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Endogenous digitalis:

pathophysiologic roles and therapeutic applications

Alexei Y Bagrov* and **Joseph I Shapiro**
AY Bagrov is an Investigator and Head of the Hypertension Unit at the Laboratory of Cardiovascular Science, Intramural Research Program, National Institute on Aging, NIH, Baltimore, MD and JI Shapiro is a Mercy Health Partners Education Professor, Chairman of the Department of Medicine and Professor of Medicine and Pharmacology at the University of Toledo College of Medicine, Toledo, OH, USA.

SUMMARY

Endogenous digitalis-like factors, also called cardiotonic steroids, have been thought for nearly half a century to have important roles in health and disease. The endogenous cardiotonic steroids ouabain and marinobufagenin have been identified in humans, and an effector mechanism has been delineated by which these hormones signal through the sodium/potassium-transporting ATPase. These findings have increased interest in this field substantially. Although cardiotonic steroids were first considered important in the regulation of renal sodium transport and arterial pressure, subsequent work has implicated these hormones in the control of cell growth, apoptosis and fibrosis, among other processes. This Review focuses on the role of endogenous cardiotonic steroids in the pathophysiology of essential hypertension, congestive heart failure, end-stage renal disease and pre-eclampsia. We also discuss potential therapeutic strategies that have emerged as a result of the increased understanding of the regulation and actions of cardiotonic steroids.

Keywords

end-stage renal disease; endogenous cardiotonic steroids; hypertension; Na^+/K^+-ATPase; natriuretic hormone

INTRODUCTION

For many years, high dietary salt intake was suspected to increase the risk of cardiovascular and renal diseases.[1,2] During the past few decades, however, the role of salt intake in the development of hypertension has shifted from a topic of debate to a well-established phenomenon.[3-6] The effect of dietary salt on cardiovascular disease is indicated by data from several large trials, such as the International Study of Salt and Blood Pressure (INTERSALT) [7] and the Dietary Approaches to Stop Hypertension (DASH) study.[8] Against this background, an understanding of the specific mechanisms underlying the deleterious effects of salt becomes critically important.

*Correspondence, Laboratory of Cardiovascular Science, National Institute on Aging, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA, bagrova@mail.nih.gov

REVIEW CRITERIA

The authors reviewed manuscripts from their personal libraries and searched PubMed for articles referring to “cardiotonic steroids”, “digitalis-like factors”, “natriuretic hormones”, “Na^+/K^+-ATPase”, “bufadienolides”, “bufanolides”, “ouabain”, “sodium metabolism”, “sodium pump signaling”, or “hypertension”. No date or language restriction was placed on the search.

Competing interests

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The topic of this Review is the role of endogenous cardiotonic steroids—also known as digitalis-like factors or inhibitors of the sodium/potassium-transporting ATPase (Na^+/K^+-ATPase)[9]—in linking dietary salt with cardiovascular and renal disease. We focus particularly on effects in humans and how therapy might be targeted and outcomes affected. Endogenous cardiotonic steroids have been the focus of research at our laboratories for the past two decades. The importance and the very existence of such factors has been a matter of controversy;[10, 11] however, notable breakthroughs in the field include the identification of specific endogenous cardiotonic steroids in experimental and clinical studies,[12-15] the detection of altered concentrations of endogenous cardiotonic steroids in different disease states, the elucidation of the roles of endogenous cardiotonic steroids in these settings,[16,17] and, in parallel, the discovery of the signaling functions of the Na^+/K^+-ATPase and its involvement in many elements of basic cell biology.[18,19] These issues, particularly the last, have been reviewed in a series of excellent papers.[16-23]

The main purpose of the present Review is to emphasize the roles of endogenous cardiotonic steroids in human health and disease—in particular with regard to the renal and cardiovascular systems—and to highlight potential therapeutic targets that have arisen as a result of research in this area. In short, we hope to establish that endogenous cardiotonic steroids have begun their journey from bench to bedside.

HISTORICAL PERSPECTIVE

In the 1960s, it became obvious that the actions of the renin-angiotensin-aldosterone system, vasopressin, and the sympathetic nervous system could not adequately explain physiologic and pathophysiologic responses to acute or chronic expansion of blood volume.[24,25] This point was elegantly demonstrated in 1961 in a classic paper by de Wardener and colleagues,[26] who demonstrated that natriuresis induced by saline infusion is maintained even if renal perfusion pressure and glomerular filtration rate are prevented from increasing. The factor responsible for this phenomenon (the so-called 'third factor') was a topic of great interest in the 1960s and 1970s.[9] Cort and Lichardus[27] and Buckalew and colleagues[28] demonstrated that volume expansion is associated with increased levels of a circulating substance that inhibits active sodium transport *in vitro*. Extremely important contributions to measuring and understanding the implications of this 'third factor' were made by Schrier and co-workers[29-31] and by Kramer and Gonick.[32] The latter group of investigators demonstrated in rats that volume expansion stimulated the production of a substance that could inhibit activity of the Na^+/K^+-ATPase in the kidney. In fact, Bricker and colleagues[33,34] incorporated the concept of a circulating inhibitor of the Na^+/K^+-ATPase in their model of renal failure progression and the pathogenesis of the uremic syndrome, termed the 'trade-off' schema. In 1980, Gruber *et al*.[35] demonstrated in dogs that plasma volume expansion was associated with elevated levels of circulating digoxin-like immunoreactive material. Shortly thereafter, Hamlyn *et al*.[36] demonstrated that plasma Na^+/K^+-ATPase-inhibitory activity correlated positively with blood pressure in a group of patients with essential hypertension. At the same time, Kojima *et al*.[37] showed that administration of an antibody to digoxin lowered blood pressure in rats with hypertension induced by administration of deoxycorticosterone and salt.

