**Neurogenic Component of Ouabain-Evoked Contractions Is Modulated by the Endothelium**

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**Abstract**

The influence of endothelium on the neurogenic component of ouabain-induced contractions in isolated perfused guinea pig carotid arteries was analyzed. Ouabain (0.1 μmol/L to 0.1 mmol/L) evoked concentration-dependent increases of perfusion pressure. Phentolamine (0.3 to 10 μmol/L) and prazosin (30 nmol/L to 10 μmol/L) (nonselective antagonist of α-adrenergic receptors and selective antagonist of α₁-adrenergic receptors, respectively) induced a concentration-dependent relaxation in segments precontracted with ouabain (0.1 mmol/L). When the arteries were preincubated with those blockers (both at 3 μmol/L) or the animals were pretreated with reserpine, the contractions to the glycoside were diminished, indicating that they are partially mediated by norepinephrine release from adrenergic nerve endings. Deendothelialization abolished the effect of adrenergic blockade on ouabain-induced contractions. On the other hand, deendothelialization did not modify significantly the effect of the neurogenic blockade on norepinephrine-induced contractions. The nitric oxide blocker oxyhemoglobin, at concentrations (10 μmol/L) that abolished endothelium-dependent relaxations induced by 3 μmol/L acetylcholine, or the cyclooxygenase blocker indomethacin (10 μmol/L) did not modify the relaxation caused by phentolamine. In bioassay experiments, 30 μmol/L phentolamine induced a relaxation on the ouabain-elicited contraction only when the glycoside was added through a donor segment with endothelium. Ouabain-induced triitated norepinephrine release was significantly reduced by the removal of endothelium but not by 1 μmol/L oxyhemoglobin or 1 μmol/L indomethacin. These results suggest that the endothelium modulates the neurogenic component involved in contractions evoked by the glycoside by a diffusible factor (or factors) whose nature is unknown; however, the factor is neither nitric oxide nor a cyclooxygenase-related compound.

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**Key Words**
- ouabain
- endothelium
- norepinephrine
- guinea pigs
- carotid arteries

Both vascular endothelium and the sodium pump have been involved in the pathogenesis of hypertension. Recently, ouabain has been proposed to be the endogenous sodium pump inhibitor associated with certain forms of hypertension. It is well known that ouabain may cause vasoconstriction by acting directly on the Na⁺,K⁺-ATPase in vascular smooth muscle (myogenic component) or by releasing norepinephrine from the perivascular sympathetic nerve endings (neurogenic component). The predominant component depends on the animal and the kind of vessels studied. In addition, ouabain may interfere with endothelium-dependent relaxations in several species, including humans, by means of interference with the endothelium-derived hyperpolarizing factor (EDHF), nitric oxide (NO) release, or NO effector mechanisms.

In previous works, we have reported that the endothelium modulates the vasoactive responses elicited by sodium pump blockade by reducing the myogenic component of the ouabain-induced contractions. This effect is clearly observed in human placental vessels, which lack autonomic innervation; but in guinea pig carotid arteries it becomes evident only after the blockade of adrenergic neurotransmission with α-adrenergic receptor antagonists or pretreatment of animals with reserpine. These latter results suggested that the endothelium also could have a positive influence on the neurogenic component of ouabain-evoked responses.

The ability of the endothelium to modulate the vascular response induced by sympathetic nerve activity already has been proposed; thus, endothelium removal increases contractions caused by nerve stimulation. However, to our knowledge there are no studies analyzing whether the norepinephrine release caused by sodium pump inhibition can be affected by the endothelium. Therefore, we undertook the present work to test the possibility of an endothelial mediation of the neurogenic component of ouabain-induced contractions, facilitating the release of norepinephrine induced by the glycoside from the perivascular nerve endings.

