Published in final edited form as:
J Cardiovasc Pharmacol. 2013 August ; 62(2): 174–183. doi:10.1097/FJC.0b013e3182955d33.

Increased Constrictor Tone Induced by Ouabain-Treatment in Rats

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Abstract

Ouabain-induced hypertension in rodents provides a model to study cardiovascular changes associated with human hypertension. We examined vascular function in rats after long-term treatment with ouabain. Systolic blood pressure (SBP) was measured by tail-cuff plethysmography in male Sprague-Dawley rats treated with ouabain (Oua, ~25 $\mu g \cdot day^{-1}$) or placebo for 8 weeks. Blood pressure increased in ouabain-treated animals, reaching 30% above baseline SBP after 7 weeks. At the end of treatment, vascular responses were studied in mesenteric resistance arteries (MRA) by wire myography. Contraction to potassium chloride (KCl) in intact and denuded arteries showed greater sensitivity in Oua-treated animals. Contraction to phenylephrine (PE) and relaxation to acetylcholine (ACh) were similar between groups with a lower response to sodium nitroprusside (SNP) in Oua-treated arteries. Sensitivity to endothelin-1 (ET-1) was higher in Oua-treated arteries. Na^+-K^+ ATPase activity was decreased in MRA from Oua-treated animals, whereas protein expression of the Na^+-K^+-ATPase α_2 isoform was increased in heart and unchanged in mesenteric artery. Pre-incubation with indomethacin (10^{-5}M) or L-NAME (10^{-4}M) abolished the differences in KCl response and Na^+-K^+ ATPase activity. Changes in MRA are consistent with enhanced vascular smooth muscle cell reactivity, a contributor to the increased vascular tone observed in this model of hypertension.

Keywords

sodium pump; blood pressure; glycosides; contraction; vascular smooth muscle cells

INTRODUCTION

Alterations in vascular smooth muscle influencing contractility can lead to increased vascular tone and hypertension (1). Ouabain is an endogenous cardiotonic steroid with important roles in health and disease, regulating from cardiovascular function to metabolism and cell growth (2). This adrenocortical hormone (3) displays central and peripheral actions

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Conflicts of Interest None conflicts of interest declared.

and ouabain plasma levels are increased in patients with essential hypertension (4) or congestive heart failure (5). The sodium pump (Na^+-K^+ ATPase) plays a vital role in the regulation of membrane potential in smooth muscle and the heart (6). The catalytic subunit of this enzyme, α, has three isoforms detected in rat: α_1 is relatively insensitive, whereas α_2 and α_3 are relatively sensitive to cardiac glycosides such as ouabain. Like other glycosides (7), ouabain inhibits Na^+-K^+ ATPase activity by binding to the α_2 subunit. It has been proposed that this inhibition alters intracellular calcium (Ca^{+2}) homeostasis in smooth muscle cells, thus enhancing the responses to contractile agonists (8-11).

Although some models of ouabain treatment display no changes in blood pressure (12-14), rodent models subjected to long-term treatment with exogenous ouabain have been extensively used to investigate the effects of increased ouabain levels on blood pressure regulation (15-19). In this rodent model, blood pressure rises after 1 week of ouabain treatment and stabilizes after 5 weeks, resulting in approximately 30% increase in systolic blood pressure (SBP). Increased sympathetic activity and impaired baroreflex have been observed in models of ouabain-induced hypertension (18;20).

Alterations observed in vascular responses after ouabain treatment depend in part on the protocol (length of treatment and doses used) and the particular circulation being analyzed. For example, the contractile response to KCl has been reported to be similar in aorta, superior mesenteric or tail arteries (21), but greater in renal vessels (22) from ouabain-treated animals. Similarly, responses to α-adrenergic agonists have been reported to be higher (22) or lower (21) in vessels of ouabain-treated animals. It has been suggested that the lower adrenergic response reported in the aorta, but not in small resistance arteries (23), is the result of increased vasodilatory mechanisms induced by ouabain treatment (24-26), whereas no differences in vasodilatory responses have been reported in renal arteries (22). Recently, in rats treated for 5, 10 or 20 weeks with ouabain (8 $\mu g \cdot kg^{-1} \cdot day^{-1}$), no differences in vasodilatory responses were observed in MRA and a greater contraction to noradrenaline was observed only after 20 weeks of treatment (27).