Interest in the concept of inhibitors of the Na^+/K^+-ATPase as the so-called third factor decreased during the 1980s and 1990s, in part because of inconsistencies within the experimental data. Most of the assays originally developed for inhibitors of the Na^+/K^+-ATPase were based on cross-reactivity of the putative endogenous factors with various antibodies to digoxin, but results of these tests varied dramatically.[38-40] Probably the most important factor causing this inconsistency is that digitalis, the prototypical inhibitor of the Na^+/K^+-ATPase, is not natriuretic in normal individuals.[41]

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Enthusiasm for the study of endogenous cardiotonic steroids has steadily increased during the past decade, mainly because several endogenous cardiotonic steroids, including ouabain,[12,42,43] marinobufagenin,[14,15] telocinobufagin[15] and bufalin,[13] have been isolated and characterized in both animals and humans (Figure 1). In addition, an effector function of the Na^+/K^+-ATPase signal cascade has been identified that does not seem to depend on inhibition of the ion transport function of the pump by endogenous cardiotonic steroids, but rather is activated by binding of cardiotonic steroids to the caveolar Na^+/K^+-ATPase in the presence of Src and the epidermal growth factor receptor (EGFR)[19,44-47] (Figure 2). Finally, as a result of the identification of specific endogenous cardiotonic steroids, reliable immunoassays have been developed.[16,48-54]

SUBTYPES OF ENDOGENOUS CARDIOTONIC STEROIDS

Endogenous cardenolides

In 1991, Hamlyn and co-workers[12] isolated from human plasma a cardiotonic steroid that was indistinguishable in every way from the plant-derived cardenolide ouabain, and was, therefore, named endogenous ouabain. Since this report was published, endogenous ouabain has been isolated from bovine adrenal gland,[42] bovine hypothalamus[43] and rat adrenomedullary cells.[55] Mass spectrometry and nuclear magnetic resonance studies have indicated that mammalian endogenous ouabain is identical to plant-derived ouabain.[42,43,55] The adrenal cortex and hypothalamus are considered to be the sites of ouabain production in mammals.[55-57] Adrenocorticotropic hormone, angiotensin II, vasopressin, and phenylephrine stimulate the release of ouabain from the adrenal cortex *in vitro*.[58,59]

Evidence suggests that digoxin is an endogenous cardiotonic steroid[60,61] that might be an endogenous antagonist of endogenous ouabain;[16,62] however, the extensive variability in the detection of digoxin-like immunoreactive material by highly specific commercial digoxin immunoassays in digoxin-naive populations[38-41,63] argues against this idea.

Endogenous bufadienolides

Amphibians produce cardiotonic steroids belonging to the bufadienolide class, which differ from cardenolides in that they possess a doubly unsaturated six-membered lactone ring (Figure 1).[64] Bufadienolide-containing preparations from frog and toad skin have been used for the treatment of congestive heart failure in traditional medicines of the Far East.[65] The highest levels of bufadienolides are detected in the skin of species that migrate from dry to aquatic environments.[66] The skin regulates water and electrolyte homeostasis in amphibians; bufadienolides and the Na^+/K^+-ATPase appear to be integral to this process.[66,67] In support of this concept, brain and skin levels of bufadienolides in toads have been shown to fluctuate in response to changes in environmental salinity.[67]

The above observations triggered a search for mammalian bufadienolides. Initially, bufalin-like immunoreactive material was detected in human bile and plasma.[68-71] Lichtstein *et al.*[13] detected bufalin derivatives in the lenses of several mammalian species by using mass spectroscopy. Other workers[14,15] demonstrated marinobufagenin in mammalian plasma and urine by the use of specific immunoassays, mass spectrometry and, subsequently, nuclear magnetic resonance spectrometry.[15] Sich *et al.*[72] reported that human plasma and bovine adrenal glands contained material that cross-reacted with antibodies against proscillaridin A, a rare example of a plant-derived bufadienolide (Figure 1). Hilton and co-workers[73] identified a bufadienolide compound in human placentae and plasma by the use of mass spectroscopy.

Marinobufagenin emerged as a candidate mammalian endogenous cardiotonic steroid largely because of the findings of studies that characterized the pharmacological properties of amphibian bufadienolides.[74-76] Our laboratory showed that venom from the toad *Bufo*

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marinus contained digoxin-like immuno-reactive material with vasoconstrictive, Na^+/K^+-ATPase-inhibiting, and positive inotropic effects.[74,75] Subsequently, this substance was identified as marinobufagenin, a steroid previously described in toads.[14,15] Other studies found that various antibodies to marinobufagenin cross-reacted with material from human, canine and rat plasma and/or urine.[14,51,63,64,76]

Komiyama *et al*.[15] used tandem mass spectrometry and nuclear magnetic resonance spectrometry to demonstrate that uremic human plasma contained increased levels of another bufadienolide, telocinobufagin. This bufadienolide differs from marinobufagenin because it has a hydroxyl at position 14 of the lactone ring rather than an epoxy group at position 14/15; the authors hypothesized that telocinobufagin is a natural precursor of marinobufagenin.[15]

ROLE OF CARDIOTONIC STEROIDS IN HYPERTENSION

Endogenous ouabain

Effects on the kidney—Endogenous ouabain does not fulfill the criteria for classification as a putative natriuretic hormone (i.e. it does not increase sodium excretion), but it does have a role in the adaptation to both sodium depletion and sodium loading. Although a few studies have shown that salt loading of normotensive rats stimulates release of ouabain,[77,78] other experiments performed in dogs,[79,80] rats[81] and humans[48,49] have not reported this finding. Among 180 patients with untreated hypertension, plasma levels of endogenous ouabain did not change during 2 weeks of salt loading (administration of 170 mmol sodium per day), but increased following 2 weeks of sodium depletion (intake restricted to 70 mmol per day).[48] In another study, salt depletion produced a four-fold rise in mean plasma endogenous ouabain levels in 13 healthy men,[50] and salt loading (171 mmol sodium daily) was associated with a 13-fold elevation in plasma endogenous ouabain levels after 3 days; levels decreased within 2 days, but remained higher than baseline.[50] This pattern of ouabain response after salt loading has also been observed in Dahl salt-sensitive rats[82,83] and in humans with normal blood pressure.[84]

