THE ENDOGENOUS OUABAIN: MOLECULAR BASIS OF ITS ROLE IN HYPERTENSION AND CARDIOVASCULAR COMPLICATIONS

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Endogenous ouabain, salt and blood pressure interactions
4. Endogenous ouabain as a risk factor for cardiac and renal complications
5. Endogenous ouabain as a target for a novel therapeutical approach
6. Conclusions and perspective
7. References

1. ABSTRACT

Elevated levels of the endogenous ouabain (EO), a closely related isomer of ouabain, are implicated in rat and human hypertension and in related cardiovascular complications. The pathogenetic mechanisms through which EO affects the cardiovascular system involve the modulation of the renal Na/K-ATPase, implicated in renal tubular sodium reabsorption, and the activation of signal transduction pathways, promoting the transcription of growth-related genes. Experimental and clinical evidence on rats and humans stimulated the pharmacological research for developing novel anti-hypertensive agents able to antagonize the cellular and molecular alterations mediated by EO. Among them, the digitoxigenin derivate, PST 2238, has been selected for its ability to antagonize the ouabain-induced effects on blood pressure and organ hypertrophy at oral doses of \( \mu \text{g/kg/day} \) in vivo. The pharmacological selectivity and safety of PST 2238 suggests that the compound may be effective for the treatment of those forms of hypertension in which renal sodium handling alterations and cardiovascular complications are associated with increased production of EO.

2. INTRODUCTION

Cardiac glycosides exert their pharmacological action by binding to the extracellularly exposed recognition sites on Na/K-ATPase, an integral membrane protein that establishes the electrochemical gradient of Na\(^+\) and K\(^+\) across the plasma membrane (1). The phylogenetic conservation of isoform-specific regions involved in digitalis binding within Na/K-ATPase (2) in distantly related species has led to the proposal that an endogenous counterpart to the plant cardiac glycoside might exist in mammals and that Na/K-ATPase represents its functional receptor. Accordingly, evidence has been collected over the years providing that mammalian fluids and tissues contain distinguishable biologically active Na/K-ATPase inhibitors whose levels might be regulated under physiological and pathological conditions (3-12). Purification and mass spectral analysis have led to the identification of two major endogenous compounds in mammals, structurally related to plant ouabain (endogenous ouabain, EO) (3-6, 10, 11) and bufodienolides (8, 9).

The existence of distinct endogenous inhibitors of Na/K-ATPase is in accordance with the hypothesis that
they may be functionally involved as tissue-specific regulators of the different Na/K-ATPase isoforms (13) and that they may be independently regulated by peculiar stimuli.

3. ENDOGENOUS OUABAIN, SALT AND BLOOD PRESSURE INTERACTIONS

For many years, our group has been involved in the attempt to elucidate the molecular mechanisms leading to hypertension. The strategy has included studies of renal function and cellular, biochemical and molecular characterization (14-19) of a genetic hypertensive rat model (the Milan hypertensive rats, MHS) (15-17, 19) and an experimental model of hypertensive rats (OS), in which hypertension has been induced by a prolonged infusion of low doses of ouabain into normotensive rats (20, 21). Elevated levels of ouabain / EO that circulate in mammals at concentrations in the sub-nanomolar range (6, 20, 22) and mutations of the gene coding for the cytoskeletal protein, adducin (17, 18), have been identified as common genetic-molecular mechanisms underlying the development of hypertension in MHS rats, as well as in a subgroup of hypertensive patients (22-24). The increased ouabain / EO levels and adducin mutations associate with enhanced expression and activity of alpha 1 Na/K-ATPase both in vivo in renal tubuli of OS (21) and MHS rats (19, 25) and in cultured renal cells, either exposed to sub-nanomolar concentrations of ouabain (21) or transfected with the mutated adducin genetic variant (18). Also, hypertensive patients carrying the adducin mutations or increased EO levels, show alteration of renal sodium reabsorption due to activation of a proximal tubular mechanism (22, 24). Recent published data are in accordance with our findings since nanomolar concentrations of ouabain have been demonstrated to induce a stimulatory effect on Na-K pump in human artery endothelial cells (26).