**Methods**

**Perfusion of Arterial Segments**

Guinea pigs of either sex weighing approximately 500 g (Dunkin-Hartley, Iffa-Credo, Domain des Oncins, France; housed at the facilities of Facultad de Medicina de la UAM, Madrid, Spain) were anesthetized with 35 mg/kg IP sodium pentobarbital and killed by bleeding. The carotid arteries were dissected into segments 1.5 to 2 cm long. Perfusion of the vascular segments was performed according to the procedure described by De La Lande and Rand, with minor modifications. Both ends of the segments were cannulated with stainless steel tubes (0.4 mm outer diameter), which were fixed, allowing the vessel to stay at the previously measured in situ length. The cannulated segments were introduced into an organ bath containing 25 mL Krebs-Henseleit solution (KHS) at 37°C continuously bubbled with a 95% O₂-5% CO₂ mixture that gave a pH of 7.4. The segments were perfused with KHS
by means of a peristaltic pump (HP-8, Gilson Medical Electronics, Villiers le Bel, France) connected to a silicone circuit, both the arterial and venous segments were immersed in a reservoir containing 22 mL oxygenated KHS at 37°C, allowing the recirculation of the perfusate. The circulating volume of KHS was 7 mL. We tested the unique point in the circuit with significant resistance to flow was the arterial segment; thus, the basal pressure of the system was related to the vascular tone, and contractions or dilations of the arterial segment produced increases or reductions of the perfusion pressure, respectively. Drugs were added to the reservoir and therefore infused and recirculated throughout the experiment.

Perfusion pressure was measured by means of a polyethylene catheter (0.5 mm outer diameter) placed in the circuit next to the proximal end of the vessel, which allows for the retrograde transmission of pressure. This catheter was connected to a transducer (P23XL, Gould Instruments, Oxnard, Calif), and pressure was registered on a polygraph (4000, Letica, Barcelona, Spain). The recording system was calibrated daily with a mercury manometer.

Preliminary experiments were performed to establish the optimal perfusion pressure needed for the vasoactive agents to elicit maximal responses. For this, the segments were subjected to different flow rates ranging from 5 to 15 mL/min, which caused basal perfusion pressures from 5 to 20 mm Hg. When a concentration-response curve to 0.1 to 30 μmol/L norepinephrine in the perfusate was plotted, the higher responses were observed at a basal pressure around 15 mm Hg, which corresponds to a flow rate of approximately 12 mL/min. Therefore, in the rest of the experiments the vessels were submitted to this perfusion pressure for a 60-minute equilibration period, and viability of the segments was systematically checked by addition of 30 μmol/L norepinephrine to the perfusate. When contraction of the vascular segments reached a stable plateau, 3 μmol/L acetylcholine was administered. The presence of functional endothelium was verified when acetylcholine reduced norepinephrine-induced tone by at least 30%. After a washout period, the drugs used in the present study were added to the perfusate. The vascular endothelium was removed from some segments by gentle rubbing or infusion of saponin (see below) for analysis of the influence of endothelium in drug-induced responses.

Bioassay Technique

These experiments were performed according to the procedure described by Rubanyi et al.7 Segments (1.5 to 2 cm) of guinea pig carotid arteries were cut under a dissecting microscope and placed in an organ bath containing 10 mL oxygenated KHS at 37°C. These segments were considered the donors and were perfused with KHS by means of a peristaltic pump (Eyela, Tokyo Rikakikai Co, Tokyo, Japan) at a constant rate of 2 mL/min. A stainless steel tube through which KHS was pumped at the same rate was also placed in the organ bath. A ring of guinea pig carotid artery, from which the endothelium had been removed (bioassay ring), was suspended immediately below the organ chamber by means of two steel stirrups passed through its lumen. One stirrup was bound to an isometric force transducer (Panlab, Barcelona, Spain) connected to a polygraph (Houston Instruments, Gistel, Belgium). The assembly of bioassay ring, stirrups, and force transducer could be moved freely below the organ chamber, allowing the preparation to be superfused with the perfusate from either the vascular segment or the stainless steel tube.