Several lines of evidence support the idea that endogenous ouabain has a prohypertensive role; these include the 'adducin paradigm',[85] the induction of hypertension in ouabain-treated rodents, the elevation of endogenous ouabain levels in hypertensive rats, and the central prohypertensive action of this hormone. In rats, chronic peripheral administration of low doses of ouabain (10-50 μg/kg per day) increased arterial pressure and induced cardiac hypertrophy.[86-91] Interestingly, administration of digoxin (200 μg/kg per day) actually reversed the ouabain-induced hypertension in these rats.[62]

A mechanism for the prohypertensive effect of endogenous ouabain has been suggested by experiments performed in Milan hypertensive rats. These rats carry a mutation in the gene that encodes the cytoskeletal protein adducin and also exhibit increased circulating levels of endogenous ouabain.[92] Both of these characteristics are associated with heightened expression and activity of the Na^+/K^+-ATPase in the renotubular epithelium; in the case of adducin, the mutation leads to an increase in the residence time of the sodium pump in the cellular membrane.[87,93] Unlike the ouabain-resistant α1 Na^+/K^+-ATPase, expressed in the renal epithelium, the α1 isoform found in the caveolae of renal tubular cells exhibits remarkable sensitivity to ouabain.[87] Subnanomolar concentrations of ouabain in the plasma of Milan hypertensive rats lead to activation of this sodium pump and stimulation of the Src-EGFR-extracellular-signal-regulated protein kinase (ERK)-dependent signaling pathway, which results in renal sodium retention and hypertension.[87]

Endogenous ouabain can also raise blood pressure by inhibiting the transport function of the α2 Na^+/K^+-ATPase in vascular smooth muscle, and thus promoting entry of calcium via the

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sodium-calcium exchanger.[88,94] Mice genetically engineered to express the ouabain-resistant α2 Na^+/K^+-ATPase do not manifest an increase in blood pressure following chronic administration of ouabain, unlike control mice with ouabain-sensitive α2 Na^+/K^+-ATPases.[88] Accordingly, vascular smooth muscle from mice with ouabain-resistant α2 sodium pumps is insensitive to the pressor effect of ouabain.[88] Furthermore, mice genetically engineered to have reduced expression of the α2 Na^+/K^+-ATPase (but not of the α1 Na^+/K^+-ATPase) become hypertensive and their arteries exhibit enhanced tone *in vitro*.[94]

Some studies have not found major differences in ouabain affinity between the caveolar and the non-caveolar Na^+/K^+-ATPase.[47] We have observed, however, that in renal proximal tubular epithelial cells both ouabain-induced signal transduction through the Na^+/K^+-ATPase-Src-EGFR cascade and ouabain-induced clathrin-mediated endocytosis of the Na^+/K^+-ATPase were limited to the Na^+/K^+-ATPase residing in caveolae.[95,96] These discrepancies might be explained by different levels of expression of the γ subunit of the Na^+/K^+-ATPase in the preparations employed, as suggested by Nguyen and co-workers.[97]

In accord with the sodium-retaining effect of endogenous ouabain on the kidney, polymorphisms of the adducin gene and raised levels of endogenous ouabain are associated with altered renal sodium reabsorption both in experimental animals and in patients with hypertension.[98] Furthermore, in Milan hypertensive rats, administration of the digitoxin derivative rostafuroxin (PST 2238)[99] antagonized the interacting effect of endogenous ouabain and mutated adducin on the caveolar renal Na^+/K^+-ATPase, lowered blood pressure and inhibited the activity of the Na^+/K^+-ATPase in the renal medulla.[87]

Effects on the brain—Substantial evidence indicates that endogenous brain ouabain contributes to the pathogenesis of salt-sensitive hypertension.[100-103] In rats, centrally administered ouabain elicits pressor and natriuretic responses, which are dependent on the activation of the renin-angiotensin system.[22,104,105] Likewise, central administration of salt and systemic salt loading both lead to increases in brain levels of endogenous ouabain in Dahl salt-sensitive rats; these increases are mediated by the brain renin-angiotensin system.[102, 103,105] Leenen and co-workers[106,107] demonstrated that an increase in the level of sodium chloride in cerebrospinal fluid precedes the development of hypertension and that sodium ions in the brain enter the intracellular space via epithelial sodium channels. Entry is modulated by central mineralocorticoid receptors.[108] Recent evidence indicates, however, that brain-specific sodium channels, rather than epithelial sodium channels, have a key role as sensors of cerebrospinal fluid sodium level.[109] The above observations indicate that endogenous ouabain might act as a central mediator of salt-sensitive hypertension (Figure 3).

Endogenous marinobufagenin

In normotensive rats, plasma levels of marinobufagenin increase in response to acute plasma volume expansion, as well as following chronic administration of a high-salt diet.[81,110,111] Enhanced production of marinobufagenin has also been demonstrated in humans with volume expansion,[51] pre-eclampsia,[52] essential hypertension, primary aldosteronism, and end-stage renal disease.[53,54] At concentrations comparable to *in vivo* plasma levels, marinobufagenin induces vasoconstriction in isolated human pulmonary and umbilical arteries[52,76] as well as substantial inhibition of the ouabain-resistant α1 Na^+/K^+-ATPase in rat aorta and rat renal medulla.[83,112] Additionally, immunoneutralization of marinobufagenin with a specific antibody reduces blood pressure and renal sodium excretion in salt-loaded Dahl salt-sensitive rats.[83,102] These rats are, in fact, the same experimental model of hypertension in which Lewis Dahl predicted the existence of an endogenous vasoconstrictive and natriuretic substance.[113]

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Interaction between endogenous ouabain and marinobufagenin

In Dahl salt-sensitive rats with salt-induced hypertension, an important interaction seems to occur between brain and peripheral cardiotonic steroids. After acute and chronic salt-loading in these animals, a transient increase in circulating endogenous ouabain precedes a sustained increase in circulating marinobufagenin.[82,83] This observation has led us to postulate that endogenous ouabain, acting as a neurohormone, triggers release of marinobufagenin, which in turn causes increases in cardiac contractility, peripheral vasoconstriction and natriuresis by inhibiting the Na^+/K^+-ATPase (Figure 3).[102,103] We have subsequently demonstrated that the greatest transient increases in brain endogenous ouabain level occur in the amygdala, hippocampus and supraoptic nucleus of the hypothalamus.[102] Endogenous ouabain in the brain activates the central renin-angiotensin system, which—possibly via sympathoactivation—in turn activates the renin-angiotensin system in the adrenal cortex.[102,103] Activation of the adrenocortical renin-angiotensin system facilitates production and secretion of marinobufagenin, which results in increased plasma and urinary levels of the bufadienolide. This sequence of events was fully mimicked by intrahippocampal administration of a very low dose (60 pg) of plant-derived ouabain.[102] More recently, we demonstrated that, similar to Dahl salt-sensitive rats, salt-loaded normotensive humans exhibit a transient increase in urinary endogenous ouabain, which precedes a more sustained increase in renal marinobufagenin excretion.[84]

