Structure-Activity Relationships for the Hypertensinogenic Activity of Ouabain
Role of the Sugar and Lactone Ring

Paolo Manunta, Bruce P. Hamilton, John M. Hamlyn

Abstract—Elevated levels of an endogenous ouabain circulate in many patients with essential hypertension. However, in contrast to ouabain, digoxin does not induce hypertension. This study investigated the hypothesis that within a single cardiac glycoside, the structural elements that induce hypertension differ from those responsible for high potency as a sodium pump inhibitor. Normal male Sprague-Dawley rats received infusions of vehicle (VEH), rhamnose (RHA), ouabain (OUA), ouabagenin (OGN), dihydro-ouabain (DHO), iso-ouabain (ISO), and a lactone ring opened analog (ORO) at 30 μg·kg⁻¹·24 h⁻¹ for 5 weeks via subcutaneous osmotic pumps. Cuff pressures were taken weekly. At the end of the study, trunk blood was harvested, extracted by C18 column, and subjected to high-performance liquid chromatography. Fractions were analyzed for OUA, OGN, and DHO by immunoassay. In OUA-, OGN-, and DHO-infused rats, 1 main peak of immunoreactivity corresponding to the infused agent was found. No evidence of in vivo conversion to OUA or DHO was found for any analog except ORO. At 5 weeks, systolic blood pressures in VEH, RHA, OUA, OGN, DHO, ISO, and ORO were 132±2.5, 133±1.5, 159±2.6,*, 154±4.9, 167±4,*,† 171±2.2,*,† and 169±2.4*,† mm Hg, respectively (*P<0.01 versus VEH and RHA, †P<0.05 versus OUA). The hypertensinogenic activity was greater than OUA in 3 analogs (DHO, ISO, and ORO) in which the lactone was saturated, conformationally restrained by linkage with the oxygen at C14, or opened, respectively. These compounds were weak inhibitors of dog kidney Na,K-ATPase. Thus, RHA and the unsaturated lactone ring are crucial to the high potency of OUA as an inhibitor of the sodium pump but appear to be unrelated to its ability to induce hypertension. The conclusion that this form of hypertension is mediated primarily by the steroid nucleus suggests also that OUA may have a mechanism of action independent of the sodium pump. (Hypertension. 2001;37[Part 2]:472-477.)

Key Words: sodium pump ■ blood pressure ■ glycosides ■ analogs and derivatives

The therapeutic properties of the digitalis family of cardiac glycosides have long been recognized.1 More recently, endogenous sodium transport inhibitors have been found in amphibians and mammals, and some circulate in elevated amounts in hypertensive animals and humans.2,3 A popular hypothesis has proposed that reduced sodium pump activity mediated by humoral inhibitors leads to secondary alterations of cellular calcium handling that underlie the sustained increase in arterial vascular resistance in hypertension.4–6 However, the significance of endogenous inhibitors of the sodium pump in hypertension has been controversial. The aforementioned hypothesis appears to account well for the short-term vasopressor action of digitalis glycosides and other cardiotonic steroids in humans.7 However, the hypothesis falls short in explaining why digitalis glycosides do not induce sustained high blood pressure among individuals undergoing prolonged therapy and why they may actually lower blood pressure in some patients with essential hypertension.8 Therefore, it was surprising when the prolonged administration of ouabain was found to induce sustained hypertension in the rat.9–13 Moreover, our interest was heightened further when, in agreement with the clinical impression, comparable infusions of digoxin given to rats not only failed to induce hypertension but lowered blood pressure.14 Because ouabain and digoxin have comparable potency as inhibitors of the isoforms of the rat sodium pump,15 we suspected that the long-term pressor activity of ouabain might be independent of its ability to inhibit the sodium pump (Na,K-ATPase). Because the sugar and integrity of the lactone ring have quantitative contributions to the high potency of ouabain as an inhibitor of Na,K-ATPase,16 we investigated a series of natural and synthetic analogs of ouabain for their effects on long-term blood pressure in normal rats. In each analog, the integrity of the steroid nucleus was maintained so the hemo-
were euthanized indicated that all pumps functioned normally. The term “open-ring ouabain” (ORO) refers to a mixture of the aldehyde and hydroxy acid forms were synthesized from ouabain (ORO) consisting of a mixture of ouabain aldehyde acid and ouabain hydroxy acid (Figure 1) in these studies. Each compound was prepared in phosphate-buffered saline (pH 7.8) and delivered via miniosmotic pumps (ALZET 2002, ALZA Corp) that were placed subcutaneously in the flank of the animal under halothane anesthesia.

