

Ouabain chronic infusion enhances the growth and steroidogenic capacity of rat adrenal zona glomerulosa: The possible involvement of the endothelin system

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Abstract. Ouabain, an inhibitor of the Na⁺/K⁺-ATPase, has been reported to affect the secretory activity of the adrenal cortex, and especially of zona glomerulosa (ZG). However, conflicting results were obtained, depending on the experimental condition used since ouabain appears to interact with angiotensin-II (Ang-II) and its action to be influenced by the electrolyte balance. Hence, we investigated the effects of prolonged (4-month) infusion with ouabain on the rat adrenal cortex. Ouabain raised the plasma concentrations of aldosterone, corticosterone and endothelin-1 (ET-1), without affecting either systolic blood pressure (SBP) or plasma renin activity (PRA). The treatment caused a marked hypertrophy of ZG and ZG cells, which mainly ensued from increases in the volume of the mitochondrial and smooth-endoplasmic-reticulum compartments, where the enzymes of steroid synthesis are located. Conversely, the volume of the lipid-droplet compartment, which stores cholesterol utilized in steroid-hormone production, underwent a striking decrease. Zona fasciculata and its parenchymal cells were not affected. Basal and maximally agonist (ACTH, Ang-II and ET-1)-stimulated *in vitro* mineralocorticoid secretion from adrenal slices was also notably enhanced by ouabain administration. Collectively, these findings indicate that prolonged treatment with ouabain selectively stimulates the growth and steroidogenic capacity of the rat adrenal ZG. The possibility that the activation of the renin-angiotensin system may be involved in this effect of ouabain is ruled out by the lack of significant changes in SBP and PRA. Instead, our results suggest the possible involvement of ET-1, the plasma level of which is elevated in ouabain-infused rats.

Introduction

Many lines of evidence suggest that the endogenous Na⁺/K⁺-ATPase inhibitor ouabain-like factor (OLF) is a novel adrenocortical hormone, that regulates the sodium pump and is possibly involved in the pathogenesis of hypertension (1). Consistent findings indicate that authentic OLF is produced by adrenal glands, and especially from the zona glomerulosa (ZG) (2-8), its secretion being modulated by angiotensin-II (Ang-II) and Na⁺ balance (9-11).

In vitro studies on the acute effect of ouabain on aldosterone secretion from ZG cells gave rather controversial results. To summarize: i) in rats, ouabain (10⁻⁴ M) was found to increase or not to affect basal, but to inhibit Ang-II-stimulated aldosterone secretion from ZG cells (12,13); ii) in humans, ouabain (10⁻⁸ M) has been reported to lower both basal and Ang-II- or ACTH-stimulated aldosterone production (14); iii) in contrast, OLF immunoneutralization was found to decrease aldosterone plasma concentration in rats kept on a low Na⁺ diet (15); and iv) in bovines, ouabain (10⁻⁸ M) has been shown to increase basal and Ang-II-stimulated aldosterone secretion from ZG cells (16).

Therefore, it seemed worthwhile to investigate the effects of long-term ouabain administration on the secretion and growth of adrenal cortex in the rat. Moreover, since stimulation of ouabain-sensitive Na⁺/K⁺-ATPase has been described as one of the possible mechanisms underlying the aldosterone secretagogue action of endothelin-1 (ET-1) (17,18), we have also studied the effect of ouabain treatment on the plasma levels of ET-1 and on the *in vitro* secretory response of adrenals to ET-1.

Materials and methods

Animals and reagents. Male Wistar-Kyoto rats (226±10 g body weight at the beginning of treatment) were obtained from Harlan-Nossan (Milan, Italy). Animals were housed in temperature- and humidity-controlled conditions, exposed to a 12-h light-dark cycle, and given rat standard chow (M. Mucedola, Settimo Milanese, Italy) and tap water *ad libitum*. The experiment protocol was approved by the local Ethics Committee for Animal Studies. ACTH, Ang-II and ET-1

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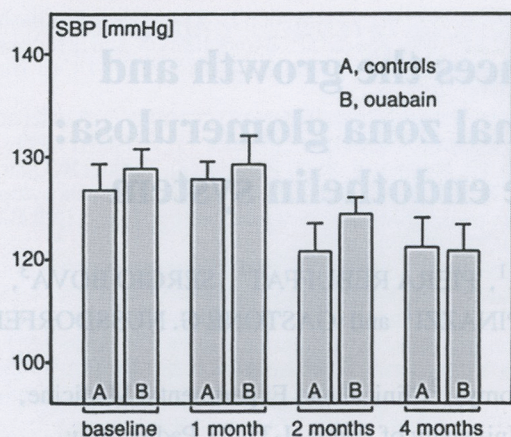


Figure 1. Lack of effect of chronic ouabain infusion on the SBP in rats. Bars are means $\pm$ SEM (n=10).

were purchased from Phoenix Pharmaceuticals (Belmont, CA). Medium 199 was provided by Difco (Detroit, MI), and glutaraldehyde by Serva (Heidelberg, Germany). Ouabain, human serum albumin (HSA) and all other chemicals and laboratory reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MO).