The endothelin system plays an important role in blood pressure regulation (28). It is composed of peptide ligands, with endothelin-1 (ET-1) being the predominant form expressed in the vasculature, and receptors (ET_A and ET_B) ubiquitously expressed throughout the cardiovascular system (28;29). Evidence indicates that ouabain treatment activates the brain (30) and vascular (25) endothelin system. Di Filippo et al 2003, showed that four weeks of ouabain treatment increases ET-1 levels and decreases ET_A receptor expression in the brain (30). At the vascular level, ouabain treatment increases ET_A receptor expression with no changes in ET_B receptor expression (25). In ouabain-treated animals, the administration of an endothelin ET_A receptor blocker prevents the development of hypertension (25;30). Na^+-K^+ ATPase inhibition by ouabain plays an important role in controlling vascular tone (31), therefore, functional vascular Na^+-K^+ ATPase activity has been studied in different models of hypertension (32;33). In hypertensive rats, changes in Na^+-K^+ ATPase isoform expression have been detected in the aorta (34); whereas, in hypertensive patients, expression of the α_2 isoform in cardiac tissue is increased (35). In ouabain-treated rats, no differences in vascular Na^+-K^+ ATPase activity have been reported in conductance vessels, however the sensitivity to inhibition by ouabain was increased in the aorta and decreased in tail arteries (21).

Our study addresses the effects of ouabain treatment over 8 weeks (25 $\mu g \cdot kg^{-1} \cdot day^{-1}$) on rat cardiovascular responses. Blood pressure was recorded and at the end of treatment small mesenteric resistance arteries (MRA) were studied using wire myography to determine: 1) contraction to potassium chloride, phenylephrine and ET-1; 2) relaxation to acetylcholine and sodium nitroprusside and 3) functional Na^+-K^+ ATPase activity. Protein expression of

the Na^+-K^+ ATPase α_2 subunit was also measured in heart tissue and main mesenteric artery in ouabain-treated animals.

MATERIALS & METHODS

Animal Preparation

12-week-old male Sprague-Dawley (SD) rats were obtained from Charles River Laboratory (Wilmington, MA). The animals were housed at a constant room temperature, humidity and light cycle (12:12h light dark), with a global 18% protein extruded rodent chow (Harlan Laboratories, Indianapolis, IN) and water available *ad libitum*. All experiments were performed in accordance with the guidelines of the Wake Forest University School of Medicine Institutional Animal Care and Use Committee.

Blood Pressure Measurements

Indirect systolic, diastolic, and mean arterial blood pressure and heart rate measurements were taken daily via tail-cuff plethysmography (Columbus Instruments, Columbus, OH), as previously described (36). Ten blood pressure readings per animal were recorded, from which the average was calculated for a single daily value over the course of 9 weeks: 1 week of baseline measurements and 8 weeks of ouabain treatment measurements.

Ouabain Pellet Implantation

After 1 week of baseline blood pressure measurements, the rats were divided into ouabain-treated and sham groups. The animals were anesthetized with 3% isoflurane, and a small incision was made on the back of the neck where a 1.5mg 60-day ouabain slow-release pellet (~25 μg of ouabain/day, Innovative Research, Sarasota, FL) was implanted subcutaneously in the ouabain-treated group (n=6). The control group (n=5) had a sham surgery performed in the same manner that the ouabain pellet was implanted.

Tissue Collections

After 8 weeks of treatment, animals were painlessly euthanized by asphyxiation with carbon dioxide (CO_2) followed by a cardiectomy. Heart ventricles and main mesenteric artery were dissected, cleaned and stored at −80°C until further use. For reactivity studies mesenteric arcades were carefully removed and third-branch mesenteric arteries were isolated and cleaned of fat and connective tissue while kept in cold Krebs-Henseleit Buffer (KHB) containing (in mmol/l): NaCl 118, KCl 4.47, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2 \cdot 2H_20$ 2.5, glucose 5.5.

Western Blotting

Collected heart tissues were homogenized and microsomes were isolated. Main mesenteric arteries were homogenized in lysis buffer (Hepes 50 mM, Nacl 150 mM, Triton X-100 1%, EDTA 1 mM, cocktail of protease inhibitors), and centrifuged at 2000xg for 5min, protein concentration was determined in supernatant using the Bradford method. For heart tissue, 100 μg of protein from control (n=5) and ouabain-treated (n=5) were used. For the mesenteric artery, 18 μg of total protein extract from arteries in control (n=4) and ouabain-treated (n=4) and 10 μg of total rat brain as positive control were used. Proteins were separated by 7.5% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% non-fat milk in Tris-buffered solution and incubated overnight at 4°C with primary rabbit polyclonal antibody anti-Na^+-K^+ ATPase α_2 at 1:1000 dilution (Millipore, Billerica, MA). Anti-rabbit HRP-conjugated antibody (1:3000 dilution) was used as secondary antibody. The immunocomplexes were detected using an enhanced chemiluminescence system and ChemiDoc^{TM} XRS Software (Bio-Rad). Optical

density relative to a standard sample, in arbitrary units (a.u.), was calculated using ImageJ Software (NIH, Bethesda, MD).

