct effect of nicotine on these cells [155]. In our experiments we were able to inhibit aldosterone secretion using nicotine concentration two orders of magnitude lower (!) than was used in nasal epithelial cells. Moreover, both the ganglionic blocker hexamethonium and mecamylamine reduced the nicotine induced inhibitory effect on the zona glomerulosa aldosterone secretion, thus suggesting role of functional nicotinic receptor in the regulation of this hormone. The ?7 receptor antagonist MLA had only moderate inhibitory effect. However, only the ?7 receptor antagonist MLA showed a dose-dependent inhibitory effect on nicotine stimulated OLF secretion by zona glomerulosa, but at the same time it potentiated the effect of nicotine in zona fasciculata/reticularis. In contrast, the ganglionic blockers seemed to have inhibitory effect on nicotine induced OLF response only in zona fasciculata and they potentiated the effect of the compound in the zona glomerulosa. These data may suggest presence of different nicotinic receptors in the distinct layers (zonae) and involvement of different receptors in the regulation of aldosterone and OLF. It was already shown, that distinct angiotensin II receptors play role in regulation of OLF (AT_2) and aldosterone (AT_1) in bovine adrenocortical cells [50].

Potentiation and inhibition of nicotinic receptors by the same compound is well known [156-159, for review see ref 150]. Although, another possible explanation for the response to the antagonists can be that the compounds we used have bound with high affinity to certain nicotinic receptors, thus increasing the concentration and enhancing the stimulatory effect of nicotine on other receptor types which are also involved in regulation of OLF secretion. Further, there are evidences for the existence

of non-classical nicotinic receptor associated with inositolphospholipid breakdown in frog [160] and for presence of noncholinergic nicotinic binding site in the rat brain [161] which add further evidences for the high diversity of this receptor family. Our results suggest, that similar to the regulation of aldosterone and OLF by angiotensin-II in bovine adrenocortical cells [51], in nicotinic regulation of these two steroid hormones different nicotinic acetylcholine receptors can be involved. However, further investigation needed to better characterize these receptors, especially those involved in regulation of endogenous OLF.

As the adrenal gland in part consists of postganglionic neurons, activation of nicotinic acetylcholine receptors in the adrenal medulla causes release of norepinephrine and epinephrine into the bloodstream [162]. Many cardiovascular side effects of nicotine (elevated blood pressure, increased heart rate) are explained as activation of these synaptic ganglionic acetylcholine receptors, mediating peripheral autonomic neurotransmission. In contrary some authors found that upon nicotine administration blood pressure rises immediately and well before any change in the circulating cathecholamines [for review see reference 163). There is some evidence also, that adrenaline response during cigarette smoking is modest [164].

Another mechanism that can be affected by nicotine is the renin-angiotensin-aldosterone system. Nicotine and related constituents of tobacco smoke were shown previously to inhibit aldosterone synthesis [146]. Consequently, activation of the renin-angiotensin system was speculated, which in turn could cause not only vasoconstriction but also vascular hypertrophy and injury [165], leading to vasculopathy and hypertension in smokers. The hypothesis had to be rejected however as in a clinical trial [164] captopril, which was used for blocking angiotensin-II generation, failed to inhibit increases in heart rate and blood pressure during smoking.

The Na^+/K^+-ATPase inhibitor ouabain was shown to increase blood pressure via various mechanisms [166], including activation of the sympathoadrenal system, vasoconstriction, and interaction with aldosterone and atrial natriuretic peptide [167]. Vizi [168] demonstrated that OLF has an important effect on neurotransmission. They showed that OLF at low concentrations potentiated noradrenaline release [169], whereas at high concentrations OLF had a direct vasoconstrictor effect. Ouabain was

also shown to augment nicotine evoked norepinephrine release in bovine adrenal chromaffine cells [170]. It also enhanced acetylcholine and nicotine induced catecholamine secretion in cat adrenal gland [171]. We showed earlier [122,139] that depending on other pharmacological agents present (ANP, angiotensin-II) and on the extracellular potassium concentration ouabain had stimulatory or inhibitory effect on aldosterone secretion. As our studies proved that nicotine is a strong stimulator of OLF secretion we suppose that elevated OLF level can contribute through the above mentioned mechanisms to the nicotine induced cardiovascular changes seen in smokers.