Experimental design. Rats were randomly assigned to receive either 100 μ g/kg per day of ouabain dissolved in 0.9% NaCl (n=10) or saline vehicle (n=10) continuously infused through a mini-osmotic pump (model 2004; Alzet, Palo Alto, CA) for 4 months (19). The pumps were implanted in a subcutaneous pocket created by making a small incision in the interscapular skin under anesthesia with ketamine (80 mg/kg) and xylazine (20 mg/kg). The incision was closed by sutures. Each rat underwent this surgical procedure four times because pumps deliver solution for only one month. Systolic blood pressure (SBP) was recorded by tail cuff sphygmomanometry (BP-Recorder; Ugo Basile Ltd., Comerio, Italy) in unrestrained rats at time 0 and after 1, 2 and 4 months of infusion. At the end of the treatment, animals were decapitated, and their trunk blood was collected in the presence of EDTA (1 mg/ml); plasma was separated and stored at -80°C . Adrenal glands were promptly removed and freed of adherent fat.

In vitro incubations. Slices of the right adrenal cortex of 5 rats in each group were put in Medium 199 and potassium-free Krebs-Ringer bicarbonate buffer with 0.2% glucose, containing 5 mg/ml HSA (20). Specimens were incubated with 10^{-8} M ACTH, Ang-II or ET-1, or without any peptide. The incubation was carried out in a shaking bath at 37°C for 90 min, in an atmosphere of 95% air-5% CO_2 . The incubation tubes were centrifuged at 4°C , and supernatants were stored at -80°C .

Morphology. The left adrenals of 5 rats in each group were fixed in Bouin's solution, embedded in paraffin and serially cut at a thickness of 6-7 μm . Sliced pieces of the right adrenals were fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon (21). Thick (0.5 μm) and thin (60-70 nm) sections were cut with an LKB Supernova Ultratome (Reichert-Jung, Wien, Austria) at the level of the ZG and zona fasciculata (ZF). Thin sections were counter-

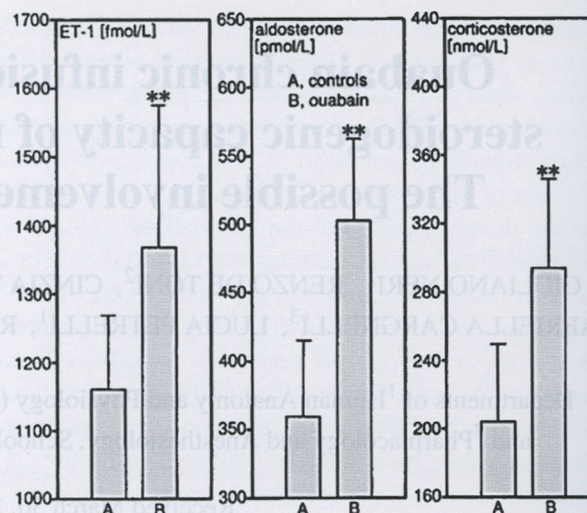


Figure 2. Effects of chronic ouabain infusion on the plasma concentrations of ET-1, aldosterone and corticosterone in rats. Bars are means $\pm$ SEM (n=10). **p<0.01 from the respective control value.

stained with lead hydroxide, and examined and photographed in a Hitachi H-300 electron microscope. The volume of ZG and ZF, and the number and volume of their parenchymal cells, as well as the volume of nuclei, were determined on light micrographs of paraffin and 0.5 μm -thick epon sections, as previously described (22). On electron micrographs of ZG cells, the volume of the mitochondrial and lipid-droplet compartments, and the surface area of mitochondrial cristae and smooth endoplasmic reticulum (SER) were evaluated by conventional stereological techniques (22).