Vascular Reactivity Experiments

Arterial segments, maximum of 2mm in length, were mounted between an isometric force transducer (Kistler Morce DSC 6, Seattle, WA, USA) and a displacement device on a myograph (Multi Myograph, Model 620M Danish Myo Technologies, Aarhus, Denmark) using two stainless steel wires (diameter 40 μm), as described previously (37;38). The myograph organ bath (5 ml) was filled with KHB maintained at 37°C and aerated with 95% O_2/5% CO_2. In a subgroup of control (n=4) and Oua-treated (n=4) animals the endothelium was destroyed by passing a human hair through the lumen as described by Pulgar and Figueroa (37). The vessels were washed and incubated for 30 min before the normalization procedure was performed. Each arterial segment was stretched in a stepwise manner and the internal circumference and corresponding wall tension at each stretch were calculated and plotted to produce a resting wall tension–internal circumference curve for that particular artery using the DMT Normalization Module (ADInstruments). Arterial segments were normalized to $0.9 \cdot L_{100}$, with L_{100} being the internal circumference the vessels would have if they were exposed to a transmural pressure of 100 mm Hg, as previously described (39). Optimal diameters (OD) were calculated as $OD=0.9 \cdot L_{100}/\pi$. MRA OD showed no differences between control and ouabain-treated animals (260±9 *vs* 255±10 μm, $p>0.05$). After obtaining the optimal diameter, a 30-min equilibration period preceded the addition of test substances.

Response to Potassium Chloride (KCl)

After equilibration, in order to test the viability of the arterial preparations and determine responses to non-receptor mediated contraction, MRA were exposed successively to increasing concentrations of potassium (K^+) in KHB. Arterial segments were exposed to nine different concentrations of K^+ (6.25–75 mM), with each dose being maintained for 2 min and then washed with KHB before the subsequent concentration was introduced. In parallel experiments, different arterial segments were denuded or pre-incubated for 15 min with the nitric oxide synthase inhibitor L-NAME (10^{-4} M), or the cyclooxygenase inhibitor indomethacin (10^{-5} M).

Response to Phenylephrine (PE)

After washing and resting for 20 min, MRA segments were exposed to a cumulative concentration-response curve of PE by exposing arteries to fourteen (10^{-8}–$10^{-4.5}$M) increasing concentrations in fourthlog steps, with each subsequent dose being introduced only after a steady response had been reached.

Response to Endothelin-1 (ET-1)

Under resting tension, MRA segments were exposed to a cumulative concentration-response curve of ET-1 by exposing arteries to nine (10^{-11}–10^{-7} M) increasing concentrations in half-log steps, with each subsequent dose being introduced after a steady response had been reached (every 4 min). At the end of the ET-1 curve, arteries were washed and allowed to recover.

Response to Acetylcholine and Sodium Nitroprusside

Arteries were washed and stimulated with a sub-maximal dose of PE, between 10^{-6} M-3×10^{-5}M, in order to attain an equivalent level of contraction. A dose response curve to acetylcholine (10^{-9} -10^{-5}M) was performed. Data of all the vessels studied were included in the analysis, denuded arteries showed less than 10% relaxation to acetylcholine. After

washing in KHB for at least 30 min, PE stimulation was repeated and after a stable contraction was reached, increasing concentrations of sodium nitroprusside (10^{-9}-10^{-3} M) were added at 3 min intervals.

Functional Vascular Na^+-K^+ ATPase Activity Assay

Functional vascular Na^+-K^+ ATPase activity was determined as previously described (40). At resting, the media in the myograph chamber was changed to KHB without K^+ (all K^+ was replaced by Na^+). After a 15 min incubation in KHB-K^+ free media, arteries were stimulated with PE (3×10^{-6} M) and when a stable contraction was reached, K^+ concentration was increased in steps at 2 min intervals by adding small volumes from a concentrated KCl solution. After full relaxation was reached, arteries were washed with normal KHB. After 15 min of resting, arteries were incubated again in KHB-K^+ free media and 10^{-4} M ouabain was added to the chamber. After 15 min of pre-incubation, arteries were stimulated with PE and the dose response to K^+ was repeated.