6.4. Role of endogenous ouabain-like factor secretion in the adrenal gland: Interaction between ouabain, atrial natriuretic peptide (ANP), angiotensin-II and potassium: effects on rat zona glomerulosa aldosterone production

6.4.1. Inhibitory action of ANP

ANP is a well known inhibitor of the aldosterone secretion. *In vitro* and *in vivo* studies demonstrated that ANP blocks the stimulation of aldosterone secretion induced by A-II, ACTH and elevation of extracellular K^+ [172,173]. The inhibitory effect of ANP is not well understood. ANP acts through a specific membrane receptor activating the guanylate cyclase or inhibiting the adenylate cyclase system. Some studies [174, 175] suggest that the calcium entry or mobilisation processes can be dissociated from the inhibitory action on aldosterone biosynthesis. However, other results [176], using Ca^{2+} -ionophore A23187, indicate that ANP may selectively and noncompetitively inhibit an intracellular step necessary for Ca^{2+}-dependent stimulation of the early pathway of the biosynthesis and/or inhibit T-type Ca^{2+} channels. Another hypothesis [177] suggests that ANP can directly bind to adrenocortical mitochondria. Thus ANP can exert its inhibitory effects directly at the mitochondrial level by blocking cholesterol transfer. The many types of inhibitory mechanism of ANP may explain why it inhibits the action of all type of agonists.

6.4.2. Stimulatory actions of ouabain, angiotensin-II and potassium

Ouabain is known to exert its action on cell function through Na^+/K^+-ATPase which is ubiquitous in the mammalian cells. The inhibition of the membrane bound enzyme results in the increase of intracellular Na^+ concentration, which in turn affects the cellular Na^+-Ca^{2+}-exchange in such a way to result in the increase of intracellular Ca^{2+} concentration. This rise in the cytosolic Ca^{2+} concentration leads to the activation of calmodulin dependent processes, which initiate the aldosterone secretory response.

Angiotensin-II exerts its steroidogenic effect through the AT_1 receptor. Via the AT_1 receptor phospholipase C becomes stimulated, which induces Ca^{2+}-release [178, 179] from $InsP_3$ sensitive vesicles (calciosomes). A-II has a sustained effect on dihydropiridine-sensitive Ca^{2+} influx and inhibits Na^+/K^+-ATPase [180, 181] depending on the extracellular Ca^{2+} concentrations.

Increase in extracellular potassium induces the opening of voltage dependent Ca^{2+} channels, to cause influx of Ca^{2+} resulting in increased intracellular calcium levels [182].

Because of A-II, potassium and ouabain exert their action through a final common pathway involving the second messenger Ca^{2+}, their effects and interactions are effected by changes in the concentration of intracellular Ca^{2+}. The rise in intracellular Ca^{2+} levels causes the increase of Ca^{2+} in mitochondrial matrix [182], which in turn, leads to the activation of dehydrogenases, and finally, the production of NADPH. NADPH is needed for the hydroxylation of different steroid hormones. At the same time, the elevated levels of intracellular Ca^{2+} activate Ca^{2+}-calmodulin dependent protein kinases which support cholesterol transfer into mitochondria. Thus the cytoplasmic $[Ca^{2+}]$ controls both the redox state and the cholesterol supply - and thus hormone secretion- of the cells [for review see 183].

Increase of intracellular $[Ca^{2+}]$ can lead to increase in aldosterone secretion. However, too high $[Ca^{2+}]_{ic}$ can cause damage to adrenal cells, reflected by the decrease in NADPH signals and decrease in steroidogenesis [184].

6.4.3. Interaction of ouabain and ANP at 3.6 mmol potassium

ANP inhibited both basal and ouabain stimulated aldosterone production at doses 10^{-11}-10^{-7}M. This phenomenon can be simply explained with the assumption that the inhibitory effect of ANP was more potent than the stimulatory effect of ouabain. We observed an interesting, but not easily understandable interaction between 10^{-3} M ouabain and 10^{-11}-10^{-10} M ANP. In the presence of 10^{-11} and 10^{-10} M ANP, 10^{-3} M ouabain increased aldosterone production more than 10^{-4} M ouabain alone. Ouabain concentration of 10^{-4} M was the most stimulatory ouabain concentration in our hands as shown in Figure 18. One explanation can be that, in order to support steroidogenesis, adrenal cells require an optimal range of intracellular Ca^{2+} levels. Because 10^{-3} M ouabain increases intracellular Ca^{2+} to levels higher than this optimal range, the result is either no effect on or inhibition of aldosterone synthesis. 10^{-11} M and 10^{-10} M ANP alone may lead to a decrease in intracellular [Ca^{2+}] and steroidogenesis, but if added together with 10^{-3} M ouabain, an optimal the cytoplasmic Ca^{2+} level is achieved, thus resulting in an increase in aldosterone synthesis. Higher ANP concentrations lower the intracellular Ca^{2+} levels, thereby diminishing aldosterone synthesis.

6.4.4. Interaction of ouabain, ANP and angiotensin-II at different potassium concentration

The interaction between A-II and K^+ was studied earlier [for review see 167]. It was shown that A-II exerts either a stimulatory or an inhibitory effect depending on the levels of potassium in superfused rat adrenal glomerulosa [173], in dispersed bovine glomerulosa cells [185] and *in vivo* in the dog adrenal cortex [186]. The biphasic action of A-II on the T-type calcium channels [185] and the connection between plasmalemmal dihydropyridine receptors (K^+) and $InsP_3$ (A-II) receptors in subplasmalemmal calciosomes are hypothesized [187]. Thus changes in extracellular K^+ concentration modify the cytoplasmic Ca^{2+} response to A-II.