Biochemical assays. Plasma renin activity (PRA) was assayed by RIA of angiotensin-I generated after incubation of plasma (Ang-I RIA kit; Phoenix Pharmaceuticals). The plasma concentration of ET-1 was measured by RIA, using an ET-1 RIA kit following the manufacturer's protocol (Phoenix Pharmaceuticals). The plasma concentrations of aldosterone and corticosterone were assayed by RIA, as previously described (23). The concentrations of aldosterone, 18OH-corticosterone and corticosterone in the incubation media were determined by quantitative HPLC, as detailed previously (24,25).

Statistics. The data obtained were averaged per experimental group, and SEM was calculated. The statistical comparison was performed by ANOVA, followed by Duncan's multiple range test.

Results

Prolonged infusion with ouabain did not change either SBP (Fig. 1) or PRA (control versus ouabain-treated rats: 5.0 ± 1.3 versus 4.7 ± 1.1 fmol/ml-h), but induced a significant increase ($\sim 18\%$) in the plasma level of ET-1, as well as in the plasma concentrations of aldosterone ($\sim 40\%$) and corticosterone ($\sim 43\%$) (Fig. 2).

Ouabain chronic treatment caused significant increases in the volume of the ZG (37%) and ZG cells (36%), without affecting ZG-cell number and nuclear volume. The ZF and

Table I. Effect of chronic ouabain infusion on the morphometric parameters of rat adrenal gland.

	Controls	Ouabain
ZG		
Volume of zona (mm ³)	2.792±0.306	3.812±0.495 ^a
Number of cells (x10 ³)	3148.1±439.4	3292.4±451.3
Volume of cells (μm ³)	709.5±82.6	962.4±97.7 ^a
Volume of nuclei (μm ³)	113.6±8.3	147.8±9.3 ^b
ZF		
Volume of zona (mm ³)	14.909±1.403	15.107±1.704
Number of cells (x10 ³)	8352.6±809.2	8159.9±851.5
Volume of cells (μm ³)	1695.7±231.9	1758.8±268.2
Volume of nuclei (μm ³)	163.2±13.8	159.7±13.0

Data are means ± SEM (n=5). ^ap<0.05 and ^bp<0.01 from the respective control value.

Table II. Effect of chronic ouabain infusion on the stereological parameters of rat ZG cells.

	Controls	Ouabain
Volume of mitochondrial compartment (μm ³ /cell)	120.6±14.5	164.7±17.9 ^a
Surface area of mitochondrial cristae (μm ² /cell)	2219.0±259.6	3063.3±347.7 ^a
Surface area of SER (μm ² /cell)	5525.8±624.2	8078.1±718.5 ^b
Volume of lipid-droplet compartment (μm ³ /cell)	40.2±4.7	18.8±2.02 ^b

Data are means ± SEM (n=5). ^ap<0.05 and ^bp<0.01 from the respective control value.

its cells were not affected (Table I). The ZG-cell hypertrophy was associated with significant increases in the volume of mitochondrial compartment (37%), and in the surface area per cell of mitochondrial cristae (38%) and SER membranes (46%). Conversely, the volume of the lipid-droplet compartment underwent a marked reduction (-53%) (Table II).

Basal secretion of aldosterone, 18OH-corticosterone and corticosterone from adrenal slices of ouabain-treated rats was significantly higher than that from adrenal slices of control animals (aldosterone, 18OH-corticosterone, from 3- to 4-fold; corticosterone, from 40 to 50%) (Fig. 3). ACTH, Ang-II and ET-1 induced similar rises in aldosterone and 18OH-corticosterone production from adrenal slices of control and ouabain-infused rats. Corticosterone secretion was enhanced by ACTH, but not by either Ang-II or ET-1 (Fig. 3).