Drugs

Nω-nitro-L-arginine methyl ester (L-NAME), phenylephrine (PE), acetylcholine (ACh), sodium nitroprusside (SNP), and ouabain were from Sigma (St Louis, MO) and stock solutions were prepared in distilled water. Indomethacin (Sigma) was dissolved in 50 mM $NaCO_3$ in KHB. Endothelin-1 (ET-1) (California Peptide Research Inc., Napa, CA) was dissolved in KHB with 1% BSA. All other chemical reagents were from Sigma.

Data Analysis

Maximal contractile responses to KCl were expressed in absolute values, whereas maximal responses to PE and ET-1 were expressed as a percent of the maximal response induced by KCl ($\%K_{MAX}$). Vasodilatory responses to ACh and SNP were expressed as % of pre-constricted tone. Na^+-K^+ ATPase activity was expressed as the difference in area under the curve (%dAUC) in the absence and presence of 10^{-4} M ouabain in the myograph chamber. Concentration-response curves for KCl, PE, ACh, SNP and ET-1 were analyzed by fitting individual experimental data to a logistic curve to determine the maximal response and sensitivity. The curve was of the form Y = bottom + (top − bottom) / (1 +10(LogEC_{50} − X)* Hill Slope)) where X is the logarithm of the concentration and Y is the response; the sensitivity values reported are derived from these fits. The contractile response to KCl was expressed in mN/mm as units of arterial wall tension (AWT) (AWT = force / 2 × length of vessel). For each arterial segment, the maximal response to KCl was calculated, and the response to contractile agonists was expressed as percent of the corresponding maximal response to KCl. Sensitivity was expressed as pD_2 ($pD_2 = -\log [EC_{50}]$) with EC_{50} being the concentration of agonist producing 50% of the maximal response. Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons was used to determine significant differences. A $p < 0.05$ was accepted as an indication of statistical significance.

RESULTS

Blood pressure measurements

Treatment with ouabain lead to an increase in systolic blood pressure (SBP) compared to control animals. The baseline SBP for the total of animals studied was 130.7±8 mmHg (mean±SEM; n=11). At the end of the ouabain-treatment, SBP values were 170±10 mmHg (n=6) for ouabain-treated and 127±10 mmHg (n=5) for the control group. The increase in SBP reached stable values after 7 weeks of ouabain treatment (130±12 *vs* 103.1±2.5% of baseline, $p<0.05$) (Figure 1).

Vascular measurements

Contractile responses

Potassium chloride: Similar maximal responses to KCl were observed between Oua and control MRA, with a greater sensitivity in MRA from Oua-treated animals ($p<0.05$, Figure 2, Table 1). Endothelial denudation increased sensitivity to KCl in control and Oua-treated arteries with the greater sensitivity being maintained in Oua-treated arteries (Figure 2B, Table 1). Pre-incubation with indomethacin decreased maximal responses in both groups with no effect on sensitivity (Figure 2C), whereas pre-incubation with L-NAME increased both maximal response and sensitivity in control, such that there is no longer a difference with Oua-treated arteries (Figure 2D). The higher sensitivity to KCl in untreated Oua MRA compared to control MRA was abolished by pre-incubation with indomethacin or L-NAME (Figure 2, Table 1),

Phenylephrine: Responses to PE showed no differences between Oua-treated and control MRA (Figure 3A). Endothelial denudation increased maximal response to PE in both groups (Figure 3B, Table 1). Indomethacin reduces maximal PE response in both groups (Figure 3C), whereas L-NAME treatment increased sensitivity in control arteries (Figure 3D and D, Table 1).

Endothelin-1: A greater sensitivity to ET-1 ($p<0.05$) was observed in arterial segments from Oua-treated animals while no differences in maximal responses were seen (Figure 4A). Endothelium denudation induced an increased sensitivity in control and Oua-treated arteries and tended to alter the maximal response to ET-1; however, this did not reach statistical significance and no differences between both groups were found (Figure 4B, Table 1). Pre-incubation with indomethacin reduced maximal responses to ET-1 (Figure 4C), whereas L-NAME treatment increased sensitivity to ET-1 in control arteries. The greater sensitivity to ET-1 was maintained after pre-incubation with Indomethacin or L-NAME (Table 1).