Several theories have been proposed for explaining the biphasic action of ouabain, including the theory of two distinct membrane receptors for ouabain-ATPase system [188]. On the basis of Spät *et al.*'s [183] theory, we suggest that one reason for the inhibitory effect of the high doses of drugs -alone or combined- is that it causes an increase in intracellular Ca^{2+} levels, which results in the decrease of NADPH.

The interaction of A-II, ANP, ouabain and potassium may be of physiological significance in the multifactorial regulation of aldosterone secretion. Thus A-II, ANP and ouabain may all act together either through the systemic circulation or as local paracrine factors.

6.5. Production of adrenocortical steroid hormones by human adenoma cells

The ouabain-like factor production of incidentally detected human adrenocortical tumour cells was studied. The occurrence of these "incidentalomas" which are associated with distinct steroid secretion patterns and sometimes with diabetes mellitus and/or hypertension is increasing proportionally to the use of radiographic imaging. Regarding to clinical data [189] approximately 1-10% of computer tomography (CT) scans and magnetic resonance images (MRI) will detect adrenal incidentalomas that are 5 mm or greater. About 80% of the adenomas are nonfunctioning and benign, however twenty percent of them are either functioning or malignant and require further evaluation and treatment to avoid medical complications. In some cases the overexpressed hormone (cortisol, aldosterone, pheochromocytoma etc) is described. We supposed that ouabain-like factor can be one reason for the above mentioned syndromes; thus we investigated the OLF production in incidentalomas.

Both the effect of ouabain on human adrenocortical cells' aldosterone secretion, and the production of endogenous ouabain-like or digitalis–like factor by human adrenocortical cells were studied mainly in tumor cell lines [136, 190-192]. There is some *in vivo* data suggesting that patient with primarily aldosteronism have significantly higher level of OLF than the healthy controls [129, 107, 193] and that after unilateral adrenalectomy plasma immunoreactive ouabain levels were decreased [129, 193]. However, the authors failed to show any ouabain-like immunoreactivity in the extracts of the adenoma tissue [129]. Another study conducted by Rossi *et al.* showed that 56 % of 17 patients with adrenal cortical adenoma and aldosterone excess had also higher plasma ouabain levels [107]. Yet, there are no reports in the literature on regulation of OLF in human adrenal cells. Healthy adrenals are extremely hard to

obtain, thus we studied two patients with incidentalomas (one aldosteronoma and the adjacent normal tissue and one incidentaloma with normal steroid secretion pattern).

We found that OLC production of the incidentaloma was 20-fold and of the aldosteronoma 40-fold higher than that of the adjacent normal tissue, which correspond to the *in vivo* data published by Masugi *et al.*, Rossi *et al.* and Naruse *et al*. [129, 107, 193]. Our data suggest that human adrenals produce ouabain-like factor and it is modulated by the extracellular potassium concentration. Thus we imply that OLF can contribute directly or indirectly to the symptoms (hypertension, diabetes mellitus) found in patients with incidentalomas. Because of the difference in OLF regulation by extracellular [K^+] in incidentalomas with low or high ouabain-producing adrenocortical cells the contribution of OLF may depend on the production of other adrenocortical hormones, like aldosterone. Further investigations needed to study correlation between symptoms and OLF level in incidentaloma patients.

6.6. Plasma levels of endogenous ouabain-like compound (OLF) and adrenomedullin (ADM) in experimental cardiac hypertrophy in rats

6.6.1. Endogenous OLF

Elevated plasma level of endogenous ouabain-like factor was described in essential hypertension, in patients with moderate to severe hypertension and in patients with congestive heart failure due to dilated cardiomyopathy [194]. Our investigation aimed to characterize the changes in plasma OLF concentration from the early stage of cardiac hypertrophy in rats. Similar investigation was conducted by Balzan *et al.* [195] to study circulating OLF in borderline to mild hypertension and in the early stage of dilated cardiomyopathy. OLF level was not significantly higher in borderline hypertension and in patient with cardiac arrhythmia. However, it was increased (52.3±25.8pM versus normal control 29.4±20.6 pM) in patients with asymptomatic left ventricular dysfunction. The fact that increases in OLF concentration was observed before the development of manifest heart failure suggested that OLF might be an early marker of dilated cardiomyopathy. Our present data show that one week after the creation of the aorto-caval shunt OLF tends to be increased; however there is not any significant left

ventricular hypertrophy present in the animals at this time. Week two may correspond to the condition characterized by Balzan: plasma OLF level increased 1.9-fold and there is a significant hypertrophy present. Our results suggest that besides its causative role in adrenal incidentaloma–induced hypertension, OLF can play a role in the compensatory mechanisms of the body to enhance contractility of the hypertrophied myocardium.