Methods
Male Sprague-Dawley rats (7 to 8 weeks old) were from Zivic Miller (Zelienople, Pa). They were maintained in an air-conditioned facility at constant temperature (23°C) on a normal rat chow diet (containing 0.5% wt/wt sodium and 1.1% wt/wt potassium) and water ad libitum. All experimental protocols described using animals in this study were approved by the Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines.

We used rhamnose (RHA), ouabain (OUA), ouabagenin (OGN), dihydro-ouabain (DHO), iso-ouabain (ISO), and an open-ring form of ouabain (ORO) consisting of a mixture of ouabain aldehyde acid and ouabain hydroxy acid (Figure 1) in these studies. Each compound was prepared in phosphate-buffered saline (pH 7.8) and delivered via miniosmotic pumps (ALZET 2002, ALZA Corp) that were placed subcutaneously in the flank of the animal under halothane anesthesia. Continuous infusions were made for 5 weeks at a rate of 30 μg·kg⁻¹·24 h⁻¹. Ouabain given at this infusion rate produces a maximal increase in blood pressure under our conditions. Control animals received vehicle infusions only. The pumps were replaced every 14 days under halothane anesthesia. Inspection of the residual infusate during replacement or at the time the animals were euthanized indicated that all pumps functioned normally.

Blood Pressure Measurements
For direct measurements of mean arterial pressure, rats were anesthetized (ketamine/xylazine 90%/10% IM) and fitted with femoral arterial catheters as described previously. After a 48- to 72-hour recovery period, direct mean arterial pressures were recorded from conscious unrestrained animals resting in their home cages. Blood pressure and heart rate were recorded on a Gould polygraph. Body weight in all the animals was measured weekly.

Indirect systolic blood pressures (SBPs) were recorded weekly by tail plethysmography with a commercial photoelectric system and a device that provided constant rates of cuff inflation and deflation (model 29, IITC Inc). In this procedure, conscious rats were restrained in acrylic animal holders for 3 to 10 minutes in a warm quiet room and conditioned to numerous cuff inflation/deflation cycles by a trained operator before data collection. Subsequently, mean values for SBP and heart rate were obtained for each rat from 4 to 6 sequential cuff inflation/deflation cycles. The onset of oscillations during cuff deflation was taken as the SBP.

Sample Collection
At the end of the study, the rats were fasted overnight, and the following morning they were weighed, lightly anesthetized with halothane, and killed by decapitation. Trunk blood was collected into heparinized containers and centrifuged, and the plasma was frozen at -20°C for assay.

