Endogenous Ouabain Is Not Ouabain

Lynley K. Lewis, Timothy G. Yandle, Philip J. Hilton, Berit P. Jensen, Evan J. Begg, M. Gary Nicholls

The concept of a circulating digitalis-like inhibitor of the sodium pump, Na^+, K^+-ATPase, evolved from studies performed in the 1960s. De Wardener et al[1] addressed the question of whether a small increase in the glomerular filtration rate together with changes in the concentration of the more recently discovered hormone aldosterone could explain the natriuresis that generally followed salt (sodium) loading. In their studies, dogs had their renal blood flow reduced significantly by constricting the aorta above the renal arteries and were given supramaximal doses of fludrocortisone, a synthetic analogue of aldosterone, and vasopressin before being challenged with intravenous saline. Their ability to develop a natriuresis clearly demonstrated that a third factor (beyond changes in glomerular filtration rate and aldosterone concentrations) was involved in the natriuresis of salt loading. Subsequent experiments demonstrated that the responsible agent could be transmitted by the plasma of the volume-expanded animal.[2] Although we may now ask whether in these experiments the effect was mediated, at least in part, by the release of atrial and B-type natriuretic peptide from the heart, it was nearly 2 decades before those hormones were discovered during which time it was demonstrated that the plasma of volume-expanded animals had the ability to inhibit the sodium pump[3]—which is not a target of atrial natriuretic peptide and B-type natriuretic peptide. Essentially parallel studies were performed by Welt and colleagues[4] in uremia where inhibition of the sodium pump of erythrocytes was demonstrated along with the ability of uremic plasma to induce such a defect in normal erythrocytes.

In 1975, it was shown that patients with essential hypertension had, as a group, reduced activity of the sodium pump of leukocytes, with corresponding elevated values for intracellular sodium.[5] This finding proved to be reproducible in various laboratories, and once again the effect was transmissible by exposing normal cells to the plasma of hypertensives. It was also shown that there was a crude correlation between the elevated blood pressure and the depression of the activity of the sodium pump as measured by the efflux rate constant.[6] It was then clear that under certain conditions humans and other animals had the ability to secrete an inhibitor of the sodium pump and that this may well be of importance in the physiology/pathophysiology of salt overload, uremia, and essential hypertension.

Long overdue is a review of De Wardener's third factor and the proposition that it or a similar compound acts by inhibition of the sodium pump in a manner akin to digitalis and related materials, and that this compound plays a role under physiological circumstances and in the pathophysiology of circulatory and renal disorders. In the current brief review, we have focussed on a narrow component of this broad field: the question of whether, as claimed below, authentic ouabain or an isomer of ouabain is present in human circulation.

By the late 1970s, the reasonable question was that, given the repeated demonstration of the phenomena noted above, what was the circulating agent involved? Despite investigations from many laboratories, the identity of the inhibitor compound(s) remained elusive until 1991 when Hamlyn and colleagues from the Upjohn Laboratories in Kalamazoo, Michigan, and the Department of Physiology at the University of Maryland addressed this issue by concentrating large volumes of human plasma and subjecting them to liquid chromatography coupled to mass spectrometry. In that year, they produced a series of articles suggesting convincingly that their highly concentrated sample contained an inhibitor of Na^+, K^+-ATPase that was structurally, biochemically, and immunologically indistinguishable from ouabain or an isomer of ouabain.[7–10] These workers went on to show that their isolated material had cardiotonic and vasotonic activity similar to authentic plant-derived ouabain.[11] They also developed an immunoassay for measurement of ouabain in plasma.[9] The *Lancet* greeted these reports with an editorial article entitled "Welcome to Ouabain – a New Steroid Hormone."[12] Studies thereafter reported that endogenous ouabain (EO), as has become the common term, was secreted by bovine zona glomerulosa cells from the adrenal cortex under the control of adrenocorticotrophic hormone and angiotensin II, the latter via angiotensin type II receptors.[13–15]

A different approach to the isolation of a circulating inhibitor of the sodium pump in humans was also pursued. Following on from a criminal trial in Canada in which a nurse was accused of causing the death of a newborn when digoxin was discovered in the plasma despite the drug never having been prescribed, Valdes et al[16] observed that immunoassayable digoxin was regularly present in neonatal plasma. Because it was unlikely (although not impossible) that the immunoassay was measuring authentic digoxin, this could imply that cord blood contained a sodium pump inhibitor. Following this lead, our group in London saw weak sodium pump inhibitory action in a large series of cord blood samples[17]; however, the amounts were