The precise molecular mechanism underlying the increased surface expression of Na/K-ATPase induced by either ouabain or adducin in renal cells is under investigation. Recent findings suggest that this alteration is associated with an increased residual time of Na/K-ATPase on plasma membrane (27), as a consequence of a reduced rate of endocytosis through clathrin-coated vesicles (28). Furthermore, adducin has been shown to directly interact and stimulate Na/K-ATPase activity in a cell free-system (29). Although the mutations in rat and human adducin occur at different sites, the mutated adducin from both species shows a higher affinity for Na/K-ATPase as compared to the corresponding wild-type variant (29). Therefore, the over-expression of renal Na-K pump represents a common biochemical alteration induced by both sub-nanomolar ouabain and adducin in rats and humans. Taken together, these findings support the proposal that ouabain / EO and adducin contribute to the increased renal tubular sodium reabsorption, and consequently to the raise of blood pressure in rats (19, 21) as well as in humans (22-24).

We have recently demonstrated (30) that sub-nanomolar ouabain in vitro activates a Src-dependent tyrosine phosphorylation of rat renal alpha 1 Na/K-ATPase, associated with an increase of Na/K-ATPase activity and appearance of a high-affinity binding site for ouabain. In vivo, after a prolonged infusion of low doses of ouabain into rats (OS), this ouabain-induced Na-K-ATPase activation occurs in the restricted membrane subdomains of caveolae. Under our experimental conditions, ouabain favors the enrichment of Na/K-ATPase in caveolae and triggers the downstream signaling pathway via the Src/EGF/PI3K module (30). In this physiological context, since the Na/K-ATPase pool localized within caveolae retains a catalytic activity, it may contribute to the in vivo hypertensinogenic effect of EO / ouabain, participating to the increased renal tubular sodium reabsorption (21). These findings also explain the reason why ouabain, at sub-nanomolar concentrations, can produce renal effects in an ouabain-resistant species, such as rats, where only the alpha 1 Na/K-ATPase isoform with a low-affinity for ouabain (10^-4 M) has been detected in kidneys.

The view that low ouabain / EO concentrations can favor hypertension by activating the Na/K-ATPase at renal level is apparently in contrast with the original hypothesis of deWardener (31) and Blaustein (32), supporting that EO is a natriuretic hormone secreted to counterbalance conditions of volume expansion and salt loading. Along this traditional view, recent data from Liu et al. (33) have demonstrated that in LLC-PK1 cells, derived from an ouabain high-affinity species, a prolonged exposure to 5x10^-8 M ouabain for 12h causes a decrease in plasmalemmal Na/K-ATPase activity and abundance. This effect is mediated through the stimulation of a clathrin-dependent endocytosis of a Na/K-ATPase/Src/EGF/PI3K complex. The Authors propose this mechanism to explain the reduction of the proximal tubular sodium reabsorption induced by the ‘endogenous Na/K-ATPase inhibitor’ in vivo (33). To reconcile these data with ours, it might be proposed that EO / ouabain has a bimodal action on Na/K-ATPase, depending upon the concentrations: low concentrations stimulate while higher concentrations inhibit the overall Na-K-ATPase activity at the plasma membrane. In fact, in our studies on rat renal cells (21), the stimulatory effect of ouabain was observed after a prolonged exposure to 10^-11 M - 10^-10 M ouabain, concentrations that are 6-7 order of magnitude lower than the ouabain inhibitory IC50 of rat renal alpha 1 Na/K-ATPase (10^-4 M) while, at 10^-7 M, a concentration that is 3 order of magnitude lower than the IC50 on the Na/K-ATPase activity. In Liu’s study (33), ouabain has been used at a concentration of 5x10^-8 M, that is 1/20th of the acute ouabain inhibitory IC50 in LLC-PK1 cells, thus being within the concentration range that inhibits the Na/K-ATPase activity.

