Effect of Ouabain on Endothelium-Dependent Relaxation of Human Resistance Arteries

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Inhibition of active sodium transport by ouabain was found to cause concentration- and time-dependent impairment of acetylcholine-induced relaxation in human resistance arteries with a significant effect at 100 pM. The reduced acetylcholine response was attributable to inhibition of the $\text{Na}^+\text{,K}^+$-ATPase but not the indomethacin-sensitive component of relaxation. Relaxation by sodium nitroprusside was not affected by ouabain, suggesting that inhibition of sodium transport, directly or indirectly, must affect synthesis or release of endothelium-derived relaxing factor rather than its effector pathway. These results do not support the existence of an additional endothelium-derived relaxing factor other than endothelium-derived relaxing factor, which is dependent on sodium pump activity. The finding that inhibition of sodium transport has a profound effect on vascular relaxation may have implications in the pathogenesis of certain forms of hypertension. (Hypertension 1991;17:619–625)

Vasomotor tone is dependent on the balance between systemic and local constricting and relaxing factors. The importance of the endothelial cell layer in the control of vasomotor tone has become apparent with the identification of endothelium-derived relaxing factor (EDRF) as nitric oxide. Using N\textsuperscript{G}-monomethyl L-arginine (LNMMA) to inhibit EDRF synthesis\textsuperscript{2,3} in vivo, it has been suggested that EDRF may be important in the control of blood pressure. Vallance et al\textsuperscript{4} have demonstrated considerable elevation in forearm vascular resistance after intrabrachial infusion of LNMMA, and elevation of blood pressure after systemic administration of LNMMA has been demonstrated in anesthetized rabbits.\textsuperscript{5} Locally acting vasodilating prostaglandins, prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) and prostaglandin I\textsubscript{2} (PGI\textsubscript{2}), are also released by the endothelium.\textsuperscript{6} Other relaxing factors have been postulated, for example an endothelium-derived hyperpolarizing factor,\textsuperscript{7} but none have been specifically identified. Locally produced and acting vasoconstrictors may also play a role. Endothelin, the novel endogenous vasoconstrictor\textsuperscript{8} released by endothelial cells, may regulate local tone but currently its physiological importance remains uncertain.

Inhibition of active sodium transport (Na\textsuperscript{+},K\textsuperscript{+}-ATPase) and raised intracellular sodium have been implicated in the pathogenesis of essential hypertension\textsuperscript{10} and pregnancy-associated hypertension\textsuperscript{11} based on the hypothesis that a circulating inhibitor of Na\textsuperscript{+},K\textsuperscript{+}-ATPase may be present and that consequent elevation of intracellular sodium could increase the blood pressure by increasing vascular smooth muscle tone.\textsuperscript{12} This has been disputed by some,\textsuperscript{13} but we have recently shown that inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase by ouabain will elevate the tone of resting human resistance arteries and will potentiate the tone of precontracted vessels.\textsuperscript{14} Several studies have also investigated the effect of inhibition of active sodium transport on endothelium-dependent and endothelium-independent relaxation in conduit vessels with conflicting results. Ouabain has been shown to prevent acetylcholine-induced relaxation in canine femoral artery\textsuperscript{15} and in rat thoracic aorta\textsuperscript{16} but to have no effect on canine coronary artery\textsuperscript{17,18} or rabbit ear artery.\textsuperscript{19} An inhibitory effect of ouabain on endothelium-independent relaxation produced by high concentrations of sodium nitroprusside has been noted by Rapoport et al\textsuperscript{20} using rat thoracic aorta.

We have recently investigated endothelium-dependent relaxation of human subcutaneous resistance arteries and have found LNMMA- and indomethacin-sensitive components.\textsuperscript{21} In the present study, we investigated the effect of active sodium transport inhibition on endothelium-dependent and endothelium-independent relaxation in human subcutaneous resistance arteries.