Thus, it seems that two scenarios involving different patterns of response to cardiotonic steroids are involved in the pathogenesis of hypertension. At least one of these scenarios—the adducin paradigm—has been demonstrated to be relevant to human hypertension: levels of endogenous ouabain become elevated in hypertensive individuals who possess the appropriate mutation or mutations of the adducin gene.[98] The clinical relevance of the other scenario, the interplay between brain endogenous ouabain and circulating marinobufagenin, remains to be established.

ROLE OF CARDIOTONIC STEROIDS IN RENAL SALT HANDLING

Although the definition of a natriuretic substance is one that increases urinary sodium excretion, the prototypical cardiotonic steroid—i.e. digitalis or digoxin—is not natriuretic at typical clinical doses.[41] Nevertheless, studies indicate that some other cardiotonic steroids do seem to function as natriuretic substances *in vitro* and *in vivo*.[114] We have observed dose-dependent and time-dependent endocytosis of the plasmalemmal Na^+/K^+-ATPase induced by cardiotonic steroids in LLC-PK1 cells; this cell line has features of proximal tubule cells.[115] In a canine cell line that resembles distal tubular cells, no such depletion was observed. Endocytosis of the plasmalemmal Na^+/K^+-ATPase has been shown to proceed via caveolae and via clathrin-coated pits following activation of phosphoinositide-3 kinase by the Src-EGFR pathway.[95,96] In an *in vivo* rat model, we noted that endocytosis induced by marinobufagenin contributed to the altered sodium reabsorption seen with increases in dietary sodium.[111] Specifically, we observed that increases in dietary sodium led to increased urinary excretion of marinobufagenin and sodium, decreased proximal tubule expression of the Na^+/K^+-ATPase, increased accumulation of the Na^+/K^+-ATPase in both early and late endosomes, and decreased sodium reabsorption. Administration of an antibody to marinobufagenin blocked endocytosis of the Na^+/K^+-ATPase and blunted the increase in urinary sodium excretion.[111]

Other work has demonstrated that cardiotonic steroids can induce decreases in the apical expression of the sodium/hydrogen exchanger 3 (NHE3) in proximal tubule cells.[114,116] Some of the long-term decrease in NHE3 expression caused by cardiotonic steroids is related to decreases in NHE3 transcription, whereas binding of ouabain to the basolateral Na^+/K^+-ATPase also seems to rapidly induce endocytosis of the apical NHE3.[117] Taken together, the above data suggest that salt loading is accompanied by an increase in the circulating level of

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marinobufagenin, which in turn induces a decrease in both basolateral and apical sodium transport in the proximal tubule, resulting in increased urinary sodium excretion.

ROLE OF CARDIOTONIC STEROIDS IN CONGESTIVE HEART FAILURE

Congestive heart failure (CHF) is associated with fluid retention and plasma volume expansion, conditions under which one would expect cardiotonic steroids to be released (Figure 3).[118] As early as 1981, Schreiber, Kölbel *et al.*[119] hypothesized that cardiotonic steroids might be involved in myocardial hypertrophy and the regulation of tissue growth. In 1988, Morise *et al.*[120] demonstrated in rats that development of CHF was associated with increased plasma activity of a Na^+/K^+-ATPase inhibitory factor. In 1990, Liu *et al.*[121] showed in a group of 50 patients that the severity of CHF was positively associated with the degree of digoxin-like immunoreactivity in plasma, as well as with erythrocyte sodium concentrations. The relationship between cardiotonic steroids, cardiac geometry and central hemodynamic parameters has been analyzed in several studies. Gottlieb *et al.*[122] found that although plasma endogenous ouabain level did not increase as cardiac failure progresses, levels were elevated in patients with severely impaired left ventricular performance (left ventricular ejection fraction less than 30%).

Manunta *et al.*[123] demonstrated that the plasma concentration of endogenous ouabain positively correlated with systolic and diastolic blood pressure in a patient group that comprised 110 normotensive individuals and 100 hypertensive individuals; levels also positively correlated with left ventricular mass index and left ventricular end diastolic volume in the hypertensive patients. Pierdomenico *et al.*[124] found that circulating endogenous ouabain levels in 92 hypertensive patients positively correlated with mean blood pressure and total peripheral resistance index, and that left ventricular end diastolic volume index, stroke index and cardiac index exhibited inverse correlations with the plasma ouabain level. In the same study, the plasma endogenous ouabain level was substantially higher in patients with eccentric remodeling than in those with normal left ventricular geometry or concentric hypertrophy.[124] In another study, the plasma ouabain level was higher in patients with left ventricular dysfunction than in normal individuals, but did not correlate with left ventricular ejection fraction.[125]

Pitzalis *et al.*[126] found that levels of circulating endogenous ouabain predicted the progression of heart failure in 140 patients with idiopathic dilated cardiomyopathy. Levels of endogenous ouabain have also been found to predict ventricular hypertrophy in patients with end-stage renal disease.[127]

In 23 consecutive hypertensive male patients with CHF, plasma marinobufagenin levels exhibited a progressive increase that paralleled the progression of CHF and the increase in plasma α-atrial natriuretic peptide.[54] Although plasma endogenous ouabain levels did not vary with the severity of CHF, it was noted that ouabain levels were substantially elevated in a subset of patients who had an ejection fraction less than 30% (AY Bagrov, unpublished data), a finding similar to that observed by Gottlieb and coworkers.[122]