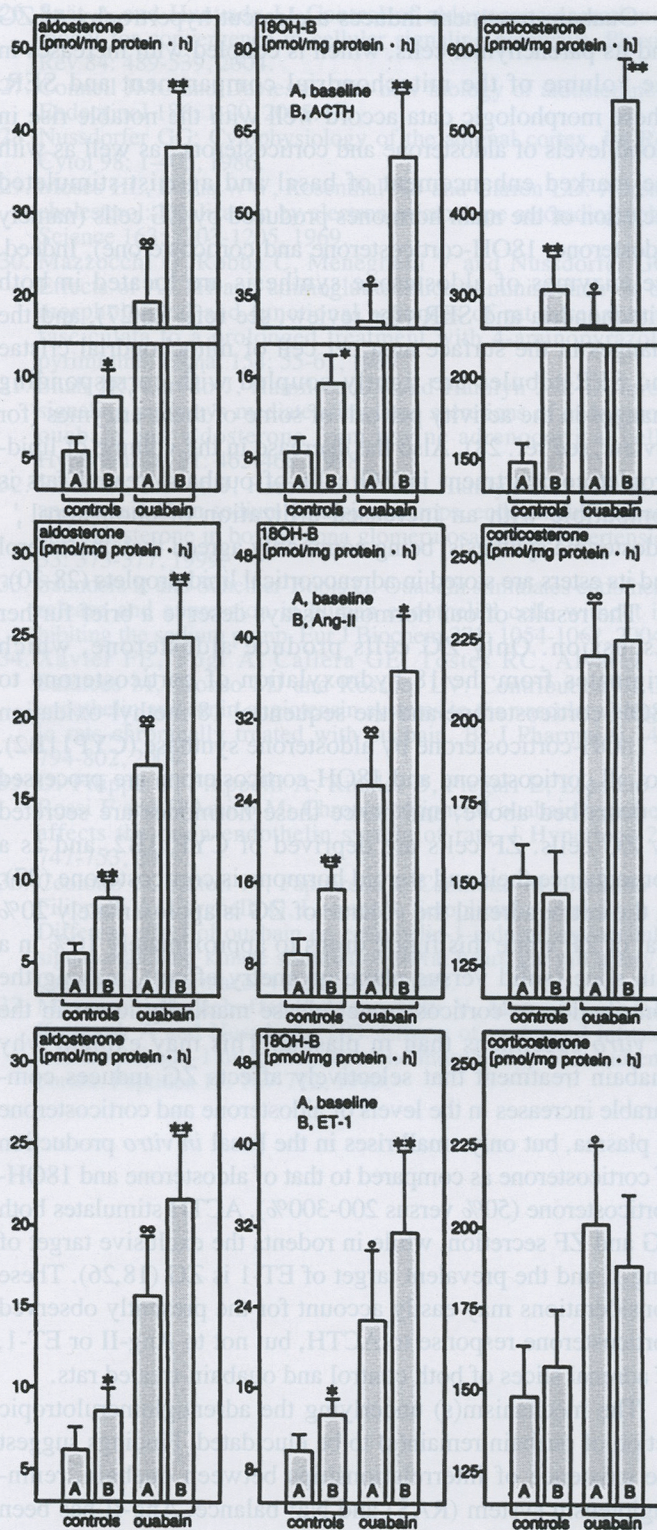


Figure 3. Effects of chronic ouabain infusion on basal and agonist (ACTH, upper panel; Ang-II, middle panel; and ET-1, lower panel)-stimulated aldosterone, 18OH-corticosterone (18OH-B) and corticosterone secretion from rat adrenal slices. Bars are means ± SEM (n=5). *p<0.05 and **p<0.01 from the respective baseline value; °p<0.05 and °°p<0.01 from the respective control value.

Discussion

Our present study provides the first evidence that prolonged ouabain treatment markedly enhances the growth and secretory capacity of rat adrenal ZG, without affecting ZF. This contention is based on both morphological and functional findings.

Ouabain treatment induces a clearcut hypertrophy of ZG and its parenchymal cells, which is coupled with increases in the volume of the mitochondrial compartment and SER. These morphologic data accord well with the notable rise in blood levels of aldosterone and corticosterone, as well as with the marked enhancement of basal and agonist-stimulated secretion of the main hormones produced by ZG cells (namely aldosterone, 18OH-corticosterone and corticosterone). Indeed, the enzymes of aldosterone synthesis are located in both mitochondria and SER (for review, see refs. 26,27), and the changes in the surface area per cell of mitochondrial cristae and SER tubules are tightly coupled with corresponding changes in the activity per cell of some of these enzymes (for review, see ref. 28). Also the decrease in the volume of lipid-droplet compartment in ZG cells of ouabain-treated rats is compatible with an increased utilization of cholesterol in aldosterone synthesis, being commonly agreed that cholesterol and its esters are stored in adrenocortical lipid droplets (28-30).

