Online Supplement

The Pump, the Exchanger and Endogenous Ouabain: Signaling Mechanisms that Link Salt Retention to Hypertension

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Assay of Endogenous and Exogenous Ouabain.

Mass spectroscopy (MS) methods reveal that the endogenous ouabain (EO) isolated from human plasma has a mass of 584.2 daltons, identical to that obtained for plant-derived ouabain.[1] Advances in MS instrumentation, coupled with improved understanding of the behavior of various ion adducts of EO in the gas phase, now enable quantitation and fingerprinting of EO using small clinically relevant volumes of blood.[2] Examples of the liquid chromatography-tandem MS-MS (LC-MS-MS) of endogenous ouabain from 0.25 ml rat plasma and plant derived ouabain are shown in Supplementary Figures 1 and 2, respectively.

Inspection of the key ion current chromatograms and the MS-MS spectra prove the presence of EO in normal rat plasma and show (in this instance) that it circulates at the high end of the subnanomolar range as documented by prior RIA and bioassay methods.[3]

References

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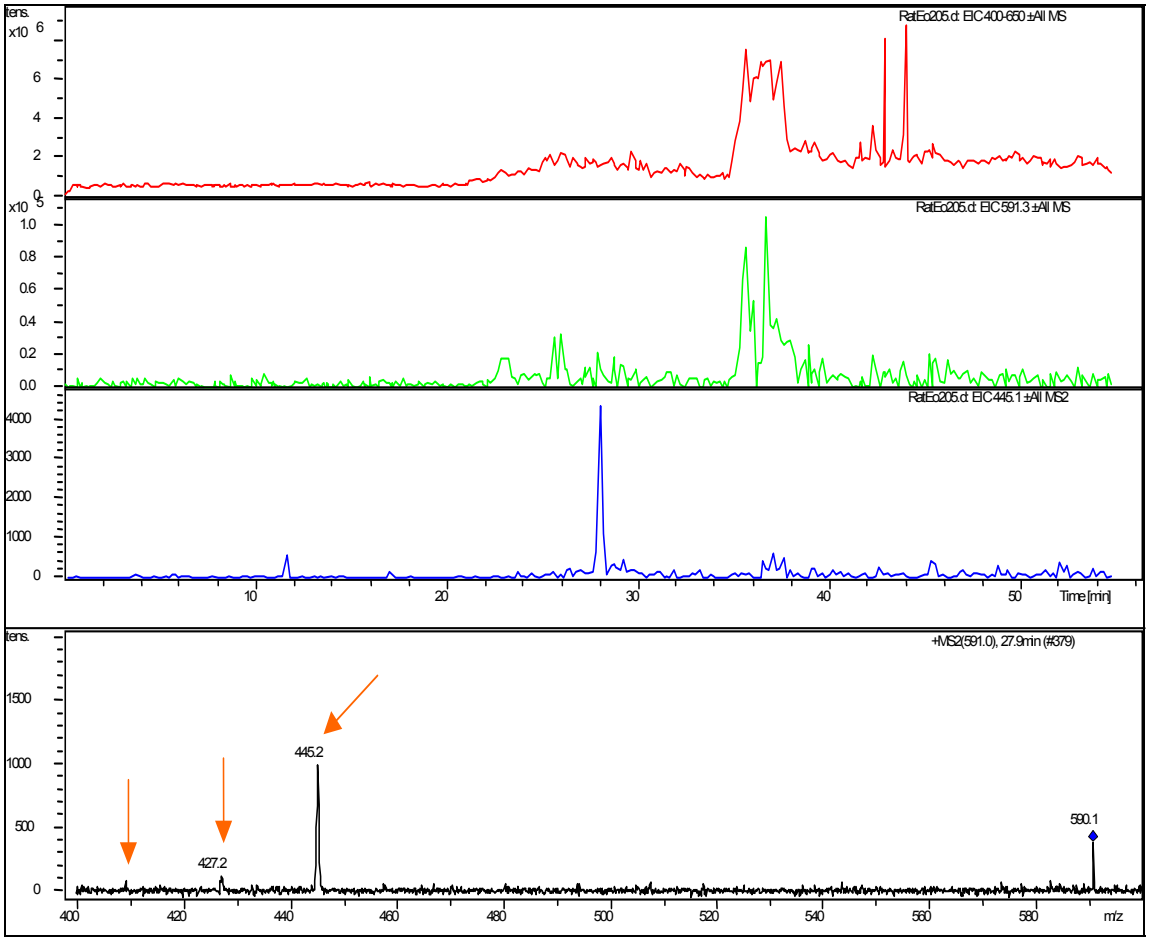
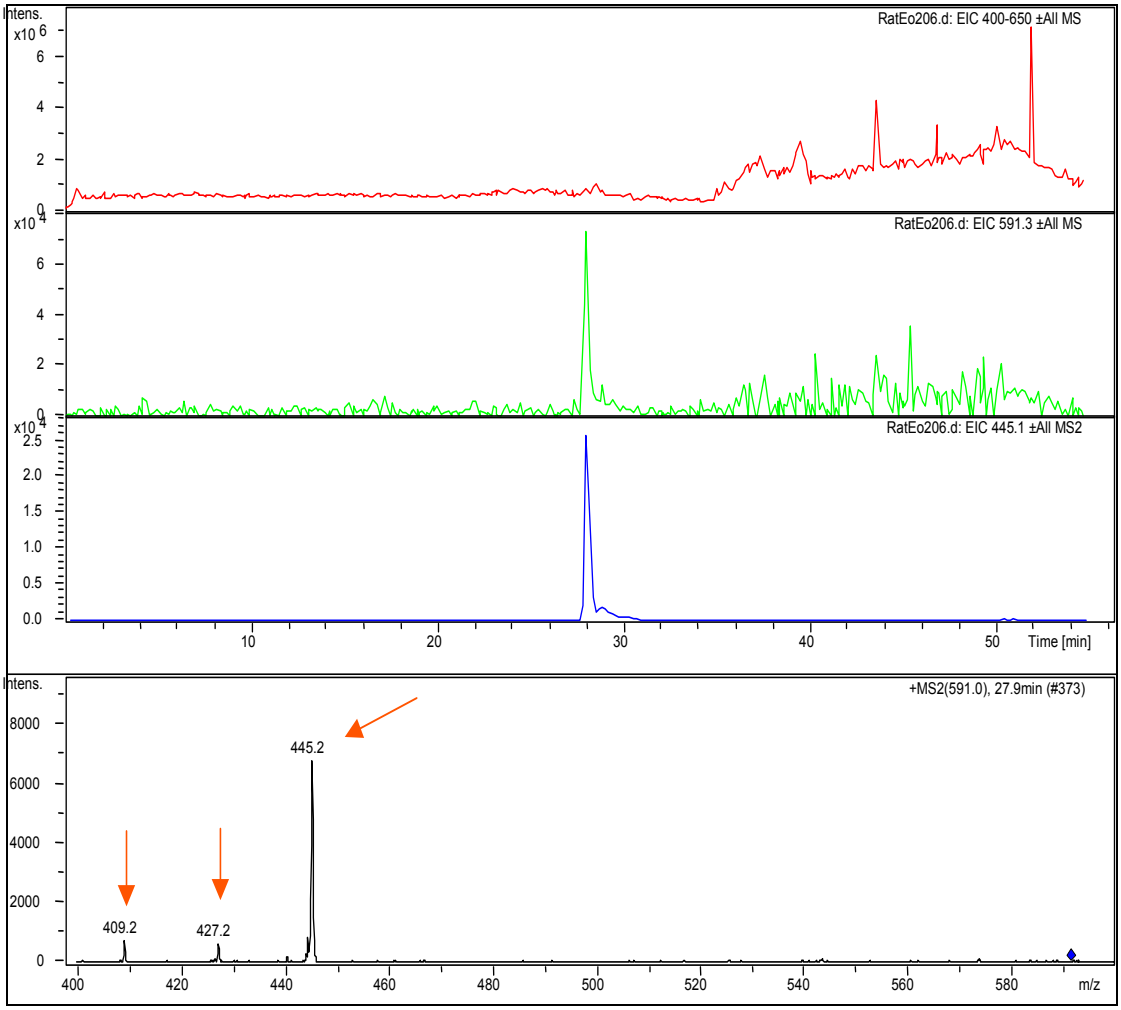
3. Ferrandi M, Manunta P, Balzan S, Hamlyn JM, Bianchi G, Ferrari P. Ouabain-like factor quantification in mammalian tissues and plasma: comparison of two independent assays. *Hypertension.* 1997;30:886-896.