Vasodilatory responses

Acetylcholine: Acetylcholine-dependent relaxation showed no differences between control and Oua-treated animals (Figure 5A, Table 2). Endothelial denudation abolished ACh relaxation in both groups (Figure 5B), whereas pre-incubation with indomethacin had no effect (Figure 5C). Pre-treatment with L-NAME greatly blocked ACh relaxation in control arteries, however the inhibitory effect was lower in Oua-treated arteries compared to control arteries (Figure 5D, Table 2).

Sodium nitroprusside: Response to sodium nitroprusside (SNP) in PE pre-constricted arteries showed lower maximal relaxation in Oua-treated arteries (Figure 6A, Table 2). Endothelial denudation increased SNP sensitivity in control arteries, and had no effect in Oua-treated arteries (Figure 6B). Indomethacin had no effect on SNP responses (Figure 6C), whereas L-NAME pre-incubation induced a greater sensitivity in control arteries (Figure 6D, Table 2).

Functional Na^+-K^+ ATPase activity—Na^+-K^+ ATPase activity was lower in MRA from Oua-treated animals than from control animals (65±2.2 vs 73.4±2.5 %dAUC, $p<0.05$, Figure 7C). Endothelial denudation had no effect on both groups, while denuded Oua-treated arteries still displayed lower Na^+-K^+ ATPase activity compared to controls. Pre-incubation with indomethacin (69.4±5.1 vs 77.2±3.6 %dAUC, $p>0.05$) or L-NAME (72.2±4.2 vs 75.6±2.3 %dAUC, $p>0.05$) abolished this difference (Figure 7C).

Expression of Na^+-K^+ ATPase α_2 isoform in heart and mesenteric vessels— Protein expression of Na^+-K^+ ATPase α_2 isoform showed greater values in heart tissue from Oua-treated animals compared to control animals (Figure 8A, $p<0.05$). In main mesenteric artery, Na^+-K^+ ATPase α_2 isoform expression showed no difference between control and Oua-treated animals (Figure 8B).

DISCUSSION

We observed an increased vasoconstrictor tone in rat small resistance mesenteric arteries induced by prolonged *in vivo* treatment with ouabain. This increased vasoconstrictor tone was expressed as an increased sensitivity to a depolarizing solution of KCl, increased sensitivity to ET-1, lower relaxation to SNP, and diminished Na^+-K^+ ATPase vascular activity. Consistent with previous reports (21;23-25;27), we found that long–term ouabain treatment increased blood pressure, reaching approximately a 30% increase over baseline values after 8 weeks of treatment. The changes we observed in the resistance vasculature suggest an increased reactivity of the smooth muscle cell that may contribute to the increased blood pressure observed in this model of hypertension.

The contractile response to a dose response curve to KCl showed no differences in maximal response between control and treated MRA. This contraction appears to be mainly dependent on smooth muscle cells and is modulated by endothelial factors since endothelial denudation increased KCl sensitivity in both groups, with Oua-treated arteries still displaying a greater sensitivity compared to control arteries. Prostaglandins are one of those endothelial factors involved in vascular control, and we observed a diminished contraction to KCl in both groups pretreated with indomethacin. The effects of blockade of prostaglandins on MRA contractile responses appears to be dependent on the protocol used to test the responses; whereas an increased effect of indomethacin after a single dose of KCl was reported (41), a detailed study of the dependence of contractile responses of rat MRA on cyclooxygenase products indicated that indomethacin decreased contraction to a dose response curve of KCl (42). These effects appear to be mediated by cyclooxygenase as well as lipooxygenase vasoconstrictor products released during contraction to KCl (42). Our results suggest involvement of vasoconstrictor prostanoids in KCl contraction, in agreement with previous findings on the effects of prostanoids on adrenergic contraction in rat arteries (43;44). Treatment with L-NAME increased sensitivity to KCl in control arteries and maximal contraction in both control and Oua-treated arteries, as expected after inhibition of nitric oxide (NO) release. The increased sensitivity in Oua-treated arteries to KCl compared to control arteries was abolished when MRA were pre-incubated with indomethacin or L-NAME. Our results suggest that in Oua-treated MRA arteries enhanced sensitivity to KCl contraction is due to increased smooth muscle cell activation and this contraction is modulated by endothelial factors. An increased endothelium-dependent modulation of contraction responses after ouabain treatment has previously been suggested in the aorta (24). Depolarization-dependent contraction is mostly dependent on Ca^{+2} influx via voltage dependent calcium channels (VDCC) (45), and plays an important role in blood pressure regulation (46) and hypertension (47). A greater response to KCl has been reported in the renal artery in ouabain-treated animals (22) and both VDCC blockers, verapamil and nifedipine, partially inhibit *in vitro* (48) and *in vivo* (49) vascular effects of ouabain respectively. Thus, our results suggest that VDCC are also modulating contractile responses in the resistance circulation of ouabain-treated animals.