Methods

Human subcutaneous resistance arteries ($n=53$), mean internal diameter $218\pm8$ μm (see below), were obtained from biopsies of anterior abdominal wall fat
taken during routine abdominal surgery on 28 normotensive patients who were not receiving medication (17 women, 11 men, mean age 48±2 years, mean blood pressure 127/79±3/1 mm Hg). The arteries were dissected from connective tissue and were mounted as a ring preparation on a myograph capable of measuring isometric tension. \(^{22}\) The arteries were bathed in physiological salt solution (PSS) at a pH of 7.4 at 37°C and bubbled with 5% CO\(_2\) in O\(_2\). The study was approved by the St Thomas’ Hospital Ethics Committee, and subcutaneous fat was harvested with the informed consent of each patient.

The vessels were equilibrated in PSS for 1 hour, and after five stretches, the passive tension–internal circumference characteristics of the arteries were determined \(^{22}\) using Laplace’s equation. An estimate of the internal circumference was then made and from that the internal diameter was determined. The arteries were stretched to achieve an internal circumference equivalent to 90% of that which they would have had when relaxed in situ under a transmural pressure of 100 mm Hg (the maximum active tension for the minimum resting tension is developed at approximately this circumference). \(^{22}\) To assess their contractile response, the arteries were then maximally contracted for a 2-minute period on five occasions with a 10-minute period between. The first, second, and fifth contractions were produced with 5 \(\mu\)M norepinephrine in 125 mM potassium solution, the third contraction with 5 \(\mu\)M norepinephrine in PSS, and the fourth with 125 mM potassium solution. Any artery failing to produce a maximum active tension equivalent to a pressure of 100 mm Hg on the fifth contraction was rejected. Tension was measured in millinewtons per millimeter length of vessel (mN/mm).

**Chemicals**

Chemicals used in the present study were ouabain (BDH Chemicals Ltd, Poole, UK), norepinephrine (Winthrop, Guildford, UK), \(N^\text{O}\)-monomethyl-L-arginine monoacetate (Novabiochem, Nottingham, UK), and sodium nitroprusside (Roche, Welwyn Garden City, UK). The arteries were bathed in PSS containing (mM) NaCl 119, KCl 4.7, CaCl\(_2\) 2.5, MgSO\(_4\) 1.17, NaHCO\(_3\) 25, KH\(_2\)PO\(_4\) 1.18, EDTA 0.026, glucose 5.5, of pH 7.4 at 37°C, bubbled with 5% CO\(_2\) in O\(_2\). The potassium solution was made with equimolar substitution of KCl for NaCl, resulting in a final K\(^+\) concentration of 125 mM.

**Experimental Method**

Acetylcholine (ACH) dose responses were constructed by adding increasing concentrations of ACh (from 10 nM to 0.1 mM) in PSS at 2-minute intervals to resistance arteries precontracted with 3 \(\mu\)M norepinephrine. Arteries were only used for one experiment involving ouabain since the effects were irreversible over the time period of the experiment.

Concentration and time dependency of ouabain-induced inhibition of the acetylcholine response. Five ACh concentration responses were performed with a 15-minute washout period between each response. After the first of these responses, arteries were divided into three groups to receive 100 pM ouabain, 10 nM ouabain, or PSS (control) thereafter. Ouabain was included with the second norepinephrine stimulating solution and thereafter (i.e., there was no preincubation in ouabain before the second ACh response).

**Evaluation of LNMMA- and indomethacin-sensitive components of acetylcholine response in the presence and absence of ouabain.** After an initial ACh concentration response, resistance arteries were incubated in 10 nM ouabain or PSS for 1 hour, and all interventions thereafter were carried out in ouabain or PSS. The ACh response was then repeated. Arteries were incubated in 10 \(\mu\)M indomethacin for 30 minutes before the next ACh response was performed. The final ACh response was carried out after 5 minutes’ rest and 10 further minutes’ incubation in 10 \(\mu\)M indomethacin and 1 mM LNMMA.