The results of our hormone assays deserve a brief further discussion. Only ZG cells produce aldosterone, which originates from the 18-hydroxylation of corticosterone to 18OH-corticosterone and the sequential 18-methyl-oxidation of 18OH-corticosterone by aldosterone synthase (CYP11B2). Not all corticosterone and 18OH-corticosterone are processed as described above, and hence these hormones are secreted by ZG cells. ZF cells are deprived of CYP11B2, and as a consequence their end steroid hormone is corticosterone (27). In the entire adrenal the volume of ZG is approximately 20% that of ZF while this figure drops to approximately 10% in a thin slice (solid versus plane geometry effect), making the contribution to corticosterone release markedly lower in the *in vitro* specimens than in plasma. This may explain why ouabain treatment that selectively affects ZG induces comparable increases in the levels of aldosterone and corticosterone in plasma, but only small rises in the basal *in vitro* production of corticosterone as compared to that of aldosterone and 18OH-corticosterone (50% versus 200-300%). ACTH stimulates both ZG and ZF secretion, while in rodents the exclusive target of Ang-II and the prevalent target of ET-1 is ZG (18,26). These considerations may easily account for the presently observed corticosterone response to ACTH, but not to Ang-II or ET-1, of adrenal slices of both control and ouabain-treated rats.

The mechanism(s) underlying the adrenoglomerulotropic action of ouabain remain(s) to be elucidated. Findings suggest the existence of interrelationships between ouabain, renin-angiotensin system (RAS) and Na⁺ balance. Ang-II has been shown to rise ouabain secretion from (10,31) and binding to bovine adrenocortical cells (32). Accordingly, Na⁺ depletion and the ensuing RAS activation has been reported to increase ouabain plasma concentration in humans (11). High Na⁺ intake, and conceivably the suppression of RAS, was found to enhance ouabain concentration in the rat adrenals (9). This last observation apparently conflicts with the above findings: however, it could be interpreted as indicating that Ang-II favors ouabain release from adrenals, but is not essential for a normal basal ouabain synthesis. Losartan, an antagonist of AT1 Ang-II receptors, was shown to prevent the secretory effect of ouabain on bovine ZG cells, leading to the conclusion that Ang-II is needed for ouabain to exert its aldosterone secretagogue action (16). Our present results argue against

the possibility that RAS activation may be involved in the effects of prolonged ouabain infusion on rat ZG, because significant changes in SBP and PRA were not observed. Instead, they appear to suggest the possible involvement of the ET system, inasmuch as chronic ouabain administration significantly increases ET-1 plasma level in rats.

This contention is in keeping with the following lines of evidence: i) ouabain was found to enhance ET-1 release from and ET-1 mRNA expression in human umbilical artery endothelial cells (HUAEC) (33), and ET-1 expression in rat aortic rings (34) and brain (35); ii) ouabain has been reported to potentiate the ET-1-induced MAPK ERK1/2 phosphorylation in the rat heart (36), and to stimulate MAPK activity and proliferation in HUAEC (33); and finally iii) ET-1 chronic administration has been shown to enhance the growth and steroidogenic capacity of rat ZG (18,37). Further studies are needed to confirm this hypothesis, involving experiments aimed at ascertaining whether i) ouabain chronic administration enhances ET-1 and ET-1 receptor expression in rat adrenals; and ii) the blockade of ET-1 receptors is able to prevent the adrenoglomerulotropic action of ouabain.

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Regucalcin is a 29 kDa protein encoded by a single gene on chromosome X(13,10). Regucalcin messenger ribonucleic acid (mRNA) and its protein are greatly present in liver and kidney cortex, and regucalcin is also found in other tissues (11,12). The expression of regucalcin mRNA is mediated through the Ca^{2+} -signaling mechanism (13,14). AP1, NF1-A1 and RGPR-p117 (novel protein) have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity (15-17).

Regucalcin plays a role in maintaining intracellular Ca^{2+} homeostasis, the inhibitory regulation of various Ca^{2+} -dependent protein kinases and tyrosine kinases, protein phosphatases, nitric oxide synthase, and the control of the enhancement of protein synthesis, nuclear deoxyribonucleic acid (DNA) and RNA synthesis in proliferative cells (6-8, 18-25). Recent study has demonstrated that regucalcin has suppressive effects on cell proliferation and apoptosis, which are mediated through many signaling factors: in cloned rat hepatoma H4-II-E cells and normal rat kidney proximal tubular epithelial NRK52E cells overexpressing regucalcin (26-28). Regucalcin has been proposed to play a role in maintaining cell homeostasis and function in many cell types (8).

We generated regucalcin transgenic (TG) rats to determine a regulatory role of endogenous regucalcin *in vivo* using a TG rat model (29). Bone loss and hyperlipidemia have been induced in regucalcin TG rats (30,31), supporting the view that regucalcin has an important role on pathophysiologic state.