We observed no differences in responses to the α_1-adrenergic agonist PE in MRA of Oua-treated animals compared to control animals. PE response was increased after endothelial denudation, whereas indomethacin lowered maximal responses to PE as described for adrenergic contraction in the same vascular bed (42). The inhibitory effect of indomethacin

on α-adrenergic contractions has been described in aorta and caudal artery in control and Oua-treated rats (24), in agreement with the inhibitory effect of indomethacin reported on adrenergic contraction (43;44). Both endothelial denudation and L-NAME treatment increased maximal responses in both groups, indicating the modulatory role that NO has in adrenergic responses. It was previously shown that ouabain treatment for five weeks alters the adrenergic responses in conductance vessels mainly as a result of increased modulation by endothelial factors (21;23;24). It appears that a longer period of ouabain treatment is required to detect effects on adrenergic responses on MRA since recent data showed greater noradrenaline contraction in MRA only after 20 weeks of ouabain treatment (27).

In conductance vessels, such as the aorta, an increased contribution of endothelial derived factors to contraction has been observed in ouabain-treated animals. Our results of similar responses to ACh in ouabain-treated and control MRA are in agreement with the absence of changes reported in ACh responses in MRA at 5 or 10 weeks of ouabain treatment (27). ACh relaxation was abolished in endothelium-denuded arteries in both groups supporting a role of endothelium-derived NO, prostacyclin and/or EDHF (50-52). In agreement with previous reports (42), indomethacin did not modify the response to ACh, indicating that prostaglandins are not involved in endothelium-mediated dilatation in these vessels. Treatment with L-NAME blocked ACh dilatation in both groups; however the remaining dilatation was greater in Oua-treated arteries compared to control arteries. Relaxation of MRA in the presence of L-NAME has been shown to be inhibited by K^+ channel blockers, such as tetraethylamonium, and ascribed to an endothelium-derived hyperpolarization factor (53). Thus, one possible explanation for the differences observed on ACh vasodilatation is that Oua-treated arteries possess an enhancement of EDHF-mediated hyperpolarization, which in turn reduces intracellular smooth muscle cell Ca^{2+} concentration causing relaxation (54) and would act as a compensatory mechanism to the increased constriction observed. EDHF activation has been showed to also modulate contraction responses; however in our experiments PE-mediated sensitivity remained unaltered, suggesting that some additional factors compensate adrenergic contractile responses in Oua-treated MRA.

Endothelium-independent vasodilatation, expressed as the response to SNP, was lower in Oua-treated arteries, suggesting that the smooth muscle portion of the nitric oxide–dependent vasodilatory pathway is affected by ouabain treatment in MRA. In control arteries, endothelial denudation and L-NAME treatment both increased sensitivity to SNP in agreement with numerous reports showing that activation of endothelium-independent relaxations may compensate for the loss of endothelial derived dilatation (55-58). This compensation appears to be dependent on an enhanced sensitivity of the soluble form of the enzyme guanylyl cyclase (sGC) in the absence of endogenous NO (55). The effects of ouabain treatment in sGC function and expression in MRA remain to be investigated.

Since earlier studies showed that ouabain stimulates expression and release of ET-1 from endothelial cells (59), and changes in central and peripheral endothelin system have been reported in models of ouabain-induced hypertension (25;30), we analyzed the contractile responses to the main agonist of this system, ET-1. Our results showed similar maximal responses and greater sensitivity for ET-1 in intact untreated MRA from ouabain-treated animals compared to control animals. Endothelial modulation of ET-1 contraction is evident in MRA since endothelial denudation, prostaglandins or NO inhibition all increased ET-1 sensitivity in control arteries. We observed decreased maximal contraction in the presence of indomethacin in both groups, similarly to what we observed for the other contractile agents, KCl and phenylephrine, and this is in agreement with previous evidences that mesenteric (60), as well as, aortic (61) reactivity to ET-1 involves the release of constrictor prostanoids that contribute to the contractile response. The increased sensitivity observed in Oua-treated arteries was not affected by indomethacin or L-NAME, indicating that prostanoids or NO