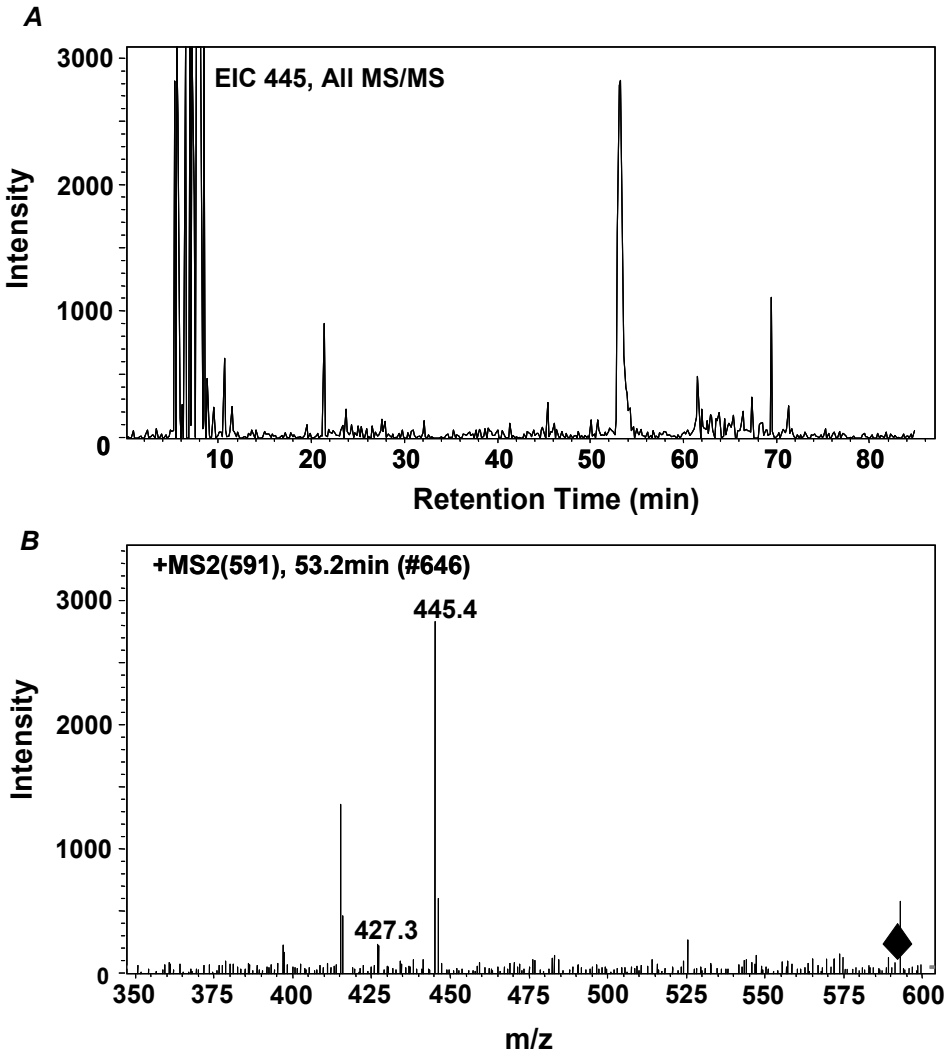


Supplementary Figure 1. Detection and Quantitation of Endogenous Ouabain in Rat Plasma by LC-MS-MS. A 10 ml of fresh rat plasma was extracted over C18 as described.[1] Following reconstitution, an aliquot corresponding to only 0.25 ml of the original plasma was injected into a capillary C-18 column attached to a liquid chromatograph (Agilent 1100) interfaced with a Bruker Esquire Ion Trap Mass Spectrometer. A solvent gradient program was used to elute the bound materials which in turn were continuously monitored for positive ion species over an abbreviated scan range (400-650 m/z). In addition, selective ion monitoring was performed for positive ions equivalent to lithiated ouabain (i.e., m/z = 591.3). The top panel (red) shows the summed MS ion chromatogram for positive ions within the scanned range (i.e., 400-650 m/z). The second panel (green) shows the extracted MS ion current chromatogram for positively charged molecular ions with m/z = 591.3 (i.e., equivalent to lithiated EO). The third panel (blue) shows the extracted MS-MS ion current chromatogram resulting from the collision induced dissociation (CID) of all ions with 591.3 m/z. Note the prominent peak eluting at 27.9 minutes; the MS-MS spectrum of that ion peak is shown in the bottom panel (black). The targeted CID of the EO parent ion at m/z 591.3 led to formation of characteristic product ions at m/z 445.2, 427.2 and 409.2 (arrows) representing the lithiated aglycone of EO

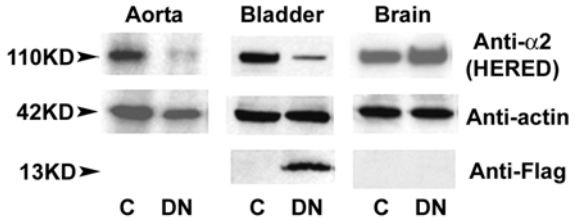
and its two dehydrated derivatives, respectively. Interpolation of the MS-MS ion current at 27.9 minutes with a standard calibration of the LC-MS-MS using ouabain under identical conditions (not shown) indicated that the EO content of the rat plasma sample was 141 pmoles/L. (Hamlyn and Manunta, unpublished).



Supplementary Figure 2. LC-MS-MS of Ouabain. Following analysis of the rat plasma sample in Supplementary Figure 1, ouabain (75 fmoles) was injected into the LC. The elution conditions, mass spectrometer settings, and ion monitoring conditions were identical to those used in Supplementary Figure 1. The top panel (red) shows the summed MS ion chromatogram for positively charged ions within the