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Endogenous Ouabain: Recent Advances and Controversies

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Abstract

In this brief article, we summarize recent reports about endogenous ouabain (EO), a cardiotonic steroid (CTS). This includes analysis of mammalian EO, the discovery of EO isomers, regulation of intracellular signaling by EO, and the roles of EO in hypertension, pregnancy, and heart and kidney diseases. Novel ouabain-resistant mice that elucidate the key roles of α2 Na^+ pumps and their CTS binding site are also discussed.

Endogenous ouabain and its isomers

EO was first identified in human plasma 25 years ago.[1, 2] Despite confirmation in humans and other mammals with mass spectrometry (MS; Figure 1; Online Supplement Figures S1–S6), nuclear magnetic resonance (NMR), and combined liquid chromatography (LC)-immunology methods,[3–6] human EO has remained controversial.[7] New analytical studies and related findings should allay skepticism. For example, employment of multistage MS (MS-MS or MS2, and MS-MS-MS or MS3) to examine the effects of pregnancy and of central angiotensin (Ang) II infusion on EO in rat plasma led to the discovery of two novel EO isomers.[8, 9] One isomer (#1) has MS2 and MS3 product ion spectra indistinguishable from those of EO, but is slightly more polar than EO; it binds to the antibody employed in our radioimmunoassay (RIA). Isomer 2 is slightly less polar than EO, has a distinct MS3 spectrum, and cross-reacts weakly in our RIA. The primary structural difference(s) between EO and these isomers may involve the steroid nucleus. Importantly, neither isomer was previously described or is detectable in commercial (plant) ouabain.[8, 9]

A recent report based on an LC-MS2 approach concluded that EO was not detected in human plasma,[10] but the LC gradient was extraordinarily short so that EO in plasma may have been missed (see Data Supplement). Further, critical data supporting their conclusion were absent from the published article,[10] and the key product ion current recording had inexplicable gaps (Figure S7) at locations where signals from EO isomers might be

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Disclosures

None.

anticipated.[11] Also, the plasma used by Baecher and colleagues[10] tested positive for EO[11] with a well-documented RIA.[8, 9, 12] These RIA data are significant because EO is routinely detected when the same sample extracts are subjected to LC-RIA and LC-MS.[5, 8, 9, 12] In contrast to MS, RIA-based estimation of EO includes the unpredictable contribution of cross-reactivity from related molecules[5, 13] such as isomers 1 and 2,[8, 9] which may vary with gender, age and disease.

The carbon isotope ($^{13}C/^{12}C$) ratio is helpful to distinguish plant versus animal metabolism. The natural abundance of ^{13}C in the bovine adrenal EO and, thus, the $^{13}C/^{12}C$ ratio determined by high resolution MS, was significantly lower than in plant ouabain.[14] EO therefore is neither a laboratory contaminant nor an ingested plant material. If adrenal EO isn't plant ouabain sequestered from the circulation,[15] it must be, either in whole (i.e., sugar and steroid) or in part (steroid, alone), an endogenous product.

What is the origin of circulating EO?

Human, bovine and rodent data indicate that the adrenal cortex contains the highest concentration of EO in the body.[1, 3] Also, adrenalectomized rats[1] and adrenal insufficiency patients[16] have exceptionally low plasma EO levels. Primary cultured bovine and human adrenal cortical cells secrete more EO than is present in the cells, indicating net synthesis.[17] Adrenal venous EO concentrations (adrenal vein cannulation) in the dog were 4–5 fold higher than that in arterial blood.[18] Similarly, in human hypertensives undergoing testing for hyperaldosteronism, the adrenal venous effluent EO concentration was 2–3 fold higher than in inferior vena cava blood.[19] In that study, MS3 analysis of the plasma confirmed that the endogenous substance was EO and an isomer (likely isomer 2). Thus, the adrenal cortex is most probably the primary source of circulating EO, and aldosterone and EO biosynthesis share a requirement for progesterone.[20] The brain is likely also a source of one isomer.[9] Regrettably, the biosynthetic pathway for EO remains unresolved. This is due, in part, to the difficulty and the resources required to elucidate an adrenal pathway whose relative carbon flux is ~20–50 fold and ~10,000 fold less than for aldosterone and cortisol, respectively.