Experimental data also indicate an association between elevated plasma levels of cardiotonic steroids and cardiovascular remodeling. In one study, normotensive Fisher 344 × Brown Norway rats subjected to a 4% salt diet exhibited increases in plasma marinobufagenin and proportional increases in cardiac weight, in the absence of hypertension.[81] Sustained ouabain infusion, sufficient to cause a two-fold elevation in plasma ouabain immunoreactivity, also induced left ventricular hypertrophy in a study of normotensive rats.[87]

A study performed in Dahl salt-sensitive rats subjected to a high-salt diet revealed coordinated shifts in the physiological function of the left ventricle (compensated left ventricular

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hypertrophy progressing to dilated cardiomyopathy), the plasma levels of cardiotonic steroids, and the relative amounts of the Na^+/K^+-ATPase α1, α2 and α3 isoforms within the left ventricular myocardium.[128] Specifically, the advancing stages of hypertrophy were associated with an elevated plasma marinobufagenin level, increased expression of the α1 Na^+/K^+-ATPase, decreased expression of the α2 Na^+/K^+-ATPase in the left ventricular myocardium, and heightened sensitivity of the cardiac sodium pump to marinobufagenin. The transition to CHF was associated with a decline in plasma marinobufagenin levels and decreased absolute levels of the α1 Na^+/K^+-ATPase in the left ventricle. Levels of endogenous ouabain rose substantially with the development of CHF, and this enhanced ouabain production was associated with increased levels of the ouabain-sensitive α3 Na^+/K^+-ATPase in the left ventricular myocardium, along with an increase in the sensitivity of the cardiac Na^+/K^+-ATPase to ouabain.[128]

Ouabain and marinobufagenin induce apoptosis and growth-promoting signaling, respectively, in cultured renal tubular cells.[129] The existence of endogenous cardiotonic steroids that have different effects on cell survival makes teleological sense, since different *in vivo* scenarios might require modulation of sodium-pump-dependent functions either with concurrent cytotoxic effects or without. Thus, the transition from compensated left ventricular hypertrophy to CHF is accompanied by a decrease in plasma marinobufagenin level, but a three-fold increase in plasma endogenous ouabain level occurs at the stage of decompensated CHF,[128] when induction of cell death might be considered desirable.[130]

ROLE OF CARDIOTONIC STEROIDS IN RENAL FAILURE

The modern concept of uremic cardiomyopathy is of ventricular hypertrophy and progressive loss of diastolic function in the context of renal disease, which can ultimately progress to eccentric hypertrophy and, rarely, to systolic dysfunction.[131] Echocardiographic studies indicate that diastolic dysfunction and ventricular hypertrophy manifest extremely frequently, whereas systolic dysfunction occurs in less than 20% of patients with end-stage renal disease. Neither the diastolic dysfunction nor the ventricular hypertrophy can be explained solely by the hypertension and anemia that generally complicate end-stage renal disease.[132] As mentioned earlier, plasma levels of telocinobufagin and marinobufagenin are substantially elevated in patients with end-stage renal disease.[15,53]

To examine the role of cardiotonic steroids in renal disease, our laboratory has established models of chronic kidney disease by the use of partial nephrectomy in the rat[133] and, more recently, the mouse.[134] Animals subjected to partial nephrectomy develop increases in marinobufagenin (similar to those seen in patients with renal disease) and diastolic dysfunction, ventricular hypertrophy, and evidence of signaling through the Na^+/K^+-ATPase-Src-EGFR-ERK cascade.[133,134] Cardiac fibrosis occurs prominently in these models[133-136] and also complicates human uremic cardiomyopathy, albeit to a lesser degree.[137] In the rat, infusion of marinobufagenin to achieve a similar elevation in plasma level to that seen after partial nephrectomy results in activation of the Na^+/K^+-ATPase-Src-EGFR-ERK cascade and development of many of the phenotypical features of experimental uremic cardiomyopathy. Active immunization against marinobufagenin attenuates most of the biochemical, physiological and morphological features of uremic cardiomyopathy in animals subjected to partial nephrectomy.[135,136]

Exposure to very small amounts of marinobufagenin (and of other cardiotonic steroids) virtually identical to the circulating plasma concentrations seen in experimental and clinical renal failure directly stimulated the production of collagen in primary cultures of cardiac fibroblasts.[134,135] Again, this effect required signaling through the Na^+/K^+-ATPase-Src-EGFR cascade. The stimulation of collagen production was associated with increased

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transcription and translation of procollagen, but no change in the stability of procollagen or collagen was identified.[134]

ROLE OF CARDIOTONIC STEROIDS IN PRE-ECLAMPSIA

Since pregnancy is associated with plasma volume expansion as a result of renal sodium and fluid retention,[138,139] it is logical to examine the role of cardiotonic steroids in pregnancy and in pregnancy-associated diseases. Graves *et al.*[140,141] were the first to demonstrate increased circulating levels of cardiotonic steroids in pregnancy and to hypothesize that cardiotonic steroids are involved in the pathogenesis of pre-eclampsia. After the introduction of assays for specific cardiotonic steroids, levels of endogenous ouabain and marinobufagenin were found to be increased by four-fold and eight-fold, respectively, in patients with severe pre-eclampsia.[52] In a study of women with milder pre-eclampsia, elevated levels of marinobufagenin accompanied inhibition of the Na^+/K^+-ATPase in erythrocytes, but endogenous ouabain levels were not markedly increased; *ex vivo* treatment of erythrocytes with an antimarinobufagenin antibody—but not an antiouabain antibody—reversed this inhibition.[142]

The role of cardiotonic steroids in pregnancy is not yet understood; however, in a study of 15 patients with pre-eclampsia, the anti-digoxin antibody Digibind® (GlaxoSmithKline, Philadelphia, PA) lowered blood pressure.[143-145] The mechanism underlying this effect is believed to involve cross-reactivity with endogenous cardiotonic steroids.[143-145] These data are in agreement with the observation of a vasorelaxant action of Digibind® in isolated perfused placentae from pre-eclamptic women[146] and of heightened sensitivity of the Na^+/K^+-ATPase to digitalis in such placentae.[147] A characterization study showed that marinobufagenin-like immunoreactive material from the plasma of pre-eclamptic women exhibits chromatographic properties similar to that of marinobufagenin produced by cultured murine adrenocortical cells.[148]