Ouabain was shown previously to induce hypertrophy in cultured cardiac myocytes, which was linked upregulation of several late response genes like skeletal a-actin, atrial natriuretic factor, myosin light chain 2, and transforming growth factor beta, and to certain signaling pathways [196]. Involvement of Src and epidermal growth factor receptor in the signal transducing function of Na^+/K^+-ATPase was reported [197]. In addition, it was shown that nontoxic concentrations of ouabain causes hypertrophy and transcriptional regulations of growth-related marker genes through Ca^{2+}-dependent signal pathways involving Ras and p42/44 mitogen-activated protein kinases [198]. Intracellular reactive oxygen species were also studied as the linkage of Na^+/K^+-ATPase to hypertrophy [199]. As these signaling events are cross-reacting with each other, complex combined action of various stimuli, including Na^+/K^+-ATPase inhibitors is necessary for the hypertrophic growth of the heart [200]. This effect on cardiac contractility is the basis of the continued therapeutic use of cardiac glycosides in the management of congestive heart failure [201]. Our present results confirm the significance of not only the exogenous digitalis, but also the importance of endogenous ouabain-like factors in the failing heart.

As we knew that the adrenal gland produces OLF, we supposed that in adrenalectomized animals (ADX alone and in ADX+Shunt) plasma OLF will be decreased. This was the case one week after the operation. However, at week two plasma OLF level normalized in adrenalectomized animals (ADX) and surprisingly it became the highest (almost 3-fold of control) in shunted and adrenalectomized (ADX+Shunt) rats. It was previously published that in patients after bilateral adrenalectomy plasma immunoreactive OLF levels were similar to those in healthy subjects, suggesting that not the adrenal is the major source of plasma ouabain [193]. As other organs were reported to produce significant amount of endogenous OLF

including the hypothalamus, hypophysis, atria, kidney and liver [7, 134], we suppose that after adrenalectomy other organs provide OLF for the increased needs of the heart.

It is known that there is increased cardiac glycoside sensitivity in compensatory hypertrophy [11]. Data are accumulating on altered expression of Na^+/K^+-ATPase ? -subunit genes in hypertrophied heart, which may be responsible for this phenomenon [202]. It was shown that in rat heart during early phase of hypertrophy expression of ? 2-subunit is decreased and in severe hypertrophy besides this finding there is an increase in ? 3-subunit expression [203]. Our recent data showing decreased plasma OLF levels four weeks after the shunt operation (compared to sham) may also suggest that there may be an increase in sensitivity to endogenous ouabain in the failing rat heart, which can lead to down-regulation of the hormone. The fact that plasma OLF is decreased in adrenalectomized animals without shunting (similar to the results one week after operation) suggest that other compensatory mechanisms induced by volume overload may also lead to down-regulation of endogenous OLF secretion.

6.6.2. Adrenomedullin (AM)

Similar to endogenous OLF, adrenomedullin secretion was shown to be elevated in patients with heart failure and with cardiac hypertrophy [204, 205]. However, it was shown that the vasodilatator adrenomedullin inhibits extracellular matrix formation in cultured cardiomyocytes and thus it may act as an inhibitor of hypertrophy in the heart [206]. Adenovirus-mediated gene delivery of adrenomedullin was shown to protect against cardiovascular and renal injuries in volume-overload hypertensive rats. It caused significant decrease in left ventricular weight and cardiomyocyte diameter and significantly attenuated hypertension [207]. Gene expression and ventricular tissue level of adrenomedullin was shown to be elevated by pressure overload and significantly correlated with the extent of cardiac hypertrophy in rats [208, 209]. However, there are some data showing that ventricular mRNA level does not increase

during the development of cardiac hypertrophy induced by abdominal aortic banding in rats [204]. Ventricular AM concentration showed significant correlation with adrenomedullin mRNA level in aortocaval shunt-induced volume overloading, but failed to correlate with it in pressure overloaded rats [210]. Recent finding which showed significant increase in left ventricular AM mRNA and left ventricular weight at 1, 2 and 3 weeks after aortocaval shunt [210] seems to support our data on correlation between plasma adrenomedullin level and left ventricular weight.

Similar to ouabain, adrenomedullin production was shown in several other organs besides the adrenal gland. Thus, AM was detected in kidney, heart, lung, spleen, brain and it was shown to be secreted by endothelial and vascular smooth muscle cells [211, 212]. Interestingly, lung and cardiac atrium AM concentration was 10 times higher in rats than in human [212]. Again, the fact that adrenalectomy did not decrease plasma adrenomedullin level suggests that, similar to ouabain, other organs' production may compensate for the missing adrenal secretion of this hormone. The rate of compensation for AM however seem to be faster than for ouabain, as we could not detect any decrease in AM plasma level one week after the operation. This is supported by the fact, that there was not any difference in plasma adrenomedullin concentration of shunted animals regardless of their adrenalectomized state at the same time. Secretion pattern of both hormones seems to be similar at week 4, suggesting that a balance developed among the compensatory mechanisms by this time.