Role of the brain in regulating circulating EO

Early work suggested that the central nervous system (CNS) influences the peripheral levels of ouabain-like substances.[9, 21] Indeed, brain ouabain-like materials are critical to the ability of low dose angiotensin (Ang) II to raise circulating EO and blood pressure (BP).[22–26] Based on new insight into CNS and vascular signaling pathways in salt-sensitive hypertension,[27, 28] the role of the brain in controlling circulating EO was recently probed with multi-dimensional MS analytical methods.[8, 9, 12] Those studies show that low doses of Ang II, acting within the CNS, up-regulate circulating EO; this, in turn, stimulates downstream arterial myocyte mechanisms that raise vascular tone and long-term BP.[9, 28] Upregulation of brain EO, *per se*, also raises BP.[29] Conversely, central blockade of aldosterone synthase, mineralocorticoid receptors (MRs), epithelial Na^+ channels (ENaCs) or brain EO prevents the sympathetic hyperactivity.[26, 27, 30, 31] These central blockers also prevent or markedly attenuate experimental forms of hypertension induced by high salt, low dose Ang II, or ouabain.[26, 27, 30, 31] The participation of EO is documented by the

demonstration that mutation of the ouabain/EO receptor site on α2 Na^+ pumps to make the pumps ouabain-resistant ($\alpha2^{R/R}$) blocks ouabain-induced and salt-sensitive forms of hypertension in mice.[32–35] Importantly, pressure overload-induced cardiac hypertrophy and failure are greatly attenuated in $\alpha2^{R/R}$ mice, whereas they are accelerated in these mice when the α1 Na^+ pumps are mutated to an ouabain-sensitive form.[35, 36] (Note: the α1:α2 expression ratio is ≈4:1 in heart and arteries.[37, 38]) Thus, in addition to hypertension, target organ damage depends, in part, on high affinity EO binding (see "*EO in kidney disease and heart failure*", below).

EO is part of a new neurohumoral pathway in blood pressure control

Compelling evidence indicates that the slow pressor effects of low doses of Ang II depend on an amplifier located in the CNS.[27, 33] The amplifier incorporates neuromodulatory components including local aldosterone synthesis, MRs, ENaCs, and increased synthesis and/or levels of EO in the brain.[39–42] Prolonged stimulation of this CNS amplifier, especially by Na^+ or low dose Ang II, increases sympathetic nerve activity (SNA), often to discrete vascular beds.[43] In addition, however, activation of the CNS amplifier raises the circulating levels of peptide hormones including ACTH, a stimulator of adrenal EO secretion,[44] vasopressin and growth hormone.[45] The relative roles of increased SNA and the humoral components is not clear.

Intracerebroventricular (icv) Ang II infusion also elevates circulating EO.[9] Sustained increases in circulating EO, *per se*, augment the expression of proteins involved in Ca^{2+} homeostasis and signaling in arterial myocytes.[46, 47] The effects of the elevated circulating EO on Ca^{2+} handling in arterial myocytes *in vivo* are fully replicated *ex vivo* with nanomolar ouabain.[46, 47] Notably, all the effects of icv Ang II on circulating EO, as well as the reprogramming of peripheral vascular function, and the elevated BP are prevented by icv administration of eplerenone, an MR blocker, as well as by inhibition of aldosterone synthase with FAD286.[9] Further, BP elevation by subcutaneous (sc) low dose Ang II + high dietary salt is greatly attenuated by immuno-neutralization of EO with fab fragments that bind ouabain with high affinity.[48] Apparently, EO itself can augment basal and stimulated vascular tone and raise BP.