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insufficient to characterize the material from that source. We argued that the likely target of the inhibitor was the placenta and attempted to extract it from this source. Multiple extractions yielded an inhibitory fraction, which, when examined by mass spectrometry, revealed a compound of mass 370 Da running in positive ion mode as material of *m/z* 371 (protonated), 393 (sodiated), and 409 (potassiated).[18,19] Accurate mass studies indicated an empirical formula of $C_{24}H_{34}O_3$, and the mass spectrometer in negative ion mode showed fragmentation to a compound of *m/z* 97 and subsequently 80. Allowing that this ion was neither a sulfate nor a phosphate resulted in the view that it represented the loss of a dihydropyrone from the original compound, suggesting that it was a bufenolide. In multiple placental tissue extractions, which showed inhibition of the sodium pump, no trace of ouabain was ever seen on mass spectrometry. Both this study and that of Hamlyn were conducted using fast atom bombardment ionization coupled with traditional magnetic sector detectors, which were considered state-of-the-art equipment at the time but, as described below, have since been repeatedly superseded in sensitivity and ease of use.

On the assumption that the ion seen by Hamlyn and colleagues was indeed ouabain, an immunoassay to this compound was developed and commercialized by Du Pont–New England Nuclear. Using this or similar immunoassays, at least 13 research groups reported plasma levels of immunoreactive ouabain in healthy volunteers, which, as we noted in an earlier article, varied widely between 2.5±0.5 (mean±SEM) nmol/L (and up to 176±68 nmol/L with exercise) and undetectable.[20] The plasma levels (and especially those achieved with exercise) reported by some groups would likely prove fatal if indeed the measured compound was authentic ouabain. With regard to these wide variations in reported levels of plasma ouabain, it is important to keep in mind the potential pitfalls of immunoassays. In particular, cross-reactivity is an everpresent risk. For example, it is possible to quote a plasma concentration of prednisolone using an immunoassay for cortisol and factoring in the known cross-reactivity and near certainty that high-dose prednisolone administration will almost totally inhibit cortisol secretion in most circumstances. Along similar lines, we have had the opportunity to study an antibody raised against ouabain that gave significant concentrations to virtually any small cyclic compound, for example, tyrosine, catecholamines, and creatinine (unpublished data). This experience suggests that the characteristics of any antibody raised against such a substituted steroid needs to be investigated thoroughly to avoid confusion as to what the resulting immunoassay is actually measuring. In this regard, 3 groups (2 from the United States and 1 from Christchurch, New Zealand) that published their data in the same year (1994) were unable to detect immunoreactive ouabain after high-performance liquid chromatography separation of human plasma,[21–23] raising the possibility that EO as measured by available immunoassays did not represent authentic ouabain.

Subsequent to the 1991 reports, data largely but not exclusively from the University of Maryland workers and their Italian colleagues have suggested that the human adrenal gland produces EO, which plays a role in the regulation of sodium balance, vascular smooth muscle tone, and arterial pressure under normal circumstances and may contribute to the pathophysiology of cardiac failure, chronic renal failure, essential hypertension,[24] as well as other disorders including pregnancy-induced hypertension and preeclampsia,[25] kidney injury after cardiac surgery,[26] Meniere disease,[27] and polycystic kidney disease.[28] Furthermore, plasma EO levels were reported to be higher in the offspring of families with a positive history of hypertension than in subjects with no such family history, to correlate with blood pressure levels, and relate to some indices of diastolic left ventricular structure and function.[29] Whereas review articles in several high-profile journals, often but not uniquely from the University of Maryland and Milan workers, have taken for granted that EO exists and is of pathophysiological importance,[24,25,29–32] questions have been raised by us and several other research groups. At an Endocrine Society meeting in 1995, opinions were divided as to whether authentic ouabain circulates in human plasma and is of adrenocortical origin.[33] This debate was aired again and in considerably more detail in 1996[34,35] and in 2009.[20,24] To summarize the major points of uncertainty in the latest of these debates,[20,24] first, serious questions were raised regarding the physical evidence obtained in the early 1990s to support the authenticity of ouabain in human plasma; second, the ability of the human adrenals to produce immunoreactive ouabain had been called into question and details of the adrenal biosynthetic pathway remained to be defined; third, the DuPont/NEN ELISA kit for measuring ouabain was withdrawn from the market because of lack of commercial interest,[24] which, we surmise, reflected widespread uncertainty as to what the assay was measuring; and fourth, the relatively sparse temporal profile of reports on EO contrasted with B-type natriuretic peptide, for example, which, since its discovery 3 years earlier than ouabain, had been the subject of a tsunami of reports based, in most cases, on well-established and widely available commercial assays. At the time of our review in 2009, we pointed to several discrepancies between the properties of the circulating sodium pump inhibitor and ouabain and suggested that the position of EO as an adrenally produced regulator of the sodium pump in human needed either to be put on a more secure footing or to lose its current status.[20]