The role of cardiotonic steroids in pre-eclampsia has been explored further in experimental animals. Salt supplementation—with drinking water containing 1.8% sodium chloride—during days 14-20 of gestation in pregnant Sprague-Dawley rats was associated with an increase in the plasma levels of marinobufagenin (but not of ouabain), an elevation of blood pressure, proteinuria, and a decrease in fetal weight, size, and number.[149] Administration of an anti-marinobufagenin antibody resulted in a 28 mmHg decrease in blood pressure and a simultaneous increase in the activity of the Na^+/K^+-ATPase in thoracic aortae.[149] In pregnant rats rendered hypertensive by deoxycorticosterone acetate and salt supplementation, uterine arteries exhibited enhanced sensitivity to the vasoconstrictor action of marinobufagenin, and an antimarinobufagenin antibody had an antihypertensive effect.[150] Thus, it appears that increases in circulating marinobufagenin levels are responsible for the Na^+/K^+-ATPase inhibition induced by preeclampsia and contribute to the pathogenesis of pre-eclampsia. Accordingly, low concentrations of marinobufagenin impair the differentiation of the cytotrophoblast *in vitro*.[151]

ROLE OF CARDIOTONIC STEROIDS IN OTHER CONDITIONS

Considering the fact that cardiotonic steroids regulate the transport and signaling functions of a key membrane enzyme, it is not surprising that the list of disorders in which these factors are implicated is not limited to essential hypertension, end-stage renal disease, pre-eclampsia, and cardiac failure. Several lines of evidence indicate the involvement of cardiotonic steroids in behavioral stress,[81,152] physiological response to exercise,[153] myocardial ischemia-induced arrhythmias,[154] manic-depressive disorder,[155,156] and ethanol addiction.[157]

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Elevated levels of cardiotonic steroids, along with perturbed function of the Na^+/K^+-ATPase, have been found in patients and experimental animals with diabetes mellitus.[158,159] More recent evidence indicates that rats with type 1 diabetes exhibit higher urinary levels of marinobufagenin and more profound inhibition of the Na^+/K^+-ATPase than rats with type 2 diabetes, implying that the degree of glycemia seen in diabetes co-varies with level of marinobufagenin excretion and extent of Na^+/K^+-ATPase inhibition.[160] Along with clinical data demonstrating that cardiotonic steroids are stimulated by oral glucose challenge,[161] this evidence implicates cardiotonic steroids in tissue glucose tolerance.

Cardiotonic steroids are also likely to be involved in the pathogenesis of cancer,[162] and the growing body of *in vitro* evidence of growth-promoting but anticancer effects of cardiotonic steroids raises the possibility of using inhibitors of the Na^+/K^+-ATPase to treat cancer.[163]

NOVEL THERAPEUTIC POSSIBILITIES

Both the ion transport function of the Na^+/K^+-ATPase and the Na^+/K^+-ATPase signaling pathway offer potential targets for therapeutic intervention (Figure 2). Although most of these targets have not yet been specifically addressed, it is interesting to note that some have been investigated, whether deliberately or inadvertently.

The development of antagonists to the Na^+/K^+-ATPase is an obvious approach to developing therapies that target cardiotonic steroids, but would not discriminate between the two mechanisms of Na^+/K^+-ATPase activity. Interesting data exist for rostafuroxin, a digitoxin derivative that is designed to serve as an endogenous ouabain antagonist.[164] Rostafuroxin has been shown to have beneficial effects in a rodent ouabain infusion model, causing a reduction in blood pressure and in left ventricular and renal weight.[87,99] The efficacy of rostafuroxin in hypertensive patients and the possible dependence of this efficacy on the adducin genotype is being assessed in the ongoing phase II multicenter Ouabain and Adducin for Specific Intervention on Sodium in Hypertension (OASIS-HT) study.[165] Spironolactone and its major metabolite canrenone are also potential antagonists of cardiotonic steroids. In experimental studies, both spironolactone and canrenone antagonized the binding of ouabain to the Na^+/K^+-ATPase as well as ouabain-induced inhibition of the pump.[166] Canrenone reduced blood pressure and restored activity of the Na^+/K^+-ATPase in a study of rats with experimental volume-dependent hypertension,[167] and has been proposed as a treatment for digitalis toxicity.[168]

In vivo immunoneutralization of cardiotonic steroids might at first appear an eccentric approach to the treatment of hypertension, and blockade of volume-sensitive hormones in a volume-contracted state may seem counterintuitive. The clinical evidence of the efficacy of Digibind® in pre-eclampsia, however, constitutes one of the most convincing arguments for a prohypertensive role of cardiotonic steroids and for therapeutic immunoneutralization.[143-145] A multicenter, double-blind, placebo-controlled efficacy study of Digibind® in pre-eclampsia (DEEP) is in progress.[169] Immunoneutralization of cardiotonic steroids might also be effective in patients with cerebral salt-wasting syndrome, a condition that frequently accompanies cerebral injury and is associated with life-threatening natriuresis. For example, in a study by Menezes and co-workers,[170] Digibind® reduced renal sodium excretion in a patient following brain tumor removal.

Another potential target for intervention is protein kinase C (Figure 2). Cicletanine, an anti-hypertensive agent that inhibits protein kinase C, is effective in the treatment of experimental salt-sensitive hypertension and attenuates inhibition of the Na^+/K^+-ATPase both *in vitro* and *in vivo*.[171] Atrial natriuretic peptide potentiates marinobufagenin-induced inhibition of the renal Na^+/K^+-ATPase *in vitro* via cGMP-dependent mechanisms, but reduces marinobufagenin-induced inhibition of the sodium pump in vascular smooth muscle.[172] Thus,

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atrial natriuretic peptide might have the capacity in some settings to antagonize the undesirable effects of cardiotonic steroids while potentiating their desired effects. As our understanding of the Na^+/K^+-ATPase signaling pathway expands, additional targets for clinical intervention will become obvious.