The demonstration that brain Ang II activates a novel long-range neurohumoral-vascular control axis that involves EO is striking. This axis amplifies the long term central effects of Ang II by recruiting CNS components (aldosterone, MRs, epithelial Na^+ channels or ENaCs, and 'brain EO')[27] and peripheral factors that include circulating EO and up-regulated expression of Ca^{2+} transport proteins in arterial myocytes.[9] Collectively, these factors contribute to the ability of chronic central Ang II and increased SNA to elevate and maintain BP. We postulate that this CNS-humoral axis is the delayed "other mechanism" that helps maintain the elevated BP when the direct vasopressor activity of circulating Ang II "plays only a minor role".[49]

The Na^+ pump is a biased receptor for EO

The physiological and pharmacological effects of the CTS have long been interpreted as the consequence of binding to a highly conserved site on the Na^+ pump catalytic (α) subunit and the block of Na^+ transport.[50] This was confirmed by studies in $\alpha 2^{R/R}$ mice[51] and mice lacking Na/Ca exchanger-1, NCX1.[52]

The groundbreaking observation that ouabain binding also activates signaling cascades added critically to the mounting evidence that ouabain is a hormone.[53] The ouabain-stimulated signal transduction is mediated by Na^+ pumps but is apparently independent of the ion transport function.[47, 54] Remarkably, recent work reveals that the ouabain binding site behaves like a "biased" receptor,[47] the first example of this phenomenon[55] in an ion transport system. Ouabain binding to arterial Na^+ pumps activates c-Src, for example, while the binding of digoxin, which is an equi-effective pump inhibitor, does not.[47] In fact, digoxin antagonizes ouabain's effects and vice-versa, both *in vivo*[56–59] and *in vitro*.[47, 60] Thus, biased signaling likely underlies the ability of ouabain and EO to induce hypertension and explains both the inability of digoxin to raise BP and its antihypertensive effect in ouabain-dependent models.[56–58]

Rostafuroxin (10 μg/kg/day), an ouabain antagonist,[60, 61] attenuates ouabain-induced hypertension in rats,[61, 62] but 5 mg/day was ineffective in unselected patients with stage I or II hypertension in the OASIS-HT trial.[63] Nevertheless, rostafuroxin effectively lowered BP in a sensitive cohort of patients with adducin variants and elevated plasma EO.[64] Importantly, rostafuroxin's affinity for its Na^+ pump binding site is relatively low: $EC_{50} \approx 1.4$ μM[60, 61] vs ouabain $EC_{50} \approx 0.5$ nM,[65] Thus, higher doses might be effective in unselected hypertensives. A new antagonist with higher affinity, that neither inhibits Na^+ transport[60, 61] nor activates signal transcription,[62] may be needed.

Role of genetics in ouabain-induced hypertension

Prolonged ouabain administration induces hypertension in many,[25, 32, 66] but not all,[7, 67] outbred rodent strains. This variable response,[7] even within a single strain,[68, 69] is neither strange nor surprising. When given high salt, excess mineralocorticoids or other hypertensinogenic substances, not all outbred rats develop hypertension; indeed, this phenotype variation was deliberately exploited to generate lines of rats with heightened or lowered susceptibility to hypertension.[70, 71] Experience with ouabain is no different. Starting from a large founding colony of outbred Sprague-Dawley rats in which high ouabain sensitivity was the dominant phenotype in both genders, minimal inbreeding led to distinct strains with ouabain-sensitive and -resistant BP phenotypes within three generations.[68, 72] The sensitive strain exhibited altered ganglionic synapse plasticity that was normalized with *in vivo* captopril.[72] Some components of the pressor mechanism of ouabain that likely function in the sensitive strain have been partially elucidated,[28, 69] whereas elevated vagal tone and increased CGRP may underlie the ouabain-resistant phenotype.[67]