are not responsible for the increased ET-1 sensitivity in Oua-treated MRA. ET-1 contraction is dependent on the action on its receptors ET_A and ET_B; ET_B is present in muscle and endothelial cells, whereas ET_A has been found only in smooth muscle cells (28). ET_A activation mediates contraction, whereas vascular ET_B receptors may mediate relaxation via NO-cGMP pathways, vasodilator prostanoids or activation of voltage-activated K^+ channels (62). Evidence also indicates that ET_B may mediate contraction (63). In MRA from hypertensive rats, an imbalance in the ET-1 contractile and dilatory responses has been described with an increase in ET_A receptors and a decrease in ET_B-mediated vasodilatation contributing to an enhanced ET-1 contraction (64). The greater ET-1 response we observed in Oua-treated MRA is in agreement with the fact that both endothelin blockers lower blood pressure (25;30) and endothelin receptor (ET_A) expression in the aorta is increased in ouabain-treated animals (25). This suggests that in the MRA, ouabain-induced changes in the endothelin system, probably involving increased ET_A-mediated signaling, are expressed. Whether these changes we observed in MRA are accompanied by changes at the level of receptor expression and/or signal transduction remains to be determined.

A key observation in the investigations about the role of the catalytic isoform of Na^+-K^+ ATPase in ouabain –induced hypertension was obtained using mice carrying a Na^+-K^+ ATPase enzyme containing an ouabain insensitive α_2 subunit (65). These animals were refractory to ouabain treatment, did not develop hypertension, and displayed an increase in vascular contractility. Since ouabain and other glycosides inhibit Na^+-K^+ ATPase (7), we sought to measure the activity of the vascular Na^+-K^+ ATPase in small MRA from ouabain-treated animals. We used the validated protocol described by Webb & Bohr (1978), in which arteries pre-constricted with phenylephrine in a K^+-free media are exposed to increasing K^+ concentrations (40). We observed a reduced vasodilatation to K^+ in MRA from ouabain-treated animals, whereas the effect of ouabain added to the assay was similar between groups. Our result of decreased Na^+-K^+ ATPase activity in ouabain-treated animals is similar to those found in vessels from spontaneously hypertensive rats (SHR) (32), and hypertensive dogs (33). As with the KCl contractile responses, the differences in Na^+-K^+ ATPase activity were maintained after endothelial denudation and abolished after pre-incubation with indomethacin or L-NAME, suggesting that ouabain-dependent effects on vascular Na^+-K^+ ATPase activity are affecting smooth muscle mechanisms and are modulated by prostanoids and NO. This is in agreement with an enhanced role for endothelium-derived factors in regulating Na^+-K^+ ATPase activity in hypertensive models (66). Previous reports in ouabain-treated animals indicate regional differences in the vascular functional Na^+-K^+ ATPase activity with increased activity in the aorta, decreased activity in tail arteries, and no changes in superior mesenteric artery (21). The reduced Na^+-K^+ ATPase activity we observed in MRA and the associated alterations in intracellular Ca^{2+} homeostasis may help explain the increased sensitivity in vasoconstrictor responses we observed.

In mice carrying the ouabain-insensitive α_2 subunit, the ouabain-induced increase in cardiac contractility is abolished (67), providing direct evidence for the mechanism used by glycosides to elevate cardiac inotropy. Additionally, previous reports show that expression of Na^+-K^+ ATPase α_2 subunit is increased in hearts of hypertensive patients (35). We observed a similar increase of the α_2 subunit in hearts from ouabain-treated animals. In the mesenteric artery, the decreased functional Na^+-K^+ ATPase activity is not a result of diminished protein levels, as we did not observe differences in expression of the Na^+-K^+ ATPase α_2 subunit; however, these determinations were performed in the main mesenteric artery and a direct comparison with small resistance vessels is needed. Elevated intracellular Na^+ and Ca^{+2} concentrations have also been reported in hypertensive patients and increased intracellular Ca^{+2} concentrations have been observed in the vasculature of ouabain-treated rats (68). The upregulation of this enzyme in heart tissue contrasts with the decreased

functional activity we observed in vascular tissue where no changes in expression were found. One possible explanation is that the presence of elevated ouabain levels and the consequent inhibition of Na^+-K^+ ATPase activity, induce a greater expression of α_2 Na^+-K^+ ATPase as a compensatory reaction to increased intracellular Na^+ concentrations. This compensatory reaction would be more evident in cardiomyocytes than in smooth muscle cells. Further research is needed to understand the relationships between Na^+-K^+ ATPase expression and activity in vascular smooth muscle cells.