**Effect of ouabain on the acetylcholine response in the presence of LNMMA.** This experiment was carried out to determine whether ouabain might cause inhibition of the ACh response over and above that caused by LNMMA. After an initial ACh response, arteries were washed and rested for 5 minutes before they were incubated in 1 mM LNMMA for 10 minutes and then a second ACh response was performed. After a 5-minute washout period, arteries were incubated in 10 nM ouabain for 1 hour, during the last 10 minutes of which 1 mM LNMMA was present. A third ACh concentration response, in the presence of 10 nM ouabain and 1 mM LNMMA, was then performed.

**Effect of ouabain on response to sodium nitroprusside.** To examine whether ouabain inhibited the effector pathway of EDRF, sodium nitroprusside concentration responses (from 1 nM to 10 \(\mu\)M) were performed before and after the 1-hour incubation in 10 nM ouabain.

**Statistical Analysis**

Results are expressed as percentage relaxations ± SEM. These were calculated by dividing the tension after the addition of acetylcholine by the tension achieved after the initial 3-minute exposure to 3 \(\mu\)M norepinephrine. Differences between means were assessed by two-tailed paired or unpaired \(t\) tests as indicated. The criterion for statistical significance was taken as \(p<0.05\).

**Results**

Results are expressed as percentage relaxations ± SEM of the precontraction. Thus, a figure of 100% corresponds to complete relaxation of the precontraction.

**Concentration and Time Dependency of Ouabain-Induced Inhibition of the Acetylcholine Response**

The maximum response to ACh in the presence of 100 pM ouabain decreased progressively throughout
FIGURE 1. Panel A: Sample trace showing an initial control acetylcholine (ACh) concentration response followed by four further ACh responses performed with 15-minute washout intervals in presence of 10 nM ouabain. For each response, artery was precontracted with 3 μM norepinephrine (NE). Panel B: After an initial ACh dose response, four further ACh responses were performed with arteries incubated in physiological salt solution (PSS) or 100 pM or 10 nM ouabain. This figure illustrates maximum relaxation response on each occasion of these three groups of arteries. *Significant differences between ouabain-treated arteries and the timed control (i.e., PSS-treated).

the period of the experiment; the fifth response fell to 53±14% (n=7). A more pronounced, but not significantly different, effect was noted in arteries (n=9) exposed to 10 nM ouabain. These results are illustrated in Figure 1. In the control group (n=6), maximum ACh-induced relaxation did not change significantly over the course of the experiment.

The size of the norepinephrine-induced precontractions preceding each ACh dose response did not change significantly either in the course of the experiments or when comparing the ouabain and control groups.

Evaluation of LNMMA- and Indomethacin-Sensitive Components of Acetylcholine Response in the Presence and Absence of Ouabain

Incubation of vessels for 1 hour in 10 nM ouabain reduced the maximum response to ACh from 92±3% to 69±8% (p<0.01, n=9). Maximum relaxation in control vessels incubated in PSS fell from 96±2% to 90±4% over 1 hour (p<0.025, n=8). (These results are illustrated in Figure 2A.) After a 30-minute incubation in indomethacin, the maximum ACh response was reduced further in both control and ouabain-treated vessels: in arteries exposed to ouabain the maximum ACh-induced relaxation fell by 17±6% versus a fall of 12±4% in control (n=9). A more pronounced, but not significantly different, effect was noted in arteries (n=9) exposed to 10 nM ouabain. These results are illustrated in Figure 1. In the control group (n=6), maximum ACh-induced relaxation did not change significantly over the course of the experiment.

Effect of Ouabain on the Acetylcholine Response in the Presence of LNMMA

The maximum relaxation response to ACh in the presence of 1 mM LNMMA did not change significantly after 1-hour incubation in 10 nM ouabain: 55±9% before incubation and 50±9% after incubation (NS) (n=5). This is illustrated in Figure 3. (Maximum relaxation response to ACh before the addition of LNMMA in these arteries was 95±2%.)

In this experiment, the size of the norepinephrine-induced precontraction did not change significantly before and after the 1-hour incubation in ouabain: 2.38±0.44 mN/mm before incubation and 2.31±0.39 mN/mm after incubation (NS) (n=5).