6.7. Plasma Levels and Urinary Excretion of Endogenous Ouabain-like Compound in Volume Expanded Conditions in Humans

6.7.1. Plasma levels and urinary excretion of OLF in patient with congestive heart failure

There are several publications in the literature describing elevated levels of endogenous ouabain-like substances in human with congestive heart failure ranging from mild hypertension and early stage of cardiomyopathy to overt congestive heart failure- as discussed before in 6.1. The fact, that patients with uncomplicated essential hypertension have elevated circulating OLF level, also greater left ventricular mass and

stroke volume besides higher diastolic blood pressure [109] suggest that endogenous OLF has a role in maintaining cardiovascular function. Studies analyzing plasma levels in patients correlated elevated ouabain concentration (negatively) also with cardiac index and mean arterial pressure [213]. However, no relation between renal function and OLF level was detected [213]. Our current data confirm that production of endogenous ouabain-like compound is increased in patients with moderate CHF and this is reflected in elevated plasma levels and increased urinary excretion. As plasma clearance and urinary/plasma OLC ratios were similar in patients with CHF and in controls, our results indicate that altered renal handling is not likely to be involved in the elevated plasma concentration. Similar to other's findings our data suggest that OLF is an important homeostatic factor in humans and that enhanced production of endogenous ouabain-like compound may contribute to the compensated state of CHF.

Recent findings indicate that there is a difference in the mechanism of development of heart failure in human compared to rats. Altered expression of Na-pump isoforms (from low affinity to high affinity isoforms) in rats during development of cardiac hypertrophy can explain the increased sensitivity of the failing heart and thus down-regulation of plasma concentration of OLF found in our study (see 6.1.). However, it is also possible that decrease in plasma OLF concentration is the primary event and to compensate the failing rat heart responds with expression of high affinity pump isoforms.

It was shown recently that there is not any significant difference in the ouabain binding affinity of the different human Na-pump isoforms [214]. Also, in hypertrophied human heart Na,K-pump concentration, measured as ^{3}H-ouabain binding site concentration [215], is reduced in left ventricle suggesting that the failing human heart sensitivity is increased to cardiac glycosides. As plasma OLF level is elevated in most CHF patients it is possible that unlike in rat, the decreased pump concentration together with the elevated endogenous ouabain is necessary to maintain cardiac function in the failing human heart. As there is not any data demonstrating lower plasma OLF level in the severe form of cardiac failure, we suppose that there are different mechanisms responsible for regulation of plasma OLF level in rats and in humans.

6.7.2. Plasma and urinary levels of OLF in diabetic patients

There are some controversial data in the literature about the possible causative role of OLF in the development of hypertension associated with diabetes mellitus. Because hypertension in diabetes was shown to be associated with increased exchangeable body sodium [216] and elevated level of plasma ANP [217], increased OLF level was postulated in hypertensive NIDDM patients. The hypothesis was supported by the observation that platelet sodium pump activity is reduced in diabetic patients [216]. Previous findings showed correlation between mean systolic blood pressure and serum OLF in hypertensive NIDDM patients whose antihypertensive treatment was withdrawn for 2 weeks [218]. In another study correlation of elevated plasma ouabain and insulin resistance was shown in NIDDM patients with hypertension; however they failed to confirm relationship between OLF and arterial blood pressure [219]. A similar study which analyzed endogenous digitalis-like immunoactivity levels in subclassified diabetic patients including: NIDDM with and without hypertension, IDDM, essential hypertension with and without hyperinsulinemia (n=8-20/ group) found the highest DLIA concentration in NIDDM patients- regardless of their blood pressure status [220]. They could not measure any difference in the plasma level of DLIA in IDDM or essential hypertensive patients compared to healthy volunteers. In addition, none of the sera studied exhibited Na^+/K^+-ATPase activity.

Our recent results showing elevated plasma OLF levels in DM patients support the hypothesis on the possible hypertensinogenic role -either as casuative agent or as a part of compensatory mechanisms- of OLF in diabetes. The finding, that normotensive and hypertensive diabetic patients showed similar urinary OLF a level to controls resembles the finding of Martinka *et al.* on plasma DLIA [220]. However, the fact that OLF concentrations were similar both in NIDDM and in IDDM patients and were comparable to controls may show that plasma rather than urinary OLF levels reflects the disease status in diabetes mellitus. The plasma OLF concentration difference between males and females may reflect the observation of Vakkuri *et al.* [135]. Namely, healthy men have higher plasma OLF concentration compared to women (12.6±1.3 pmol/l versus 9.4±0.7 pmol/l).

