Is Ouabain the Endogenous Digitalis?

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The possibility that an endogenous ligand might exist for the cardiac glycoside binding site on the Na⁺,K⁺-ATPase (i.e., the "sodium pump") has been a source of much speculation, resulting in intensive efforts over the past decade by many laboratories to identify an "endogenous digitalis-like hormone."1–6 Several arguments have been propounded in support of this hypothesis. The first and perhaps most compelling is the fact that the binding site for cardiac glycosides is highly conserved throughout the phylogeny of eukaryotes. The possibility that the conservation of the specific protein conformation responsible for binding of cardiac glycosides merely ensures successful ion translocation, having by design nothing to do with the binding of a putative cardiac glycoside-like autacoid, is unsatisfying. This explanation, for example, ignores the rationale followed in the identification of the endogenous opioids, which clearly act at receptors that also bind compounds derived from plants, although this example may be less relevant than it appears for reasons to be discussed below. Other explanations aside from a circulating digitalis-like hormone have been elaborated to explain the conservation of the cardiac glycoside binding site on the sodium pump, although these remain purely speculative.6

The second major reason for the continuing interest in an endogenous digitalis is the hypothesis that such a hormone, if it were to exist, could have important effects on sodium homeostasis and systemic vascular resistance. Neither of these actions, ironically, has anything to do with the usual indications for administration of digoxin to patients, i.e., its antiarrhythmic and positive inotropic actions on the heart, although the article by more than 30 years ago. The discovery of atrial natriuretic peptide and its congeners, none of which acts directly by inhibiting Na⁺,K⁺-ATPase, does not exclude the possibility of a second natriuretic hormone system. Speculation that a circulating digitalis-like hormone or autacoid would also induce vasoconstriction, either as a result of reduced calcium efflux or increased calcium influx via Na⁺–Ca²⁺ exchange or of increased calcium influx via voltage-sensitive Ca²⁺ channels after inhibition of Na⁺,K⁺-ATPase, provided a link between abnormal sodium homeostasis and inappropriate vasoconstriction and a unifying hypothesis for the pathogenesis of essential hypertension. This hypothesis was initially synthesized separately by Haddy and Overbeck6 and by Blaustein9 in the mid 1970s and has since provided much of the impetus for many reports that have offered either direct evidence of a digitalis-like factor in plasma and other biological fluids or indirect evidence of abnormal sodium pump function in tissue or red blood cells from patients or experimental animals with hypertension.

Thus, the key arguments in support of the existence of an endogenous form of digitalis have been the presence of a highly conserved "binding site" on the α-subunit of Na⁺,K⁺-ATPase and the fact that a circulating inhibitor of the sodium pump would provide a convenient and inclusive explanation for a number of pathophysiological phenomena. An obvious criticism of this hypothesis has been the claim that any circulating digitalis-like hormone would indiscriminantly inhibit sodium pump function in all cells, but this point has been rebutted, at least in part, by the discovery of multiple isoforms of the α-subunit of Na⁺,K⁺-ATPase, each characterized in part by its different affinity for ouabain binding.10,11 This has raised the possibility that the α-subunit isoforms represent "receptor subtypes," the expression of which is now known to be differentially regulated in each tissue and throughout ontogeny.

Interesting as these arguments may be, we believe that they rest on inappropriate analogies and unproven assumptions. The central hypothesis that the plasmalemmal Na⁺,K⁺-ATPase functions both as the receptor for a circulating digitalis-like factor and as the effector protein directly responsible for generating an intracellular signal has little precedence among the known hormonal signal transduction mechanisms. Hormones, whether autocrine, paracrine, or endocrine, are typically recognized by specific plasma membrane or intracellular proteins that subsequently result in the generation of second messengers or a change in the expression of selected genes. Whatever intracellular signal results, the transduction mechanism is always subject to modulation by a number of factors that often

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Gottlieb et al in this issue of Circulation7 does address a potential role for an endogenous ouabain in the physiological response to heart failure. The possibility that a circulating inhibitor of Na⁺,K⁺-ATPase would act as a natriuretic hormone, presumably by inhibiting the sodium pump on the basolateral membranes of renal tubular epithelial cells, has been most persuasively argued by de Wardener,1 one of the first physiologists to have postulated the existence of natriuretic hormones...
lead to either amplification or inhibition of the original hormonal signal. Some neurotransmitters, such as acetylcholine at nicotinic receptors, do act directly to alter plasmalemmal ion flux by inducing a transient conformational change in an integral membrane protein, but these extracellular signals are always confined to restricted, specialized plasmalemmal domains, often with high regional concentrations of an appropriate degradative enzyme, such as acetylcholinesterase in the example of the nicotinic receptor.