scanned range (i.e., 400-650 m/z). The second panel (green) shows the extracted MS ion current chromatogram for positively charged molecular ions with m/z = 591.3 (i.e., equivalent to lithiated ouabain). The third panel (blue) shows the extracted MS-MS ion current chromatogram resulting from the collision induced dissociation (CID) of ions with 591.3 m/z (the m/z of lithiated ouabain)-. A prominent ion current peak eluted at 27.9 minutes and the MS-MS spectrum of that ion peak shown in the bottom panel (black) reveals product ions at m/z 445.2, 427.2 and 409.2 (arrows) equivalent to the lithiated aglycone of ouabain and its singly and doubly dehydrated derivatives, respectively. (Hamlyn and Manunta, unpublished).



Supplementary Figure 3. Endogenous ouabain in human plasma determined by LC-MS-MS. *A*. LC-MS-MS of human plasma following LC separation and collision-induced dissociation of lithiated molecular ions at m/z 591 ± 0.5; the extracted ion current chromatogram for lithiated product ions was monitored at m/z 445.[3] The prominent product ion current at 53 min corresponds with the elution of the EO aglycone, ouabagenin, following dissociation of EO. *B*. Mass spectrum of the product ions eluted at 53 min. The diamond at m/z 591 corresponds to the target ion (lithiated ouabain/EO). Product molecular ions at m/z 445.2, and 427.3 correspond to the lithiated aglycone of EO and its mono-dehydrated derivative, respectively.



Supplementary Figure 4. Immunoblots of aorta and bladder smooth muscle, and brain, membranes from wild type (C) and smooth muscle-specific $\alpha 2$ dominant negative ($\alpha 2^{SM/DN}$) mice (DN). An α2(1-120)Flag construct, the N-terminal 120 amino acid residues of the $\alpha 2$ Na^+ pump, under the control of a smooth muscle myosin heavy chain promoter, was expressed in the $\alpha 2^{SM/DN}$ mice. The construct expression was detected with anti-Flag antibodies in smooth muscle (bladder; insufficient aorta protein was available), but not in brain. The Na^+ pump $\alpha 2$ subunit (detected with anti-$\alpha 2$ HERED antibodies) was down-regulated in both smooth muscles, but not in brain. Lane protein content was controlled with α-actin (Song, Chen, Kotlikoff and Blaustein, unpublished).