A resting tension of 1 g (optimal resting tone determined by previous assays with 75 mmol/L K+ was applied to the bioassay ring, which was superfused with KHS passed through the stainless steel tube (direct superfusion) for a 90-minute equilibration period. During this time the resting tension was readjusted every 15 minutes until the tone became stable. After this period the bioassay ring was moved to below the outlet from the donor segment. To check the viability of the preparation, we added 10 μmol/L norepinephrine to the perfusate, and when the contraction reached a stable plateau, we administered 10 μmol/L acetylcholine through the donor segment or directly to the ring to verify the presence and absence of endothelium in the donor segment and bioassay ring, respectively. During the experiments, drugs could be added through the donor segment or directly to the bioassay ring.

Tritiated Norepinephrine Release

Carotid guinea pig arteries were cleaned of blood traces and divided into segments of similar length (0.5 cm long), which were pooled and separated into groups of similar weight. The endothelium of one group of vessels was removed. Each group was tied to a rigid nylon fiber and immersed for 15 minutes (equilibration period) in 5 mL oxygenated KHS at 37°C. Later, the vessels were incubated for 60 minutes in 5 mL KHS with tritiated norepinephrine ([3H]norepinephrine) (0.2 μmol/L; specific activity, 9 Ci/mmol). Afterwards, the tissues were washed for 100 minutes by their successive introduction into 10 vials with 5 mL KHS at 10-minute intervals so that the spontaneous tritium release could reach a steady-state level. Subsequently, the vessels were immersed into vials with 3 mL KHS containing 0.1 mmol/L ouabain or 75 mmol/L K+.

Aliquots of 0.5 mL from each vial were collected and added to 2 mL of liquid scintillating cocktail (Ready Solv HP Beckman, Beckman Instruments, Fullerton, Calif), and radioactivity was measured in a liquid scintillation counter (Beckman LS 2800). Ouabain- or K+-induced tritium release was calculated as the percentage of basal release (100%). The interference of drugs on this evoked release was analyzed by their addition to the incubation medium 10 minutes before.

Removal of Vascular Endothelium

Deendothelialization was done by gentle rubbing or intraluminal perfusion of 0.3 mg/mL saponin in KHS for 1 minute.28 The latter procedure produced complete endothelial denudation without damage to vascular contractility, as documented by the maintenance of vasoactive responses to 75 mmol/L K+ or 0.1 to 30 μmol/L norepinephrine. Deendothelialization was also tested in some cases by histological methods.29 No differences were observed between vessels denuded by mechanical and chemical procedures.

Solutions, Drugs, and Statistical Evaluations

The millimolar composition of KHS was NaCl, 119; KCl, 4.6; CaCl2·2H2O; MgSO4·7H2O, 1.2; NaHCO3, 25; glucose, 11.1; and Na2EDTA, 0.03.

Distilled water was used to dissolve drugs, except yohimbine, indomethacin, and norepinephrine, which were dissolved in ethanol (99.5%), distilled water with 1.5 mmol/L Na2SO4, and saline-ascorbic (0.9% NaCl and 0.1% ascorbic acid, wt/vol) solutions, respectively. Stock solutions were kept at −20°C. Ouabain and prazosin solutions were protected from light. Reserpine (3 mg/kg, total dose) was administered intraperitoneally to the guinea pigs 48 and 24 hours before the experiment. Control and experimental responses were obtained from separate vascular preparations, because ouabain was not removed from the vessels after repeated washout periods. Oxyhemoglobin was prepared by reduction of commercial bovine hemoglobin with sodium dithionite, which was subsequently dialyzed. Concentration of oxyhemoglobin was determined spectrophotometrically.30

Acetylcholine chloride, bovine hemoglobin, norepinephrine hydrochloride, reserpine, saponin, yohimbine chloride, and ouabain octahydrate were from Sigma Chemical Co, St Louis, Mo; indomethacin was from Merck, Darmstadt, Germany; phen tolamine mesylate was from CIBA-GEIGY, Basel, Switzerland; prazosin hydrochloride was from Pfizer Inc, New York, NY; and [3H]norepinephrine chloride was from New England Nuclear, Boston, Mass. Results are expressed as mean ± SEM. Deviations from the mean were statistically studied using ANOVA and
Results

Reactivity Experiments in Perfused Segments

Cumulative concentrations of ouabain (0.1 μmol/L to 0.1 mmol/L) added to the perfusate at intervals of 15 minutes induced concentration-dependent increases in perfusion pressure in guinea pig carotid arteries up to 22±1.6 mm Hg. Endothelium removal did not modify the contraction induced by ouabain (results not shown).