The ouabain-induced stimulatory, or inhibitory effect, on Na/K-ATPase activity may be related to the confinement and anchoring of Na/K-ATPase to different membrane compartments. As a consequence, ouabain may regulate the rate of membrane cycling of Na/K-ATPase, either by increasing or decreasing the Na-K pump residual time on plasma membrane. Evidence has been provided that ouabain favors the recruitment of a Src-activated Na/K-ATPase complex either to caveolae
EO in hypertension and cardiovascular disease

signaling sub-domains (30) or to clathrin vesicles (33). Although it is still unclear how low or high ouabain concentrations may switch the molecular control of the Na/K-ATPase trafficking, a differential targeting of Na/K-ATPase might result in opposite effects. While it is well established that clathrin vesicles are involved in the endocytosis process of Na/K-ATPase (33), participating to the detoxification mechanism consequent to high ouabain concentrations (34), only recent findings have shed light on the possible contribution of caveole subdomains to the process of stabilization and activation of Na/K-ATPase on cell membrane (30).

Further observations derived from rat and human studies have contributed to the understanding of the molecular mechanism of EO in vivo in relation to salt balance. Our group has demonstrated that an acute and chronic restriction of salt intake, but not an acute salt loading, is responsible for a significant rise of plasma EO in humans (22) and in MHS rats (Ferrandi M, personal communication). These findings suggest that EO, at circulating sub-nanomolar concentrations, does not behave like a natriuretic hormone in vivo but rather participates to the conservation of body sodium. Therefore, EO may be involved in the re-establishment of the hydro-saline homeostatic equilibrium, through its ability to enhance renal Na/K-ATPase activity (21).

Furthermore, a recent study has approached the complex interplay among EO, salt intake and blood pressure in a general population at large (35). A significant interaction between EO and urinary sodium excretion has been reported in relation to blood pressure. At moderate salt intake, higher levels of EO appear to be associated with higher levels of blood pressure. Conversely, at higher salt intake, an opposite relationship is observed (35). Based on these recent findings it appears that a complex, although not fully elucidated, relationship exists between EO and the homeostatic regulation of blood pressure in response to changes in salt intake.

4. ENDOGENOUS OUABAIN AS A RISK FACTOR FOR CARDIAC AND RENAL COMPLICATIONS

In approximately 30% of patients with uncomplicated essential hypertension, plasma EO levels are increased (36). Patients with high levels of EO show an increase of left ventricle mass index and stroke volume with a decrease of heart rate as compared to subjects with normal EO levels (36). However, in the advanced phases, plasma EO levels are inversely correlated with stroke volume (37) and, in cardiomyopathic patients, are inversely correlated with ejection fraction and associated with negative prognostic values (38). These recent findings, obtained on hypertensive subjects investigated at different stages of the disease, together with others (9-11, 39, 40), indicate that EO, in addition to directly influencing blood pressure, may be involved in the development of cardiovascular complications (hypertrophy, heart failure, myocardial infarction), associated with hypertension. EO may therefore play a novel and direct role in vivo as a pro-hypertrophic hormone and thus may affect cardiovascular function and structure being responsible for a cardiac remodeling that contributes to an increased risk of morbid events.

Recent studies carried out on cultured rat cardiomyocytes and renal tubular cells are in agreement with this proposal since they have provided evidence that ouabain behaves like a growth-promoting hormone (41, 42). These findings indicate that ouabain, by binding to Na/K-ATPase, activates a complex intracellular signaling cascade via the Src-EGFr-ERK pathway that finally promotes the transcription of growth-related genes involved in the hypertrophic response.

In order to provide a direct demonstration that the ouabain / Na/K-ATPase signaling effects in cultured cells are also relevant for the cardiovascular effects of EO in vivo, we have evaluated the effects of a chronic infusion of a low dose of ouabain in OS rats (30), as previously mentioned. Ouabain, at sub-nanomolar concentrations, causes in vivo, besides hypertension, cardiac and renal hypertrophy (30). At molecular level, ouabain hypertrophic effect has been related to the enrichment of alpha 1, beta 1, gamma a Na/K-ATPase subunits in the caveole subdomains, together with the activation of the Src-EGFr-ERK signaling pathway. The effects of ouabain appear to occur through the interaction with the high-affinity Na/K-ATPase binding site detected in caveole (30). These findings reinforce the original hypothesis that ouabain in vivo, even at sub-nanomolar concentrations, may confer a signal transduction function to Na/K-ATPase.