Steroid Assays
For OUA and DHO, 5 mL of thawed plasma was mixed with 5 mL of H₂O, and its pH was carefully adjusted to 7.8 with 1.0 mol/L acetic acid. For measurement of plasma OGN, 5 mL of plasma was mixed with 5 mL of H₂O containing 0.1% redistilled trifluoroacetic acid (TFA). Plasma extracts were passed over prewashed 200-mg octadecylsilane (C18) disposable Bond Elut columns (Analytichem International). Unbound materials were eluted with 3×4-mL water washes followed by 4 mL of H₂O containing 2.5% acetonitrile. OUA and DHO were eluted with 4 mL of H₂O containing 20% acetonitrile and 0.1% TFA (vol/vol). OGN was eluted with 4 mL of H₂O containing 25% acetonitrile and 0.1% TFA (vol/vol). In each case, the eluates were vacuum dried, reconstituted, and subjected to reverse-phase chromatography on C18 in an acid-free (OUA, DHO) or acidic (OGN) mobile phase (0.1% TFA). Fractions were collected, dried, and reconstituted in the appropriate immunoassay buffer. OUA and OGN were measured by radioimmunoassay with a rabbit polyclonal OUA antiserum (No. R8) of high titer (1:10⁴) described previously. The differential cross-reactivity of the antiserum for OGN (40%) was used to calculate its concentration. The OUA antiserum has no significant cross-reactivity (<0.01%) for the common adrenal, testicular, and ovarian steroids. The interassay and intra-assay coefficients of variation for the OUA immunoassay in these experiments were 5.9% and 11.4%, respectively. DHO was measured with an ELISA similar to that described previously for OUA. Instead, a rabbit polyclonal antiserum (R10) of high titer (1:10⁵) for DHO was used. The antiserum was generated against DHO conjugates by a sequential immunization protocol. The differential cross-reactivity of the antiserum for OUA and OGN was 0.15% and 2%, respectively. The common adrenal, ovarian, and testicular steroids gave <0.05% cross-reactivity.

Na,K-ATPase Assay
Measurements of ATPase activity were made at 37°C in 1 mL of an ATP regenerating cocktail whose activity was followed optically by the oxidation of NADH (ε = 6220 at 340 nm). The final assay mixture contained (in mmol/L or as appropriate) 20 KCl, 100 NaCl, 6 MgSO₄, 5 EGTA, 3 ATP-Na₂, 2 phosphoenolpyruvate-monocylohexylammonium, 0.3 NADH, 100 TES-Tris (pH 7.8), lactate dehydrogenase 11 IU, and pyruvate kinase 12 IU. ATPase activity was determined continuously by following the decrease in absorbance at 340 nm with a Beckman Du8b spectrophotometer. Under optimal conditions, ~98% of the total ATPase activity (measured specific activity ~4 μmol·min⁻¹·mg⁻¹) was inhibited by 10⁻³ mol/L OUA. The maximal capacity of the regenerating system was >4200 times the maximal ATPase activity.
For assay, dog kidney Na,K-ATPase was suspended at $\sim$1.8 mg/mL in 10 mmol/L TES-Tris (pH 7.8) containing 5 mmol/L MgCl$_2$ and 5 mmol/L Tris-phosphate. Each OUA analog was preincubated with the ATPase at the indicated concentrations for 2 hours at 37°C. Subsequently, 10-$\mu$L aliquots were added to the regenerating assay containing the same concentration of the analog. The change in absorbance was used to determine the steady-state rates of NADH oxidation.

**Statistical Analysis**

All data are expressed as mean±SEM. Statistical significance was determined with a 2-tailed test comparing the means of independent sample groups. ANOVA was used for comparisons among groups, and repeated-measures ANOVA was used for within-group comparisons. The Tukey multiple range test was used to determine the significance of the F ratio. The level of significance was $P<0.05$; statistics were computed by Systat.

**Materials**

Ouabain octahydrate, OGN, and DHO were from Sigma Chemical Company. ISO and ORO were synthesized by previously published methods.$^{21,22}$ Other reagents were American Chemical Society grade. The structures of ISO and ORO (Figure 1) were determined by UV absorbency spectra (Beckman Du8B) and their 600-MHz $^1$H nuclear magnetic resonance (NMR) spectra (Bruker DMX-600).

**Results**

**Structural Analyses of ISO and ORO**

The UV spectra for ORO showed a broad absorbance that declined slowly from 200 to 260 nm, with no peak at 220 nm. The UV spectrum for ISO was narrower, declining from 200 to 230 nm, with no peak at 220 nm (not shown). The majority of the $^1$H NMR resonances (25°C in D$_2$O) and hence the overall spectra for ISO and ORO were generally similar to those produced by OUA. In ISO, the characteristic resonance ($\delta=6.05$ ppm) of the single $^1$H at position 22 was absent, consistent with the UV spectra and indicating full saturation of the lactone. The $^1$H resonance at position 21 was shifted, consistent with tethering of the lactone ring to the oxygen at position 14.