In segments precontracted with 0.1 mmol/L ouabain, the administration of cumulative concentrations of the nonselective α-adrenergic antagonist phentolamine (3 nmol/L to 10 μmol/L) induced a concentration-dependent reduction of the perfusion pressure; these relaxant responses were abolished by removal of the endothelium (Fig 1). The same concentrations of the selective α1-antagonist prazosin also produced relaxant responses (Fig 2), whereas the α2-antagonist yohimbine (10 nmol/L to 100 μmol/L) did not modify the response elicited by the glycoside (results not shown). Preincubations of the vascular segments for 10 minutes with phentolamine or prazosin (both at 3 μmol/L) or reserpinition of the guinea pigs markedly reduced the pressure increases evoked by ouabain only when the endothelium was present (Fig 3). Nevertheless, the contractions induced by cumulative concentrations of norepinephrine (0.1 to 30 μmol/L) were antagonized by both phentolamine (1 and 3 μmol/L) and prazosin (0.1 and 1 μmol/L) in segments with and without endothelium (Fig 4).

To evaluate the role of NO- or cyclooxygenase-related compounds, we preincubated the vessels with the NO inactivator oxyhemoglobin or indomethacin, respectively. Oxyhemoglobin (10 μmol/L) but not indomethacin (10 μmol/L) induced a contraction of 17.8±1.07 mm Hg. Relaxations induced by phentolamine (3 μmol/L) in arteries precontracted with 0.1 mmol/L ouabain were not antagonized by oxyhemoglobin (10 μmol/L), whereas the endothelium-dependent relaxation evoked by acetylcholine (3 μmol/L) in the
same conditions was completely abolished by this agent (Fig 5). On the other hand, indomethacin (10 μmol/L) did not modify the vasodilations induced by either phentolamine or acetylcholine (Fig 5).

Bioassay Experiments
We also analyzed the effects of the endothelium on the vascular responses to ouabain by means of bioassay experiments to evaluate the possibility that a substance released by the endothelium was the mediator of this phenomenon. First, both donor segments (with endothelium) and bioassay rings (without endothelium) were obtained from carotid arteries of control guinea pigs. In these conditions, the addition of 0.1 mmol/L ouabain through the donor elicited contractile responses in the bioassay ring that were reduced by 30 μmol/L phentolamine, whereas the contraction evoked by the direct addition of ouabain into the ring was resistant to the blockade by the α-adrenergic antagonist (Fig 6A, 6B, and 6E). When the donor segment was deendothelialized, the relaxant action of phentolamine was abolished (Fig 6Q). To exclude the possibility that norepinephrine released from the donor segment could explain these results, we performed analogous experiments using as donor segments carotid arteries from reserpinized guinea pigs; in this case, the results obtained were similar to those observed with control donors (Fig 6D and 6F).

[3H]Norepinephrine Release
The tissue tritium content after incubation with [3H]norepinephrine averaged 10 018±930 and 11 727±930 cpm/mg in guinea pig carotid arteries with (n=5) and without (n=5) endothelium, respectively. When the vessels were incubated with 75 mmol/L K+ for 3 minutes, a peak of tritium release was obtained, which was similar in both types of segments (with endothelium: basal values, 323.8±27.9 cpm/mL; after 75 mmol/L K+, increase by 328.3±28.3%, n=5; without endothelium: basal values, 383.4±35.3 cpm/mL; after 75 mmol/L K+, increase by 358.3±34.9%, n=5). The administration of 0.1 mmol/L ouabain induced a time-dependent tritium release in the control tissues that reached a peak at 35 minutes. Endothelium removal significantly reduced the radioactivity released by the glycoside, but the tritium release evoked by subsequent K+ addition in the same vessels was similar to that found in control vessels (Fig 7A, see legend). Pretreatment of normal arteries with oxyhemoglobin or indomethacin (both at 1 μmol/L) did not decrease the secretory response elicited by ouabain or K+ (Fig 7B and 7C). Moreover, pretreatment with oxyhemoglobin produced a tendency toward an increase in this secretory response, although without statistical significance (P=.087).