Effect of Ouabain on Response to Sodium Nitroprusside

The maximum relaxation response to sodium nitroprusside did not change after 1 hour of incubation in 10 nM ouabain: 95±2% before incubation and 97±1% versus after incubation 97±1% (NS) (n=8). (See Figure 4.)

As previously, in this experiment the size of the norepinephrine-induced precontraction did not change significantly: before incubation 1.70±0.13 mN/mm versus after incubation 1.57±0.12 mN/mm (NS) (n=8).

Discussion

Ouabain has previously been shown to inhibit ACh-induced relaxation of canine femoral artery rings; ACh-induced relaxation was significantly reduced after 15 minutes of preincubation in 2 μM
Our results demonstrate that ouabain will produce time-dependent and concentration-dependent inhibition of the ACh-induced endothelium-dependent relaxation response. We have found substantial immediate inhibition of this response using 10 nM ouabain and a less pronounced (although not statistically different) effect with concentrations as low as 100 pM. This sensitivity of human resistance arteries to ouabain is consistent with earlier work from this laboratory in which it was found that 10 nM ouabain caused a significant rise in tension of resting vessels after 6 hours (not significant at 2 hours) and caused rapid potentiation of tone in submaximally precontracted vessels. This earlier work was in contrast to studies in rat resistance arteries and may be attributable to cardiac glycoside insensitivity in rats.

At both concentrations of ouabain used in this study (i.e., 100 pM and 10 nM), no significant effect on the magnitude of the response to norepinephrine was demonstrated. We have previously found that incubation for 1 hour in 10 nM ouabain did not significantly alter the concentration response of these arteries to norepinephrine although this was not the case for higher concentrations of ouabain (unpublished observations from our laboratory). Other workers have not looked at the effect of low concentrations of ouabain on the norepinephrine response.

Inhibition of ACh-induced relaxation may be due to an effect of ouabain on the prostaglandin-dependent component or on the EDRF component of the response. In the latter case, it might interfere with EDRF synthesis or release or by affecting the cyclic GMP-dependent pathway leading to EDRF-induced relaxation in vascular smooth muscle cells. LNMA inhibits the synthesis of EDRF and does not affect its effector pathway in smooth muscle. Using indomethacin to inhibit the prostaglandin component and LNMA to inhibit the synthesis of EDRF, the effect of ouabain on the ACh response was examined. The experiments with indomethacin and LNMA together demonstrated that ouabain does not inhibit any component of relaxation other than the prostaglandin or EDRF-dependent pathways since the total inhibition of relaxation was no different in control or ouabain-treated vessels. The residual relaxation, persisting in the presence of LNMA, indomethacin, and ouabain, may reflect incomplete inhibition of these pathways or the release of additional factors, which are independent of these inhibitors. Examining the indomethacin-sensitive component, it appears that the size of the prostaglandin-mediated component of relaxation was not affected by ouabain. In contrast, the experiments in which the inhibitory effect of ouabain on ACh-induced relaxation in the presence of LNMA was determined, suggest that this effect was due entirely to an action of ouabain on the LNMA-sensitive (i.e., EDRF-mediated) pathway.
EDRF causes smooth muscle cell relaxation by increasing cyclic GMP production through stimulation of guanylate cyclase as does the nitric oxide donor sodium nitroprusside. Sodium nitroprusside therefore bypasses the endothelial cell layer, but causes vasodilation by the same final pathway as does EDRF. The response to sodium nitroprusside was not diminished in the presence of ouabain, thus it appears that in human subcutaneous resistance arteries, ouabain must interfere with either the synthesis or the release of EDRF by the endothelial cell layer. Our findings with sodium nitroprusside are in contrast to those of Rapoport et al., who found that ouabain will inhibit the relaxant response to high concentrations of sodium nitroprusside. They have also described a functional link between the Na⁺,K⁺-ATPase and the guanylate cyclase effector pathway in ACh-induced relaxation. Our results with sodium nitroprusside, however, suggest no important functional relation between the inhibition of Na⁺,K⁺-ATPase and guanylate cyclase activity in human subcutaneous resistance arteries.