CONCLUSIONS

Understanding of the role of cardiotonic steroids in health and disease has progressed tremendously during the past few decades, with the discovery of several endogenous cardiotonic steroids in mammals and the elucidation of the roles of these molecules in a wide range of diseases; however, current knowledge is only the tip of the iceberg. We expect that many additional therapeutic avenues will open up as the capacity to measure levels of endogenous cardiotonic steroids improves, the understanding of the biosynthesis, metabolism and regulation of these molecules deepens, and the knowledge of the role of Na^+/K^+-ATPase signaling in health and disease becomes more detailed.

KEY POINTS

- Several endogenous cardiotonic steroids, or digitalis-like factors, have been isolated and characterized in humans; of these, ouabain, from the cardenolide class, and marinobufagenin, from the bufadienolide class, are the most extensively studied
- In addition to inhibiting the ion transport function of the sodium/potassium-transporting ATPase, binding of endogenous cardiotonic steroids to this sodium pump can activate signaling via the Src-epidermal growth factor receptor pathway
- In Dahl salt-sensitive rats with salt-induced hypertension, endogenous ouabain acts as a neurohormone and stimulates the release of marinobufagenin, a natriuretic and vasoconstrictor
- Endogenous cardiotonic steroids are implicated in congestive heart failure, pre-eclampsia and diabetes mellitus
- Potential therapeutic approaches to targeting endogenous cardiotonic steroids include immunoneutralization, receptor antagonism and protein kinase C inhibition

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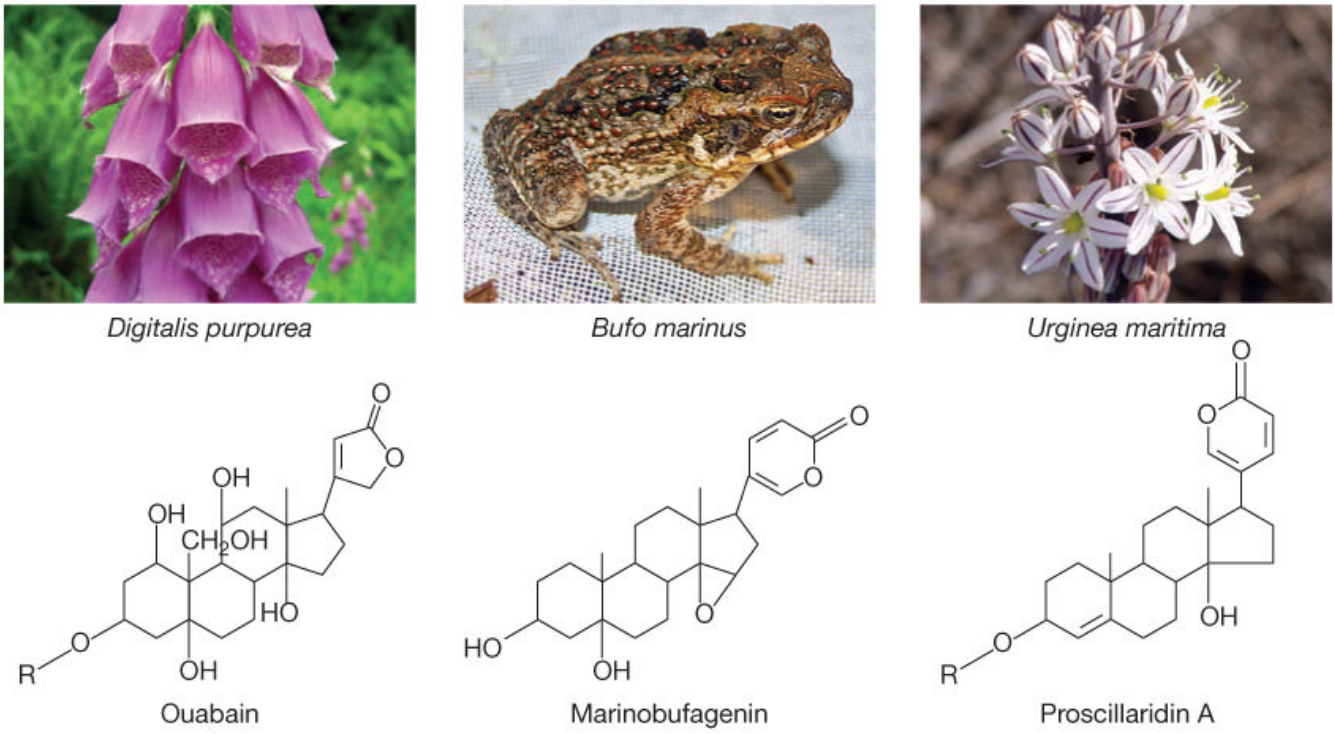

Figure 1.

Chemical structures of selected cardiotonic steroids. Ouabain belongs to the cardenolides, a class of cardiotonic steroids found in the common foxglove (*Digitalis purpurea*) and in other plants. The bufadienolides marinobufagenin and proscillaridin A are found in the cane toad (*Bufo marinus*) and sea squill (*Urginea maritima*), respectively. The image 'Urginea maritima flowers' by Júlio Reis is reproduced under the Creative Commons Attribution ShareAlike 2.5 License (http://creativecommons.org/licenses/by-sa/2.5/). The image of *Bufo marinus* is reproduced under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/licenses/by/3.0/).