Discussion
The present study shows that ouabain induces concentration-dependent contractions in perfused guinea pig carotid arteries. Contractile responses induced by sodium pump blockade have been reported in other vascular preparations.8-11 We have previously shown that endothelium prevents the effects of ouabain on vascular smooth muscle by the release of a diffusible factor.17-18 When we used noninnervated vessels (human placental vessels), deendothelialization induced a potentiating effect on ouabain-evoked contractions.17
However, when we used well sympathetically innervated vessels (guinea pig carotid arteries), this potentiating effect was not observed, as the responses in vessels with and without endothelium were similar.18 This fact suggests that endothelium may modulate not only the myogenic but also the neurogenic component of ouabain-induced contractions. Phentolamine (a nonselective α-adrenergic blocker) and prazosin (a selective α1-adrenergic receptor antagonist) but not yohimbine (an α2-adrenergic blocker) induced concentration-dependent inhibition of ouabain-evoked contractions. Such findings indicate that in vessels with intact endothelium the action of the glycoside is mediated at least partially by norepinephrine released from perivascular sympathetic nerve endings (neurogenic component), which activates postsynaptic α1-adrenergic receptors. The neurogenic component of ouabain has been described in other vascular preparations.7,8,10,11,34-37 However, the relaxations evoked by phentolamine and prazosin on ouabain-induced contractions in guinea pig carotid arteries were completely abolished after the removal of endothelium. Similarly, preincubation of arteries with these blockers or the reserpinization of the animal reduced ouabain-induced responses only when endothelium was present.

To obtain additional confirmation of endothelial influence on ouabain-evoked contractions, we designed bioassay experiments. The vasoconstriction induced by direct addition of ouabain to the bioassay denuded ring was insensitive to phentolamine. However, when the glycoside was administered through a donor segment with intact endothelium, the α-adrenergic blockade significantly reduced the glycoside-induced responses. This antagonism was not observed when deendothelialized vessels were used as donors. The possible norepinephrine release from donor segments evoked by ouabain, which could be acting in the bioassay ring, was discarded by using arteries from reserpinized guinea pigs. The results obtained with these reserpinized donor segments were similar to those found with untreated donor segments. All the above data strongly support the hypothesis that when ouabain is administered through a donor segment with an intact endothelium, an endothelial factor (or factors) is released from this donor vessel, and the contraction induced in the bioassay denuded ring becomes partially mediated by norepinephrine, because phentolamine elicited a relaxation that was not observed when the glycoside was added directly or through a deendothelialized donor segment. This norepinephrine, which subsequently interacts with the vascular smooth muscle α-adrenergic receptors, seems to be released from the adrenergic innervation of the bioassay ring, because the reserpinization of donor segment did not abolish the effect of adding the ouabain to the bioassay ring. That is, we suppose that the interaction of the glycoside with the endothelial cells releases some factor (or factors) that diffuses to adrenergic endings and mediates neurotransmitter release.

The influence of endothelium on the ouabain-evoked norepinephrine secretion was directly observed by the ability of the glycoside to release tritium from carotid arteries preincubated with [3H]norepinephrine. Ouabain produced a time-dependent tritium secretion in a way similar to that reported in other vessels after sodium pump blockade.7,35,38 This ouabain-induced radioactivity release was significantly reduced in deendothelialized arteries, which agrees with reactivity results. This reduction was not due to an alteration of sympathetic nerve endings, because the subsequent vessel exposure to K+ induced a peak of tritium release similar to the control peak, which was not affected by endothelium removal. The secretion of norepinephrine produced by K+ is due to direct depolarization of adrenergic nerve endings and calcium-dependent neurotransmitter release by exocytosis.39 Therefore, from these results it is reasonable to conclude that the reduction of ouabain-induced norepinephrine release in denuded vessels is reflecting the lack of the endothelial mediation on these responses.