Certainly, the number and variety of factors involved in polyclonal cell-cell signaling are large and rapidly growing and have included the identification of novel chemical signals. Endothelium-derived relaxation factor (EDRF), for example, the predominant form of which has been identified as nitric oxide, the "endogenous organic nitrate," is now recognized to be a nearly ubiquitous autocrine and paracrine signaling factor, the molecular receptor for which is the soluble guanylate cyclase found within most cells. Unlike the proposed digitalis-like factor, however, EDRF is short-lived, results in the generation of an intracellular second messenger, cyclic GMP, and clearly does not act as a circulating hormonal signal. Even the endogenous opioids, a class of transcellular signaling peptides (the discovery of which has often been cited as an appropriate analogy for endogenous digitalis-like factors), act via conventional plasmalemmal receptors linked to GTP-binding, protein-regulated intracellular signaling pathways. Thus, the concept of an endogenous digitalis as a transcellular chemical signal acting directly on an integral membrane protein that serves as both its receptor and primary effector mechanism, particularly such a ubiquitous protein as the Na\(^{+}\),K\(^{+}\)-ATPase, which is ultimately responsible for maintaining transmembrane ion gradients in every cell, would appear to violate most design criteria for known hormonal signal transduction mechanisms in multicellular animals. The mechanism proposed for digitalis-like factors, however, is characteristic of many drugs, venoms, and toxins.

Perhaps most discouraging for many laboratories involved in the search for the endogenous digitalis was the absence of any definitive characterization or identification of potential candidate compounds from animal tissues despite years of effort. Some chemicals present endogenously within certain tissues, such as vanadate and amphiphilic lipids, had been identified as contributing to sodium pump inhibitory activity in biological samples, but these and other nonspecific factors are unlikely to be physiologically relevant regulators of Na\(^{+}\),K\(^{+}\)-ATPase.\(^{12}\) Therefore, the report by Hamlyn, Blaustein, and colleagues at the University of Maryland in collaboration with Ludens and coworkers at Upjohn Laboratories\(^{13}\) that the digitalis-like activity present in human plasma was, in fact, ouabain or a very similar steroidlike compound provided important validation for the concept of an endogenous cardiac glycoside-like hormone. They have reported that levels of endogenous ouabain in human plasma appear to be sufficiently high (i.e., 0.1–8.8 nM) to result in physiologically relevant inhibition of Na\(^{+}\),K\(^{+}\)-ATPase and that the highest tissue levels of ouabain were found in the adrenals, as might be expected for a steroid hormone.\(^{13}\) Importantly, they also demonstrated that plasma levels of endogenous ouabain were increased in some forms of experimental hypertension in the rat, as predicted by the general theory of an endogenous cardiac glycoside-like hormone. Although a number of other laboratories are actively pursuing the identity of digitalis-like activities present in plasma, none have definitively characterized the chemical compound(s) responsible or yet confirmed that at least part of the digitalis-like activity could be caused by ouabain or a ouabainlike steroid.

To evaluate studies that purport to measure digitalis-like activity or a ouabainlike compound in any clinical or biological specimens, the reader must consider critically the specific assay techniques used and the extent to which any preparative or analytical chemical separation steps have been validated. Digitalis-like "activity" is usually detected in biological samples by one or more of the following techniques: inhibition of ATP hydrolysis by partially purified Na\(^{+}\),K\(^{+}\)-ATPase under defined conditions that support ouabain binding; inhibition of "ouabain-sensitive" activity on monovalent cation transport in whole cells, usually erythrocytes or leukocytes; inhibition of radiolabeled ouabain or digoxin binding to partially purified Na\(^{+}\),K\(^{+}\)-ATPase; a positive inotropic effect in cardiac muscle and/or vasoconstriction in vascular smooth muscle consistent with a digitalis-like effect; and recognition in competitive binding assays by antibodies directed at antigenic determinants of authentic cardiac glycosides, so-called "digoxin-like immunoactivity." None of these techniques are absolutely specific for cardiac glycosides, and all may be affected nonspecifically by common components of plasma, such as amphiphatic lipids.

The majority of reports in the literature, including the article by Gottlieb and colleagues\(^{*}\) in this issue of Circulation, have used cardiac glycoside-like immunoactivity (i.e., radioimmunoassay or ELISA) to quantify the digitalis-like activity present in plasma. As recently emphasized by Naomi and colleagues,\(^{14}\) this approach often leads to widely disparate results, depending on the specific antisera and immunoassay technique being used. In the report by Gottlieb et al, rabbit anti-ouabain antiserum was used, the ouabain binding affinity of which was not reported but which was stated to have only minimal affinity for authentic digoxin, which all of the heart failure patients in this study were receiving. Plasma samples in this study were also treated initially by a preparative chromatographic step that had been documented previously to remove plasma components that could nonspecifically interfere with ouabain-antiouabain antibody binding or the secondary antibody-peroxidase reaction in the ELISA assay.