Hypertension mediated by ouabain-sensitive α2 Na^+ pumps in the brain

Liddle's syndrome is a salt-sensitive hypertension due to enhanced ENaC activity caused by loss of regulation by the ubiquitin ligase, NEDD4-2.[73] A mouse model, NEDD4-2 knockout, NEDD4-$2^{-/-}$, with up-regulated renal ENaCs, exhibits mild salt-sensitive hypertension.[74] Brain ENaCs are also up-regulated, and salt-sensitive hypertension is prevented by icv infusion of very low dose benzamil, an ENaC blocker[75] that inhibits the CNS neurohumoral pathway.[9] Further, although icv Na^+-rich cerebrospinal fluid induces hypertension in wild-type and NEDD4-$2^{-/-}$ mice,[75] the hypertension is prevented by expression of ouabain-resistant α2 pumps, i.e., in $\alpha2^{R/R}$ and NEDD4-$2^{-/-}$-$\alpha2^{R/R}$ mice.[34, 75] Thus, an EO-like compound and CNS, as well as renal, ENaCs, and α2 Na^+ pumps apparently participate in the hypertension of Liddle's syndrome. This complements prior studies showing that α2 ouabain binding site integrity[76, 77] and its ligand[77–79] are essential for other forms of experimental hypertension.[28]

Paradoxical effects of EO in pregnancy and preeclampsia

Normal pregnancy is a volume expanded state in which plasma ACTH, renin, aldosterone and antidiuretic hormone (ADH) are elevated.[80] In view of the increased volume and reduced vasoreactivity in pregnancy, it is surprising that excess mineralocorticoid triggers a preeclampsia-like state in rats.[81] Further, excess ADH increase in early pregnancy may predict preeclampsia in humans.[82, 83] This suggests that fluid volume in pregnancy is more relevant than previously appreciated: circulating volumes in women destined to become preeclamptic appear to be inappropriately elevated very early in pregnancy.[84] The mechanisms by which early volume overexpansion might trigger vascular changes that lead to preeclampsia require investigation.

Circulating ouabain–like materials rise progressively in normal pregnancy, and decline after delivery.[85] The earlier reports were recently confirmed with advanced analytical methods: in addition to circulating EO, one of the newly-discovered isomers was markedly elevated in pregnancy.[8] Based on the emerging pressor mechanism of ouabain,[28] the elevated EO in pregnancy was expected to reprogram vascular function by increasing the expression of arterial myocyte Ca^{2+} transporters, e.g., NCX1 and TRCPC6. Upregulation of these proteins is triggered by the prolonged elevation of circulating ouabain in normal non-pregnant rats.[8, 86, 87] In the high EO state of pregnancy, however, expression of NCX1, which mediates Ca^{2+} influx and tone in arterial myocytes, was reduced. In other words, normal pregnancy is a high EO state with apparent resistance of the arteries to the pressor action of circulating EO. Indeed, even supra-physiological circulating levels of ouabain failed to raise BP in pregnancy.[8] The mechanism of ouabain-resistance is likely to be significant in elucidating the decline of vascular reactivity in pregnancy. Nevertheless, the low BP in pregnant $\alpha2^{R/R}$ mice indicates that the integrity of the α2 Na^+ pump ouabain binding site provides a small stimulus to BP in the 3rd trimester of pregnancy.[88]

Does elevated EO and/or EO resistance have any role in preeclampsia? Circulating EO is linearly related to BP in preeclampsia,[89, 90] suggesting that the mechanism underlying ouabain resistance is impaired so that the already elevated EO could raise BP in a dose-

dependent manner. Surprisingly, however, in pregnant rats with reduced uterine perfusion pressure and hypertension, prolonged exogenous ouabain administration (additional to the already elevated EO) lowered circulating sFLT1 (soluble fat mobilizing substance-like tyrosine kinase-1) and reduced BP.[91] Thus, in this preeclamptic model in which EO is believed to be elevated, ouabain behaved as an antihypertensive and had a net effect on BP that resembled that of digoxin in ouabain-dependent hypertension. The mechanism of this paradoxical and beneficial effect requires investigation. Nevertheless, it now appears that, contrary to earlier ideas, EO upregulation in preeclampsia is of potential benefit to mother and fetus.