Some authors suggest that the endothelium modulates the efficacy of adrenergic agonists and antagonists, probably by a mechanism that involves changes in receptor reserve.42 Thus, in rat aorta the endothelium removal modifies the nature of the antagonism exerted...
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Figure 6. Tracings and plots show effects of phenolamine (Phent) on bioassay denuded rings of guinea pig carotid arteries precontracted with ouabain. A and B: Representative tracings (n=6) show glycoside-evoked contraction and phenolamine-induced relaxation of bioassay ring when both drugs are administered through a donor segment (A) or directly (n=5) on the bioassay denuded ring (B). C and D: Representative tracings (n=6) show effects of phenolamine added to bioassay rings precontracted with ouabain administered through donor segments without endothelium (C) or with endothelium taken from reserpinized guinea pigs (n=5) (D). E: Quantitative representation of experiments A and C; F: Quantitative representation of bioassay experiment when vessels from reserpinized animals were used as donor segments. Results (mean±SEM) are expressed as percentage of residual contractions induced by phenolamine in vessels precontracted with 0.1 mmol/L ouabain (1093±289.6 and 1505±339.8 mg for control and reserpinized vessels, respectively) administered by a donor segment with (E+) or without (E-) endothelium. Number of segments used is in parentheses. *P<.05.

by prazosin in norepinephrine-induced contractions. Nevertheless, this does not appear to be the case in the present results, because the concentration-dependent contractions evoked by norepinephrine in perfused guinea pig carotid arteries were antagonized by phenolamine or prazosin in segments with or without endothelium, suggesting an endothelium independence on the interaction between norepinephrine and α-antagonists. On the other hand, endothelial α2-adrenergic receptors that mediate NO release could be stimulated by norepinephrine released from sympathetic nerve endings. However, the involvement of these receptors appears unlikely in the present experiments because yohimbine did not exert any effect on ouabain-evoked contractions.

The nature of the endothelium-derived substance has not been elucidated, but NO or substances related to cyclooxygenase probably can be excluded. Thus, the inactivator of NO, oxyhemoglobin, at concentrations abolishing acetylcholine-induced endothelium-dependent relaxation, did not affect the vasodilation evoked by phenolamine in vessels precontracted with ouabain; the same occurred with the inhibitor of cyclooxygenase, indomethacin. Moreover, indomethacin did not modify tritium release induced by 0.1 mmol/L ouabain. Oxyhemoglobin not only did not diminish tritium release but showed a tendency, although without statistical significance, to increase it, producing the opposite effect to that expected if NO were the mediator in these ouabain-induced responses.

Some authors have postulated the existence of the EDHF, which may participate in endothelium-dependent relaxation evoked by acetylcholine. One of the proposed mechanisms of this EDHF is the activation of Na⁺,K⁺-ATPase of the smooth muscle. This assumption comes from experiments showing that ouabain inhibits the effect of EDHF. It could be possible that the endothelial factor mediating the modulatory effect of the endothelium on ouabain-evoked contractions was EDHF. However, evidence seems to discard this possibility. The effect of EDHF is transitory, whereas our experiments suggest that the activity of the endothelial factor in evidence in our experiments acts in a long-lasting manner. Moreover, we have shown that acetylcholine-induced relaxation is totally abolished in vessels preincubated with oxyhemoglobin (which is supposed to inhibit NO-mediated acetylcholine-induced relaxations) and precontracted with ouabain (which is supposed to inhibit EDHF-mediated acetylcholine-induced relaxations), whereas phenolamine-induced relaxation is maintained, suggesting that the endothelial factor that mediates the norepinephrine release stimu-
The endothelium of guinea pig carotid arteries possesses opposite effects on the neurogenic and myogenic actions of the glycoside; neurogenic contractions are facilitated by endothelial cells, whereas myogenic contractions are inhibited. Both effects seem to be due to released factor (or factors) from endothelium, the nature of which is unknown, although cyclooxygenase products or NO-related substances do not appear to be implicated. The physiological and pathophysiological significance of these findings remains to be established.

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