The mechanism whereby inhibition of sodium transport might reduce EDRF production has not been investigated in this study but may be associated with the effect of ouabain on intracellular calcium in endothelial cells. Both the enzymatic synthesis of EDRF from L-arginine and the release of EDRF from the endothelium are dependent on a rise in intracellular calcium. Endothelial cells appear not to have voltage-dependent calcium channels, and potassium-induced depolarization leads to a decrease rather than to a rise in intracellular calcium in cultured endothelial cells. Similarly, ouabain-induced depolarization might cause inhibition of the ACh response by preventing intracellular accumulation of calcium necessary for the synthesis and the release of EDRF. If sodium-calcium exchange should exist in these cells, raised intracellular sodium secondary to ouabain-induced inhibition of Na⁺,K⁺-ATPase might be expected to inhibit or reverse this antiporter and lead to a loss of sodium-dependent calcium efflux and to raised intracellular calcium. EDRF production in this situation would increase. The evidence for a significant role for sodium-calcium exchange in the regulation of endothelial cell calcium is, however, conflicting.

Following from the observation that ACh causes relaxation accompanied by hyperpolarization associated with potassium efflux, it has been suggested that an independent endothelium-derived hyperpolarizing factor might exist. Further observations that 1) nitric oxide does not alter the membrane potential of canine arterial smooth muscle, 2) hemoglobin and methylene blue inhibit endothelium-dependent relaxation but not hyperpolarization, and 3) sodium nitroprusside causes relaxation without increasing rubidium efflux, have lent support to this suggestion. Hyperpolarization with consequent muscular relaxation has been attributed to potassium efflux from the vascular smooth muscle cells through specific ATP-dependent channels.

More recently, however, Tare et al, have demonstrated that ACh-induced hyperpolarization and relaxation of guinea pig uterine arteries can both be reduced by LNMMA, suggesting that the hyperpolarization may in fact be attributable to nitric oxide. In support of this, they also found that sodium nitroprusside and nitroglycerine can both cause hyperpolarization. In addition, although methylene blue and hemoglobin may completely inhibit mechanical relaxation, neither can completely block EDRF-induced stimulation of guanylate cyclase to form cyclic GMP. In the presence of these agents, hyperpolarization could follow from the presence of minimal amounts of EDRF.

Whether the result of nitric oxide or of a separate hyperpolarization factor, it has been suggested that endothelium-dependent hyperpolarization may result from stimulation of Na⁺,K⁺-ATPase. In support of
this, Feletou and Vanhoutte\textsuperscript{18} have demonstrated that ACh-induced hyperpolarization is sensitive to ouabain in canine coronary artery. In the same tissue, however, Chen et al\textsuperscript{17} have shown this not to be the case, and a similar observation has been made by Suzuki\textsuperscript{19} using rabbit ear artery. More recent work has confirmed that the hyperpolarization appears to be mediated by ATP-dependent potassium channels.\textsuperscript{32} It might be anticipated that ouabain would inhibit these potassium channels and could therefore reduce endothelium-dependent relaxation by this mechanism.\textsuperscript{29} In our experiments, however, we have shown that ouabain does indeed reduce mechanical relaxation but not by any pathway other than that mediated by EDRF. Thus, if a separate hyperpolarizing factor does exist and if it is influenced by Na\textsuperscript{+},K\textsuperscript{+}-ATPase, it cannot be contributing significantly to mechanical relaxation in this preparation.

In conclusion, very low concentrations of ouabain profoundly impair endothelium-dependent control of vascular tone in human subcutaneous resistance arteries. It seems that this effect is mediated by an action of ouabain on the synthesis or release of EDRF. Because EDRF plays an important physiological role in the local control of vasomotor tone, these results may have significant implications for the understanding of the pathogenesis of certain forms of hypertension in which a circulating inhibitor of active sodium transport has been implicated. This (or one of these) endogenous inhibitors has recently been revealed as ouabain or a very similar substance.\textsuperscript{34} There may also be implications for the use of cardiac glycosides in patients with impaired perfusion of particular vascular beds.

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