However, none of the more rigorous analytical procedures used in the initial report by Hamlyn et al\(^{13}\) were used here. Indeed, Gottlieb et al\(^{*}\) have assumed that the plasma component(s) that demonstrate ouabainlike immunoreactivity in their patients are identical to the compound isolated from over 80 l of human plasma after analytical high-performance liquid chromatography (HPLC) and then characterized both by mass spectrometry and by each of the assay procedures for digitalis-like "activity" described above. Although these more rigorous techniques are cumbersome and inappropriate for routine analytic use, it is our opinion that they must continue to be used at this early stage of investigation to positively identify the compound responsible for digitalis-like activity in biological samples; other-
wise, techniques that sacrifice selectivity for sensitivity and convenience, such as radioimmunoassay, are inap-
propriate in this context.

Although the collaborative effort that resulted in the identification of ouabain in human plasma represents
a milestone in the search for the endogenous ligand for the cardiac glycoside binding site on the sodium pump,
we believe that several important questions remain to be clarified before ouabain can be elevated from its
characterization as plant toxin to human hormone.

First, although addressed to a limited extent by Hamlyn and colleagues in their original report and companion
publications, the possibility that ouabain or a similar compound was a trace contaminant in their original
plasma samples has not been excluded. This would require positive identification of ouabain by HPLC/ mass
spectroscopy in the plasma of patients (or experimental animals) maintained on a synthetic diet or, most
convincingly, in defined medium conditioned by isolated, superfused adrenocortical cells or by adrenocor-
tical tissue maintained in culture. Such experiments should also address the important question of whether
the specific enzymes required for synthesis of the peculiar steroid structure of the cardiac glycosides, in which
the A: B and C: D ring junctions are cis as opposed to trans as is invariably the case in all known steroids
including cholesterol and all steroid hormones, are present or inducible in mammalian tissues. An addi-
tional, more general concern is the fact that steroid hormones, virtually without exception, act by binding to
specific intracellular receptor proteins that subsequently alter transcription of selected genes. As reviewed
above, the proposed mechanism of action of a putative digitalis-like factor, if it exists as a circulating
inhibitor of Na⁺,K⁺-ATPase, would clearly be unique.

All of these theoretical criticisms become moot, however, if such a factor can be positively identified in
biological samples and conclusively shown to be both endogenous in origin and present in physiologically
relevant concentrations either locally within a specific tissue or circulating in plasma. Even assuming, however,
that a circulating ouabainlike hormone was being de-
tected in the plasma of patients with congestive failure
by the ELISA used in the report by Gottlieb et al., several points should be noted regarding these data.
The reported correlations between plasma ouabain levels and either cardiac index or mean arterial press-
ure, although statistically significant, indicate that the variance in these physiological parameters accounts for
only 25% and 36%, respectively, of the variance in plasma ouabain concentration. As the authors note,
there was unexpectedly no correlation between indexes of intravascular volume status and plasma ouabain
levels, as would have been expected for a circulating
digitalis-like natriuretic hormone. It is also puzzling that
no correlation could be identified between renal func-
tion and plasma levels of ouabain, as clearance of exogenousy administered ouabain is known to be pre-
dominantly via the kidneys. Indeed, we have shown in both dogs and humans that there is a typical first-
order decline in plasma levels of ouabain after bolus in-
jections, as detected by radioimmunoassay using oua-
bain-specific antibodies, with no evidence in either
plasma or urine of endogenous ouabain production. Finally, two patients identified in this report who were
also receiving therapeutic doses of digoxin had plasma
levels of ouabain that clearly would have been toxic had it been administered exogenously. Indeed, plasma levels
of the combined cardiac glycosides overlap those seen in
patients after suicidal ingestions, and yet no evidence of clinical toxicity was reported.

Nature continues to provide exceptions to the con-
ventional wisdom in molecular pharmacology, and the
endogenous ouabain may be such an exception. Never-
theless, we feel that it is still too early to begin describ-
ing the role of a candidate compound such as ouabain as
a new hormone or autacoid within a given patient
population without convincing evidence of an endoge-
nous synthetic pathway and without continuing a rigor-
ous characterization and validation of the assay pro-
ducedures used. Although Gottlieb, Hamlyn, and coworkers
have gone further toward this goal than most laborato-
ries in this field, in our view, the existence of ouabain as
an endogenous digitalis-like hormone (and particularly
the assertion that it is an "essential homeostatic regu-
lator in humans") remains speculative.

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