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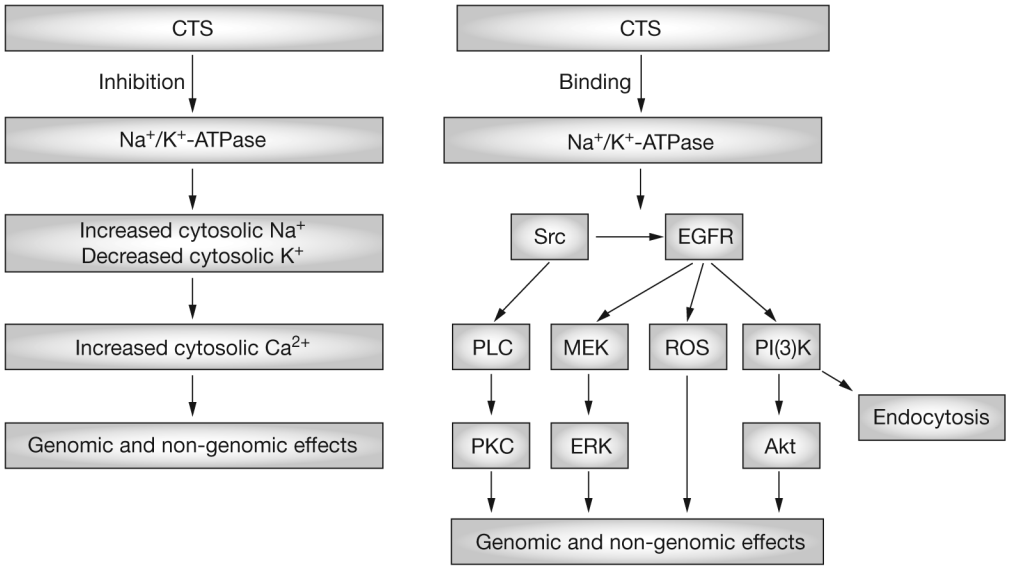

Figure 2.

The two pathways via which binding of cardiotonic steroids to the Na^+/K^+-ATPase exerts genomic and non-genomic effects. In the classic 'ionic' pathway (left), inhibition of the pump function of the Na^+/K^+-ATPase by CTS results in an increase in cytosolic sodium concentration and a decrease in cytosolic potassium concentration. These changes induce an increase in cytosolic calcium level, which in turn activates a variety of pathways with genomic and non-genomic effects. The pump function of the caveolar Na^+/K^+-ATPase might be more sensitive to CTS than that of the noncaveolar Na^+/K^+-ATPase. The 'signaling' pathway (right) involves the association of Src with the Na^+/K^+-ATPase in a caveolar domain. Binding of CTS to the Na^+/K^+-ATPase activates Src, which in turn transactivates the EGFR and PLC, leading to a cascade that involves generation of ROS, activation of ERK through activation of MEK, activation of Akt (protein kinase B) via PI(3)K, stimulation of endocytosis and activation of PKC. These steps induce the genomic and non-genomic effects of CTS. Note that both the classic and signaling pathways allow intervention at the level of the binding of CTS to the Na^+/K^+-ATPase, by immunoneutralization or pharmacological antagonism; however, the signaling pathway presents several additional targets for interference, such as Src activation and transactivation of the EGFR, PLC activation, activation of MEK, generation of ROS and activation of PI(3)K. Modulation of the signaling pathway at the level of PKC, ERK and Akt might also be possible. Abbreviations: CTS, cardiotonic steroids; EGFR; epidermal growth factor receptor; ERK, extracellular regulated kinase; MEK, mitogen-activated protein kinase; Na^+/K^+-ATPase, sodium/potassium-transporting ATPase; PI(3)K, phosphoinositide-3 kinase; PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species.

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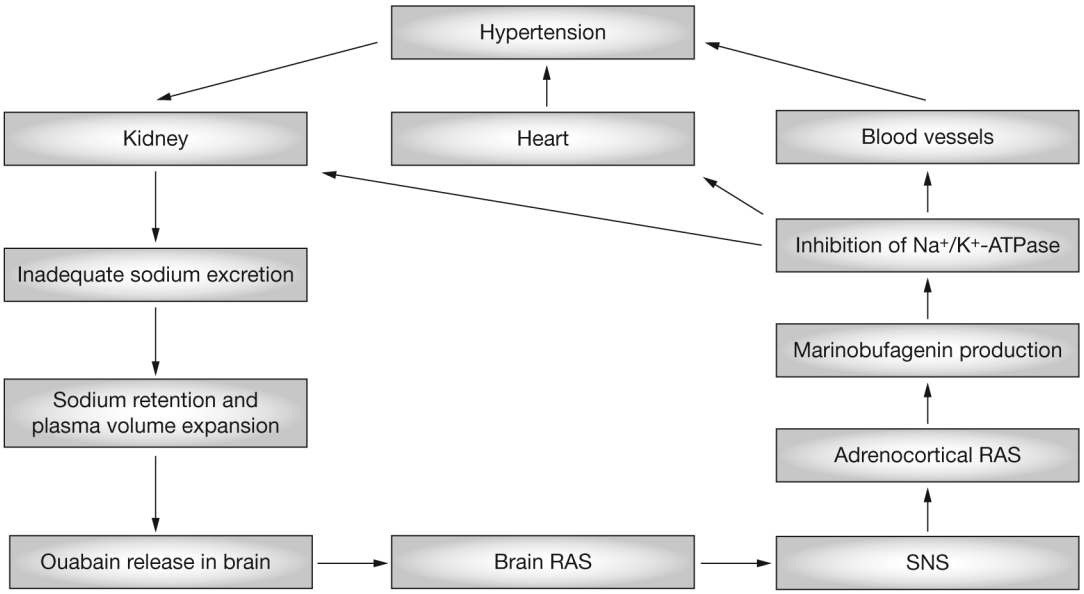

Figure 3.

Interactions between brain endogenous ouabain, the central renin-angiotensin system, and circulating marinobufagenin in the pathogenesis of salt-sensitive hypertension. In salt-loaded Dahl salt-sensitive rats, sodium retention occurs because renal sodium transport is impaired. Sodium retention stimulates the release of endogenous ouabain in the hippocampus, hypothalamus and pituitary. Brain endogenous ouabain stimulates the RAS in the hypothalamus and pituitary, which activates the SNS. These events stimulate the RAS in the adrenal cortex, and activate adrenocortical production of marinobufagenin. Marinobufagenin, a natriuretic and a vasoconstrictor, induces natriuresis via inhibition of the renotubular Na^+/K^+-ATPase. Excessive release of marinobufagenin leads to inhibition of the Na^+/K^+-ATPase in vascular smooth muscle cells—which potentiates vasoconstriction—and in the heart—which increases cardiac contractility—causing hypertension. Abbreviations: Na^+/K^+-ATPase, sodium/potassium-transporting ATPase; RAS, renin-angiotensin system; SNS, sympathetic nervous system.