6.7.3. Plasma and urinary level of OLF in diabetic pregnant women

Several studies investigated the plasma digitalis-like immunoreactivities in pregnant women [135, 221-224]. Correlation between amnionic fluid "OLF" (measured by digoxin RIA) and diastolic blood pressure was reported previously [224]. Ouabain-like sodium pump inhibitory activity was also measured and proved to be lower in normotensive pregnants compared to women in pre-eclampsia [225, 226]. There was a significant correlation between diastolic blood pressure and the factor plasma concentration. However, data on endogenous OLF in pregnant individuals are sparse. Using radioreceptor assay, plasma ouabain concentrations in non-pregnant women were found to be 204 pmol/l versus up to 2000 pmol/l in pregnant individuals [224]. Recent work of Vakkuri *et al.* revealed that plasma ouabain concentration is increasing during pregnancy, and decreases close to control (non-pregnant) plasma level 3-5 days after delivery [135]. Further, there was a significant rank correlation between systolic but not diastolic blood pressure and plasma OLF.

However, there are no previous works reporting plasma immunoreactive OLF in diabetic pregnant individuals. As we used the same OLF immunoassay as Vakkuri *et al.*, our results are comparable. Our data, showing higher level of OLF in third compared to second and first trimesters of pregnancy, were consistent with their previous finding. Further, we demonstrated that not only plasma but also urinary OLF is increasing during pregnancy, and that diabetes seems to augment this process. A clinical study conducted previously showed that in type I diabetes poor glycaemic control is associated with pre-eclampsia but not with pregnancy induced hypertension [227].As we could not find any correlation between glycosylated hemoglobine (HbA_{1C}) and plasma/ urinary OLF levels in our study either, our data correlate very well with those result. We suggest that elevated OLF level in diabetic pregnancy may rather be a sign of later pre-eclampsia than pregnancy-induced hypertension. Further, the same study found that in diabetic pregnants, who had had IDDM for less than 15 years, the prevalence of pre-eclampsia was the same as in the healthy control group. Thus, our result showing that plasma OLF is more elevated in gestational diabetes than in pregnants with IDDM may explain why gestational diabetes predispose more often to pre-eclampsia [228].

6.7.4. OLF level in premature and mature newborns

Previously, Yun *et al.* studied contribution of endogenous steroids to digitalis–like immunoreactivity in cord blood [229]. Balzan *et al.*. showed that the compound identified from healthy newborn plasma using anti-ouabain antiserum had an HPLC retention time similar to authentic ouabain [230]. Their result also indicated that newborn plasma contains higher OLF than adult plasma, however they presented data only on a limited number of subjects (n=3).

There are no previous reports studying correlation between gestational age of newborns and their plasma immunoreactive OLF levels. Our recent findings showed that endogenous ouabain concentration is increasing with maturity of the infants and it reaches up to 20 times higher concentration compared to healthy adults at 37 weeks. As high ouabain concentration was found in the amnionic fluid also, association of OLF with the fetoplacental unit was suggested [135, 224]. Maternal plasma OLF level showed a continous increase during pregnancy [135], and it only decreased 3-5 days after delivery. Interestingly, we detected a rapid drop in OLF plasma level in infants born after 37 weeks compared to the OLF level in preterm newborns. If we suppose that most of the OLF is produced by the placenta during pregnancy, of which there is no data currently available, we would expect the same tendency in changes of OLF concentration in both pregnants and newborns. Pregnancy represents a physiological volume overloaded state. As the aging of placenta may lead to decreased production of hormones, we suppose that this may trigger compensatoric OLF secretion of other organs in pregnants. As infants do not have extended plasma volume their bodies may not need to produce compensatoric OLF. As drop of the plasma OLF is very close to the birth, it would be interesting to study whether endogenous ouabain can even have a role in initiating parturition.

7. SUMMARY AND CONCLUSION

In 1998 a satellite meeting was dedicated to discuss findings and problems of the last 40 years of research related to endogenous digitalis-like substances. Data on endogenous digitalis-like substance, endogenous OLF, marinobufagenin and possible new compounds as natriuretic candidates were reviewed. The results were published in Clinical and Experimental Hypertension 20(5&6), in 1998. One of the biggest areas of debate was how to compare data measured by different laboratories: i.e. how to adequately measure endogenous cardenolide(s) level. Previously, investigators used several methods for endogenous DLF measurement, which included studying blood pressure and diuresis in whole animals [231], neurotransmitter release from tissue [168], phosphoinosite hydrolysis [232], erythrocyte ^{86}Rb uptake and ^{3}H-ouabain binding [233]. Highly sensitive radioimmunoassays were also applied with specific antibodies [36, 134]. And however in some cases researchers used biophysical assays like nuclear magnetic resonance spectroscopy and analyzed HPLC retention time also to further support their data [7, 95, 122, 123, for review see reference 234], in most of the cases validity of the results depended on the specificity of the antibody used. Measuring compound levels by antibodies with different specificity and cross-reactivity makes data by various laboratories hard to compare, especially if the group did not use any other methods along with their immunoassay to support their results. The question became especially critical after it was shown that there are several compounds with different biological and biophysical properties. As the earlier observations measuring endogenous DLF were made by using mainly digoxin/ digitoxin assays, those results do not reflect the endogenous OLF level. Thus we have only a few valid observations [7, 95, 122, 123] characterizing the nature and the role of ouabain-like factor(s).