The 1-dimensional $^1$H NMR spectra of ORO suggested a mixture, and 2-dimensional $^1$H-$^1$H total correlation spectroscopy (TOCSY) spectra (not shown) revealed the aldehyde and hydroxy acids.$^{21}$ The aldehyde acid was detected by a $^1$H resonance ($\delta=9.67$ ppm) that showed nuclear Overhauser enhancements to $^1$H assigned at C20, 22(2H) and 17 ($\delta=2.74$, 2.47, 2.31, and 1.48 ppm, respectively). The hydroxy acid was detected by a single $^1$H resonance at position 22 ($\delta=6.03$) with nuclear Overhauser enhancements to $^1$H assigned to positions 21(2H) and 17 ($\delta=5.07$, 4.95, and 3.13 ppm, respectively). In both the hydroxy and aldehyde analogs, the C18 methyl proton resonance was shifted ($\delta=1.1$ and 1.15 ppm, respectively) compared with OUA ($\delta=0.85$ ppm), consistent with reduced shielding due to hydrogen bonding with oxygen on position 21 of the open lactone ring. Proton integration studies suggested that the ratio of the hydroxy and aldehyde acids was $\sim$3:1.

**Effects on Blood Pressure**

Figure 2 shows the effects of prolonged infusion of the various OUA analogs used on blood pressure over the 5-week period. SBP rose progressively in each group of steroid-infused rats and reached a plateau by day 28. The rise in SBP was considerably faster and more robust in rats infused with ISO and DHO. SBPs in the vehicle or RHA-infused groups did not change significantly over the 5-week study. Figure 2 also shows mean intra-arterial pressure in each group just before animals were euthanized. Relative to the vehicle controls, mean arterial blood pressure was significantly greater in all groups of steroid-infused rats.

Throughout the various experiments, the body weights of the animals in the different groups were not significantly different: vehicle, 454±6 g; RHA, 458±12g; OUA, 471±11 g; OGN, 474±9 g; DHO, 469±9 g; ISO, 474±8 g; and ORO, 477±6 g. All animals appeared healthy, and no differences in behavior were observed. The heart rates of the rats in each of the groups were similar throughout the study; heart rates on day 35 in vehicle, RHA, OUA, OGN, DHO, ISO, and ORO groups were 336±8, 344±11, 329±7, 327±7, 319±11, 320±7, and 322±6 bpm, respectively.

**Plasma Steroid Levels**

Figure 3 shows the approach and results for the reverse-phase chromatography of the plasma. In the plasma from vehicle controls, only small amounts of immunoreactive endogenous OUA (EO) and its dihydro analog (EDHO) were found when acid-free conditions for extraction and HPLC were used (Figure 3A). Under acidic conditions (Figure 3C), EO and EDHO could be readily resolved as peaks at 20 and 18 minutes, respectively, and measured. Therefore, the use of acid-free conditions for these studies enabled us to avoid ex vivo relactonization of the hydroxyouabain component of...
ORO, and it was possible to measure the infused DHO (18 minutes) and OUA (20 minutes) without interference from their endogenous counterparts (Figure 3C). However, under acid-free conditions, unexpectedly severe and variable tailing of OGN was found in our HPLC system (Figure 3B). Thus, acidic extraction and HPLC conditions were necessary for the measurement of this genin (Figure 3D).

The Table presents the plasma levels of OUA, OGN, and DHO in trunk blood from each of the infused groups at euthanization. The plasma levels for the rats infused with OUA, OGN, and DHO were similar and typically 6- to 8-fold above the level of EO found in the group infused with OGN. No measurements of ORO or ISO were made. As noted above, EO and EDHO were observed in significant amounts only in the samples from the OGN-infused rats because of the extraction conditions used.