5. ENDOGENOUS OUABAIN AS A TARGET FOR A NOVEL THERAPEUTICAL APPROACH

The role of EO in the pathophysiology of hypertension and related cardiac and renal complications opened a new pharmacological field aimed at developing a novel class of antihypertensive agents able to antagonize the functional and molecular effects produced by EO and adducin both in rat and human hypertension. Along this line, our research group has synthesized and screened hundreds of original molecules. One of these molecules, PST 2238 (21, 25, 43-45), has been selected and developed. At oral doses of μg/kg/day, PST 2238 reduces blood pressure and normalizes renal Na/K-ATPase activity in the experimental OS (21) and genetic MHS rats (25).

The selective ability of PST 2238 to correct the EO and adducin-dependent alterations of Na/K-ATPase has been further proven in cultured renal cells either exposed to sub-nanomolar ouabain concentrations or transfected with the mutated adducin variant (21, 25). In these cells, PST 2238 normalizes the Na/K-ATPase function by re-establishing the normal internalization process of Na/K-ATPase via clathrin-coated vesicles (27). Furthermore, it does not affect the Na/K-ATPase activity and its endocytotic process in normal control cells.

Interestingly, PST 2238 displays an antihypertensive activity in other rat models, such as the
deoxycorticosterone acetate-salt and the reduced renal mass hypertensive rats (43), both characterized by volume expansion, low renin and increased EO levels. The selectivity of the antihypertensive effect of PST 2238 is sustained by the absence of activity on blood pressure and renal Na/K-ATPase in normotensive rats (21, 25) and in SHR rats (44), a model in which EO (46) seems not to be involved in the etiology of the disease.

In addition to its ability to antagonize the ouabain pressor effect, PST 2238, administered to OS rats at oral doses of µg/kg/day, also reverts the ouabain-induced cardiac and renal hypertrophy. The compound prevents the ouabain binding to the high-affinity site of alpha 1 Na/K-ATPase within rat renal caveolae and thus interrupts the ouabain-activated signaling pathway mediated by the Src / EGFr / ERK module (30).

The pharmacological selectivity of PST 2238 is further supported by the absence of interaction, both in vivo and in vitro, with a panel of receptors involved in blood pressure regulation or hormonal homeostasis control and by the lack of undesired cardiac and hormonal effects, typical of digitalis or diuretics, including cardiac pro-inotropic or arrhythmogenic activity and the stimulatory effect on the RAS and lipiddic and glucidic asset (44, 45).

Safety represents a further peculiar characteristic of PST 2238, as demonstrated by a safety ratio in animal models higher than 1:10000 (44) and a good tolerability in phase 1 clinical studies in healthy volunteers.

Phase 2 clinical studies are currently in progress. In a preliminary study on 42 never-treated hypertensive patients, PST 2238, given at 0.5 mg/day for three months, significantly reduces blood pressure. Despite the small sample size, PST 2238 efficacy seems to be influenced by the level of salt intake and by the polymorphism of the genes coding for adducin and the enzymes involved in EO biosynthesis (Manunta P, Tripodi G, unpublished data).

6. CONCLUSIONS AND PERSPECTIVE

A bulk of experimental and clinical evidence supports the notion that EO plays a pathogenic role in hypertension and related organ complications. These effects occur through a complex interaction between genetic – molecular mechanisms regulating renal sodium reabsorption and the environmental variable of salt intake. A new therapeutic approach based on tailored drugs (47) able to treat selectively individual patients carrying these specific pathogenic mechanisms is now developing and represents an innovative pharmacological strategy for the treatment of hypertension.

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EO in hypertension and cardiovascular disease

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EO in hypertension and cardiovascular disease


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