Another important issue addressed was the extraction and purification procedures used. These steps ranged from tissue homogenization in organic solvents or water, acetone or methanol extraction, gel filtration and affinity chromatography [for review see reference 234]. Again, suggestions were made on the standardization of the procedures, which would further help to distinguish among endogenous digoxin- and ouabain-like factors, and bufadienolides.

To address these questions we have developed a very sensitive ouabain immunoassay. This assay together with reverse phase high performance liquid chromatography was used in our work to measure endogenous ouabain levels from adrenal tissue, human and rat plasma and cell culture supernatant. Using a modified extraction protocol established by others [7, 135], namely C_{18} chromatography and acetonitrile elution, we were able to show presence of endogenous OLF. Further, using carefully characterized antibodies we got similar results in radioimmunoassay as the leading laboratories regarding the modulation of OLF by angiotensin-II, ACTH in *in vitro* experiments and the plasma concentration of endogenous ouabain both in physiological and pathophysiological states [7, 30, 48, 135]. These data confirm that with carefully designed and performed measurements results of various research groups could be compared.

The significant results of our work include raising a highly specific antibody that we were able to use for immunohistochemistry. With the help of this antibody we provided data about the localization of ouabain-positive cells, not published previously in the literature. Also, using ^{125}I-ouabain we have developed a radioimmunoassay sensitive enough to measure physiological plasma ouabain concentrations. Our *in vitro* experiments using dispersed rat adrenocortical cells provided evidence that not only the glomerulosa but also the fasciculata/reticularis cells produce OLF. Further, we showed that besides ACTH extracellular potassium concentration modulates OLF secretion both in the human and rat adrenals. We also proved that the regulatory role of angiotensin-II is not as prominent in rat as it was shown to be in bovine cells previously. As “the major regulator” of endogenous ouabain we showed the striking stimulatory effect of nicotine and the nicotinic receptor agonist eserine on rat adrenocortical cells.

Studying the role of endogenous ouabain-like factor in the adrenals we found that ouabain interacts with ANP angiotensin-II at different extracellular potassium concentrations on the adrenocortical aldosterone secretion. These novel findings could help to explain the interaction of these hormones in both physiological and pathophysiological states. Further, they provide mechanistic details on the vasoactive effects of exogenous nicotine and suggest involvement of other endogenous substances

in the development of vasculopathy besides angiotensin-II, in smokers. **Figure 30** incorporates our new findings into the known regulatory network of vasoactive substrates in the adrenals.

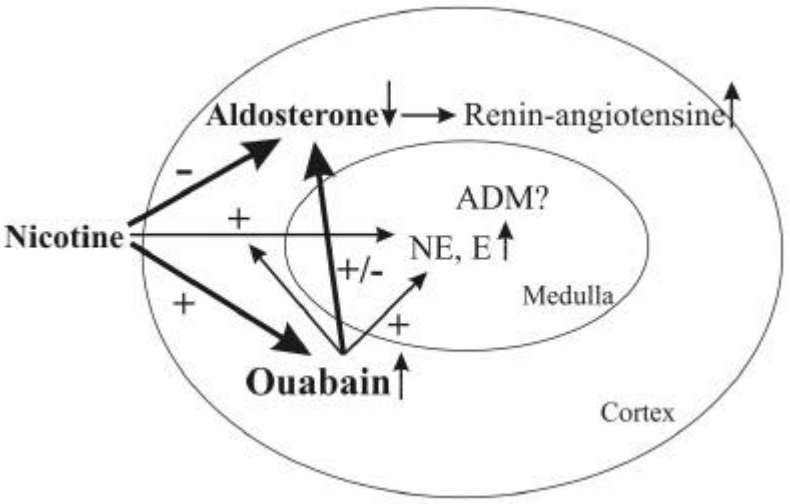

Figure 30. Regulatory network of vasoactive substances in the adrenal gland (NE: norepinephrine, E: epinephrine, ADM: adrenomedullin).

Our *in vivo* experiments aimed to clarify the role of endogenous OLF besides adrenomedullin during the development of cardiac hypertrophy. The important conclusion of our experiments is that however in physiological state the major source of these hormones are the adrenals, both hormones are substituted after adrenalectomy from other organs to provide positive inotropic substances to the failing heart, thus proving their compensatory role during the development of cardiac hypertrophy. As we found elevated plasma OLF concentrations also in patients with moderate form of congestive heart failure, our data support the hypothesis that elevated endogenous OLF may contribute to the compensated state of this disease.

Studying OLF levels in diabetes mellitus we presented significant evidence 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, we provided explanation why gestational diabetes predisposes more often to pre-eclampsia.

Another novel finding of our work is providing evidence for the correlation between gestational age and plasma immunoreactive OLF levels in newborns. As a further interesting observation we showed that mature infants have lower OLF level at birth than premature newborns, which can provide important new information for newborn physiology.