At the opposite end of the pregnancy spectrum, recent studies link low circulating EO levels with impaired fetal growth and development: In pregnant mice, anti-ouabain antibodies reduced circulating EO, decreased offspring body weight, and impaired kidney and liver growth. Further, during human pregnancy, circulating EO among women with small-for-gestational age neonates was lower than in women with normal-for-gestational age newborns.[92] Ouabain is recognized as a growth promoter, but these new results are the first to suggest that relative lack of EO increases the risk for impaired fetal development. In this context, the aforementioned ouabain resistance of pregnancy makes sense: the elevated circulating EO could exert a growth promoting effect while its hypertensinogenic activity was deactivated. Further evidence that EO is a growth factor in pregnancy is that malnutrition delayed the formation of functional nephrons in the fetus and increased susceptibility to renal injury and disease later in life. Administration of ouabain to malnourished pregnant rats protected fetal kidney development.[93]

EO in kidney disease and heart failure

Acute kidney injury (AKI) is a frequent complication that increases the morbidity and mortality of cardiac surgery. EO can behave as an adrenal-derived stress hormone and has been associated with adverse cardiovascular outcomes in clinical studies. In data from two centers (626 patients), preoperative EO was the strongest predictor of surgery-induced AKI at both centers.[94] Also, the addition of preoperative plasma EO levels to an accepted clinical model for predicting AKI significantly improved predictability.[95] Further, a rat model of ouabain-induced hypertension exhibited reduced creatinine clearance, proteinuria, and impaired podocyte nephrin expression; thus, elevated EO *per se* may be a direct cause of podocyte damage.[94]

EO, which may contribute to renal failure[96] and may be linked to cardiomyopathy in chronic kidney disease,[62, 97, 98] also appears to be a valuable biomarker of heart failure. In 845 patients undergoing elective cardiac surgery, plasma EO was correlated negatively with left ventricular ejection fraction, and positively with cardiac end-diastolic diameter and plasma NT-proBNP. Higher EO levels immediately postoperatively were associated with increased 30-day perioperative mortality.[99] Thus, both pre- and post-operative EO levels identify patients with more severe cardiovascular presentation and those with a higher risk of morbidity and mortality following cardiac surgery.[99]