So what advances have been made since the debate in 2009? We think there are 3 noteworthy observations to be made. First, there has been no substantial progress to address the question as to whether or not immunoreactive EO is produced by the adrenal glands in humans, and an adrenal biosynthetic pathway for EO remains to be established. The observations by 2 research groups in the mid-1990s, which disputed an adrenal origin for immunoreactive ouabain,[21,36] remain unchallenged. Some 23 years after the existence of EO was reported and in an age when the entire genome is sequenced and genes can be identified computationally, its adrenal biosynthetic pathway remains undiscovered. This raises questions regarding the biosynthetic machinery. Furthermore, involvement in mammalian metabolism of rhamnose, the sugar moiety of ouabain, is most unusual. Second, as mentioned already, articles continue to be published largely but not exclusively by workers at the University of Maryland and their colleagues claiming that EO not only exists but is of pathophysiological importance.[24,25,29–32,37,38] It is a rare article amongst such papers that makes mention, even in passing, that there is some uncertainty

regarding the structure and source of EO.[39] Third and most importantly, as discussed below, recent evidence from German workers using techniques not available in the early 1990s contradicts the original reports in 1991 that authentic ouabain exists in human plasma.[40]

As an aside and in relation to the potential pathophysiological role of EO, it is worthy of note that, whereas some researchers have reported clearcut biological effects of administered authentic ouabain in various animal models and human, in particular to raise arterial pressure, other workers, including those in Christchurch, have failed to see such effects.[20] In this regard and as summarized by Ferrari et al,[41] the drug rostafuroxin that selectively displaces ouabain from the Na^+, K^+-ATPase receptor has been reported to lower arterial pressure in Milan hypertensive rats and select humans, and in deoxycorticosterone acetate salt hypertensive rats, it reduced blood pressure while also ameliorating endothelial dysfunction and oxidative stress in resistance arteries.[42] These observations raised the possibility that if indeed EO exists and contributes to the development or maintenance of hypertension, inhibitors of EO mechanisms of action might provide a new class of antihypertensive drugs.[42] When compared with placebo, however, rostafuroxin had no effect on systolic or diastolic pressure (using both office and 24-hour ambulatory recordings), plasma renin activity, 24-hour urinary sodium, and aldosterone excretion or plasma immunoreactive EO levels in 435 patients with systolic hypertension,[43] although a subset of patients with a specific genetic profile was said to have shown an antihypertensive effect (data not shown). Although the outcome of this trial does not disprove the existence and pathophysiological importance of EO in the majority of patients with essential hypertension, it does not support the underlying premise. Additional studies are underway on the effects of rostafuroxin on arterial pressure in a subgroup of essential hypertensive patients with a specific genetic profile.[43]

Returning to the issue of whether or not authentic ouabain exists in the human circulation, mass spectrometry has undergone a series of remarkable changes since the early 1990s, particularly in its sensitivity or limit of detection. It is now possible using these purely physical techniques to detect a molecule such as ouabain down to below even the lowest (nonzero) concentrations previously reported by immunologic assays. A recent article from workers in Germany has documented their attempt to do just that using state-of-the-art mass spectrometry.[40] Their technique was rigorously validated and benefited from an internal standard of D3 ouabain where 3 of the hydrogen atoms are replaced by deuterium, giving a compound of virtually indistinguishable physical and chemical characteristics but with a mass of 587 as opposed to 584 Da for the natural compound. Ouabain was undetectable in any sample of plasma from either control subjects or patients with heart failure.[40] These data, produced by particularly experienced workers, cannot be dismissed. When added to earlier observations by ourselves and several other research groups, it seems highly likely that the plasma EO concentrations quoted by immunoassay do not represent authentic ouabain.

Proving that something does not exist, especially in a complex milieu such as plasma, is no easy matter. Although none of the findings noted above can be taken alone as definitive evidence against the existence of circulating EO, we think that the new report by Baecher et al[40] together with earlier observations and the unknowns enumerated above render it untenable and misleading to persist with the term EO. We endorse the view of the workers at the University of Maryland and their colleagues in 2009 that more experimental work is needed to allay lingering concerns[24] and challenge those who remain convinced that EO exists in human to provide incontrovertible evidence to this effect. Ideally all compounds with an inhibitory action on Na^+, K^+-ATPase detected in plasma or tissue, including the 370 Da compound isolated from human placenta and a volume-expanded patient,[18,19] should now be studied using the latest technology to determine their structure. Unfortunately, moves toward this end are compromised, while the existence of EO continues to be taken as fact.

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Disclosures

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