In conclusion, our data showed that using well-controlled assays, comparable results could be generated among research groups. Further, 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 secretion, and provided data for its role in diabetes, during development of cardiac hypertrophy, and in newborns.

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10. PUBLICATIONS

List of publications related to the thesis

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11 ABSTRACT

The aim of the present work was to study regulation of endogenous ouabain-like factor production. We raised a specific antibody against ouabain-BSA conjugate in rabbit. Using this antibody we showed presence of OLF immunopositive cells in rat adrenals, and developed a highly sensitive ouabain radioimmunoassay. Our *in vitro* experiments using dispersed rat adrenocortical cells provided evidence that not only the glomerulosa but also the fasciculata/reticularis cells produce OLF. Further, we showed that besides ACTH the extracellular [K^+] modulates OLF secretion both in the human and rat adrenals. As "the major regulator" of endogenous ouabain we showed the striking stimulatory effect of nicotine on rat adrenocortical cells. Studying the role of endogenous OLF in the adrenals we found that ouabain interacts with ANP and angiotensin-II at different extracellular [K^+] on the aldosterone secretion, which could help to explain the interactions of these hormones in both physiological and pathophysiological states. 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. 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.

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 secretion, and provided data for its role in diabetes, during development of cardiac hypertrophy, and in newborns.

ÖSSZEFOGLALÁS

Kísérleteink célja az volt, hogy tanulmányozzuk az endogén ouabain-szeru anyag (OLF) elválasztását fiziológiás és patofiziológiás állapotokban. Eloállítottunk egy specifikus ouabain-elleni antitestet nyulakban. Ennek az antitestnek a segítségével kimutattuk immunpozitív sejtek jelenlétét patkány mellékvesekéregben és kifejlesztettünk egy érzékeny ouabain rádióimmunoesszét. In vitro kísérleteinkben patkány mellékvesekéreg sejteken kimutattuk, hogy nemcsak a glomerulóza, hanem a faszcikuláta/ retikulárisz sejtek is termelnek endogén ouabaint. Bizonyítottuk, hogy az ACTH mellett az extracelluláris [K^+] is befolyásolja az OLF elválasztását mind patkány mind emberi mellékvesében. Kimutattuk továbbá, hogy az OLF elválasztásának legfobb serkentoje a nikotin. Az OLF mellékvesesejtekre kifejtett parakrin hatását vizsgálva kimutattuk ouabain, a pitvari nátriuretikus peptid, és az angiotenzin-II aldoszteron elválasztásra gyakorolt kölcsönhatását különbözo extracelluláris [K^+] jelenlétében. Volumenterheléses patkánykísérleteinkben kimutattuk, hogy a szívhipertrófia kialakulása során az endogén OLF és adrenomedullin termelését mellékveseirtott állapotban egyéb szervek biztosítják. Ez a megfigyelés a pozitív inotrop hormonoknak a szívelégtelenség kompenzált állapotának fenntartásában betöltött fontos szerepét támasztja alá. Az endogén ouabain volumenterheléses állapotokban való vizsgálata során az átlagosnál magasabb plazma OLF szintet mutattunk ki enyhe kongesztív szívelégtelenségben szenvedo betegekben, aminek szerepe lehet a betegség kompenzált állapotának fenntartásához. Kimutattuk, hogy diabétesz tovább fokozza a terhesség során élettanilag is kialakuló plazma és vizelet OLF szint emelkedést. Mivel a plazma endogén ouabain szintjében bekövetkezo emelkedés gesztációs diabéteszben kifejezettebb volt, mint IDDM-ben szenvedo terhesekben, adataink magyarázatot nyújthatnak arra a megfigyelésre, miszerint gesztációs diabéteszben gyakrabban alakul ki pre-eklampszia. Újszülöttek plazma OLF szint jének mérése során összefüggést mutattunk ki a gesztációs kor és a plazma endogén ouabain szintje között. Megfigyeltük, hogy érett újszülöttek születési plazma OLF szintje alacsonyabb a koraszülöttekénél, ami fontos új információ lehet az újszülött fiziológia szempontjából.

Eredményeinket összefoglalva megállapíthatjuk, hogy új adatokat szolgáltattunk az endogén ouabain-szeru anyagot termelo sejtek szervezeten belüli elhelyezkedésérol, az OLF elválasztásának mellékvesén belüli szabályozásáról, valamint a ouabain egyéb vazoaktív anyagokkal való interakciójáról. Bebizonyítottuk, hogy az OLF elválasztás legerosebb serkentoje a mellékvesében a nikotin. Kimutattuk pre-eklampsziára hajlamosító esetleges szerepét terhességi diabéteszben,

szívhipertrófia kialakulása során, továbbá szívhipertrófia kompenzált állapotában. Megfigyeltük plazmaszintjének újszülöttkori változását.