Potency of OUA Analogs and Digoxin as Na,K-ATPase Inhibitors

The concentration-activity curves for inhibition of Na,K-ATPase by the analogs are shown in Figure 4. In each case, the data were well described ($r^2$.0.9) by a single class of inhibitory binding sites, with Hill coefficients ranging from 0.93 to 1.05. The most potent compound tested was OUA, with an apparent EC50 of 4.8 nmol/L. The corresponding EC 50 values for digoxin, OGN, DHO, ORO, and ISO were 7.86, 42, 423, 812, and 2023 nmol/L, respectively. The numerical values for the rank order for potency normalized as a percentage to OUA were 100%, 61%, 11.4%, 1.1%, 0.6%, and 0.24%, respectively. RHA showed no inhibitory activity in this assay system.

Figure 5 shows the relationship between the EC 50 values for the various OUA analogs and digoxin as inhibitors of Na,K-ATPase versus the change in SBP. A positive correlation was found, indicating that the weaker inhibitors of Na,K-ATPase were more effective at raising SBP. The change in SBP for digoxin was from a previous study.23

Discussion

The major new results of the present study include the following observations: First, the prolonged subcutaneous infusion of OUA and a series of OUA analogs all induced the progressive development of hypertension in normal rats. Significantly, the aglycone, OGN, was equally effective in raising blood pressure as OUA itself, as was DHO, in which the sugar is retained but the lactone ring is saturated. In addition, 2 analogs of OUA in which the lactone ring was either opened or unsaturated and tethered to the oxygen at position 14 were highly effective in raising blood pressure.

Figure 3. Reverse-phase HPLC of rat plasma. Chromatography was performed on a Beckman 342 with a 5-µm 10×250-mm Ultrasphere ODS column and general solvent, flow, and gradient conditions as noted previously.19 A, Elution of OUA immunoreactivity under acid-free conditions. B, Elution of OGN (♦), DHO (◊), and OUA (•) from steroid-infused rats under acid-free conditions. C, Elution of EDHO (♦) and EO (◊) in the presence of 0.1% TFA. D, Elution of OGN (♦), EDHO (♦), and EO (◊) in the presence of 0.1% TFA. In A, C, and D, 5 mL equivalents of extracted plasma were injected. In B, 5 mL equivalents of the extract from each infusion group were combined. Note the different scaling of the y axes.

Figure 4. Inhibition of Na,K-ATPase by digoxin, OUA, and related analogs. Each point is the mean of triplicate values. Data were fit by computerized nonlinear regression (solid line) to the Hill equation.

OUA, OGN, and DHO were similar and typically 6- to 8-fold above the level of EO found in the group infused with OGN. No measurements of ORO or ISO were made. As noted above, EO and EDHO were observed in significant amounts only in the samples from the OGN-infused rats because of the extraction conditions used.

Plasma Steroid Levels in Rats Infused With OUA and Related Compounds

<table>
<thead>
<tr>
<th>Infusion Group</th>
<th>Steroid Measured, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>OUA &lt;0.05, OGN &lt;0.05, DHO &lt;0.05</td>
</tr>
<tr>
<td>RHA</td>
<td>OUA &lt;0.05, OGN &lt;0.05, DHO &lt;0.05</td>
</tr>
<tr>
<td>OUA</td>
<td>4.6±0.7†, OGN &lt;0.05, DHO &lt;0.05</td>
</tr>
<tr>
<td>OGN§</td>
<td>0.65±0.26§, OGN &lt;0.05, DHO 4.9±0.6†</td>
</tr>
<tr>
<td>DHO</td>
<td>OUA &lt;0.05, OGN &lt;0.05, DHO 5.4±1.2†</td>
</tr>
<tr>
<td>ISO</td>
<td>OUA &lt;0.05, OGN &lt;0.05, DHO &lt;0.05</td>
</tr>
<tr>
<td>ORO</td>
<td>0.54±0.27*, OUA &lt;0.05, OGN &lt;0.05</td>
</tr>
</tbody>
</table>

All measurements were made after acid-free extraction and HPLC with specific immunoassays unless noted. §Sample extraction and chromatography performed in the presence of 0.1% TFA. In this group, the concentrations reported for OUA and DHO reflect their endogenous counterparts ordinarily not seen in acid-free methods. Thus, the plasma levels of OUA and DHO after infusion of the other OUA analogs do not include their endogenous components.