Conclusion

During the last five years, numerous notable advances have been made in the understanding of EO, its receptor and the downstream effects of activation of EO in the brain and periphery. While many important questions remain to be investigated, compelling evidence indicates that EO is a significant entity in physiology and contributes to the pathogenesis of many common diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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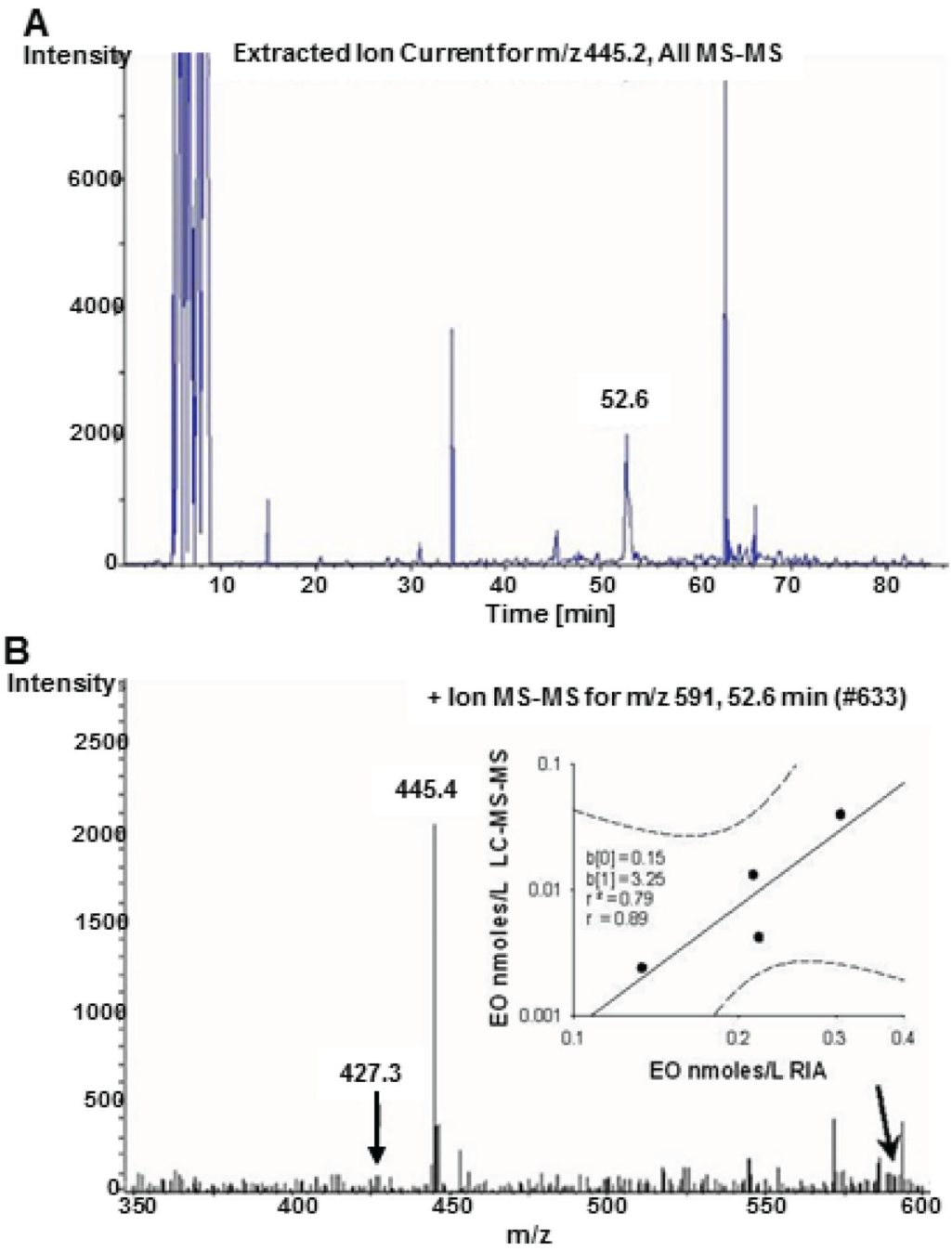

Figure 1.

Endogenous ouabain determined by LC-MS-MS in plasma from patients with cardiomyopathy. **Panel A.** Capillary LC-MS-MS product ion chromatogram for a plasma extract from a patient with cardiomyopathy. The ion current peaks represent positively charged molecular product ions with an $M+Li^+/z$ ratio equivalent to the lithiated aglycone of ouabagenin (m/z 445.4). Under the slow LC gradient conditions employed, the specific ion current peak at 52.6 minutes is the lithiated aglycone of EO and matches the retention time for EO in this system. **Panel B.** MS-MS spectrum for the ion current peak at 52.6 minutes.

Arrow points to m/z 591 which corresponds to the lithiated EO parent ion. **Inset**: Correlation (r=0.89) between plasma EO determined by RIA and LC-MS/MS from four cardiomyopathic patients. The slope of the relationship indicates that EO *per se* explained ~15% of the RIA signal in this patient group (see Online Supplement for further information). Dashed lines are the 95 % confidence interval. From Pitzalis et al.[12] with permission.