*P<0.05 vs vehicle, †P<0.01 vs vehicle controls. All n=8.
Second, the various analogs used covered a >400-fold range in terms of potency as inhibitors of Na,K-ATPase. However, their ability to induce hypertension was similar and was not related to their potency as Na,K-ATPase inhibitors.

Taken together, the results show that the structural elements (ie, the sugar and unsaturated lactone ring) that affect the inhibitory potency of OUA on Na,K-ATPase are not crucial to its effects on long-term SBP or mean blood pressure. Therefore, it appears that the steroid nucleus accounts for the long-term hemodynamic effects of OUA in the rat.

Secretion of a putative endogenous dihydro-ouabain (EDHO) has been described recently from adrenocortical cells. Using a specific immunoassay, we detected this material and EO in rat plasma but only after extraction and HPLC under acidic conditions. This reveals that circulating EO and EDHO differ in some way from their isolated forms, and one possibility is that they may circulate as their hydroxy acids, which would be very weak inhibitors of Na,K-ATPase. Overall, our results challenge the hypothesis that inhibition of the sodium pump is the initial event that leads to a sustained rise in blood pressure.

Elevated circulating levels of EO and OUA have been shown to correlate with blood pressure in human and rat studies. Renal vascular resistance is increased and renal function is reset in rats with OUA-dependent hypertension, events that may be mediated by central nervous system mechanisms. Therefore, our observations concerning the relationship and specificity of OUA-like steroids with the low nanomolar concentrations in the circulation, makes it unlikely that OUA interacts with classic steroid receptors. Further studies will be needed to determine the features in the steroid nucleus of OUA that underlie its effects on long-term blood pressure.

The fourth possibility is that the dichotomy is only an apparent one. For example, the rank order of analogs from the dog kidney Na,K-ATPase studies might not apply to the rat. There is general agreement that the rat α-1 isoform is highly OUA resistant, so the rank issue concerns primarily the α-2 and α-3 isoforms. The response of the latter enzymes to the analogs used is not known. However, studies of the structure-activity relationships for inhibition of Na,K-ATPase by large numbers of cardiotonic steroids suggest that a similar macroscopic pattern applies generally across different species.
and tissues in spite of large differences in affinities. Therefore, the rank order pattern for the OUA analogs observed with the dog enzyme is likely to apply, at least in a broad way, to each of the rat isoforms. In addition, and consistent with this view, similar inflections in the dose-inhibition profiles were generated in response to OUA and digoxin in rat brain membranes, in which 3 of the Na,K-ATPase isoforms are present. Thus, there is no indication of any significant preference by any single isoform that might explain the opposite effects of OUA and digoxin on blood pressure in the rat.

In summary, OUA and a series of structurally related analogs all induced sustained hypertension in normal rats. The observation that the hypertensinogenic activity of the analogs was independent of their potency as inhibitors of the dog kidney Na,K-ATPase raises the possibility that sodium pumps may not be the initial target in the mechanism by which OUA induces sustained increases in blood pressure. Rather, the results presented suggest that the hypertensinogenic activity of the analogs arises from a novel mechanism linked with the steroid nucleus. Moreover, the rat appears to be an especially informative model in which to explore the mechanism.

Acknowledgments

This study was supported in part by a fellowship from the American-Italian Society of Nephrologists (P.M.), the Veterans Administration (B.P.H.), the American Heart Association, and the National Institutes of Health (J.M.H.).

References

Structure-Activity Relationships for the Hypertensinogenic Activity of Ouabain: Role of the Sugar and Lactone Ring
Paolo Manunta, Bruce P. Hamilton and John M. Hamlyn

Hypertension. 2001;37:472-477
doi: 10.1161/01.HYP.37.2.472

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/37/2/472

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/