CONCLUSION

We have demonstrated an increased response to constrictor agonists and decreased endothelium-independent relaxation in the resistance vasculature of animals treated with the glycoside ouabain. This was accompanied with an increased expression of the α_2 Na^+-K^+ ATPase subunit in hearts and decreased Na^+-K^+ ATPase activity in MRA from treated rats. Our findings are consistent with enhanced vascular smooth cell reactivity to constrictor agents and may help explain the vascular changes that contribute to the increased blood pressure reported in this hypertension model.

Acknowledgments

This research was supported by grants NIMHD MD 002303, NIGMS 5T34GM070416 and HL51952.

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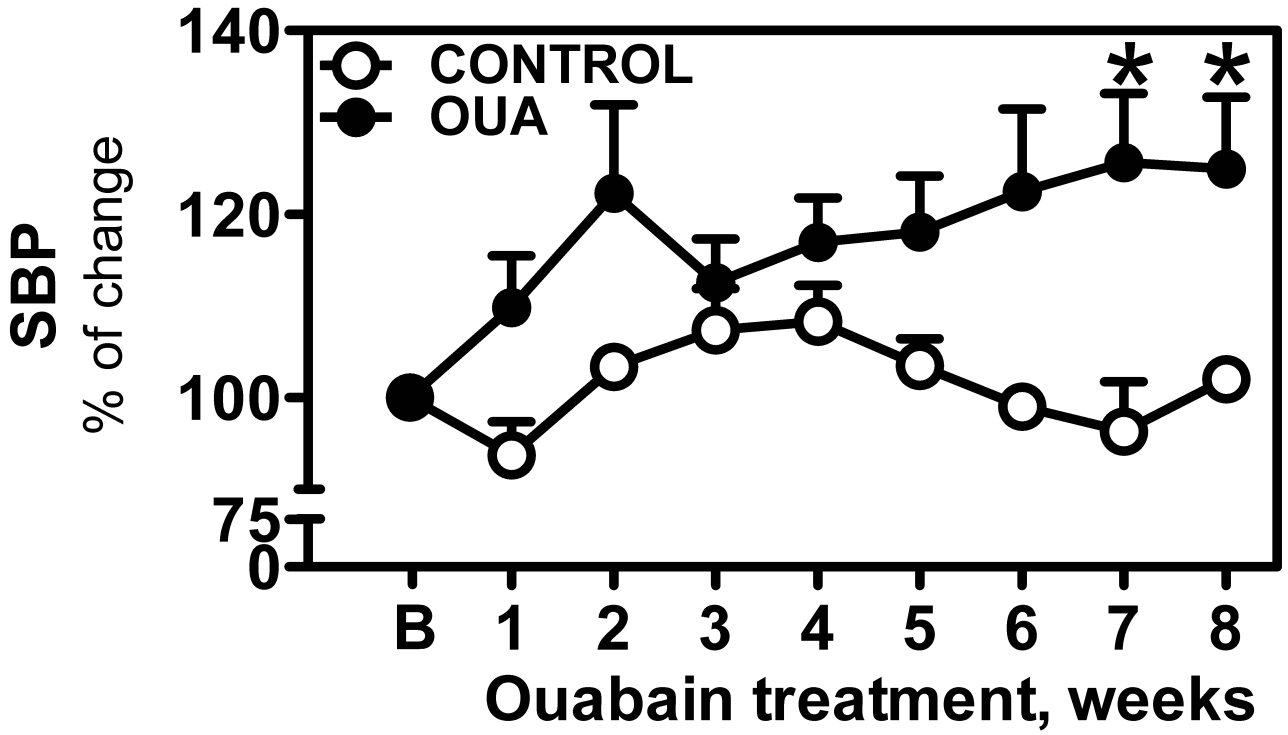

Figure 1. Systolic blood pressure during Ouabain treatment

SBP was measured daily via tail-cuff plethysmography as described in Materials & Methods in control (○, n=5) and Oua-treated rats (●, n=6). Data are expressed as mean±SEM. * $p<0.05$ *vs* control.

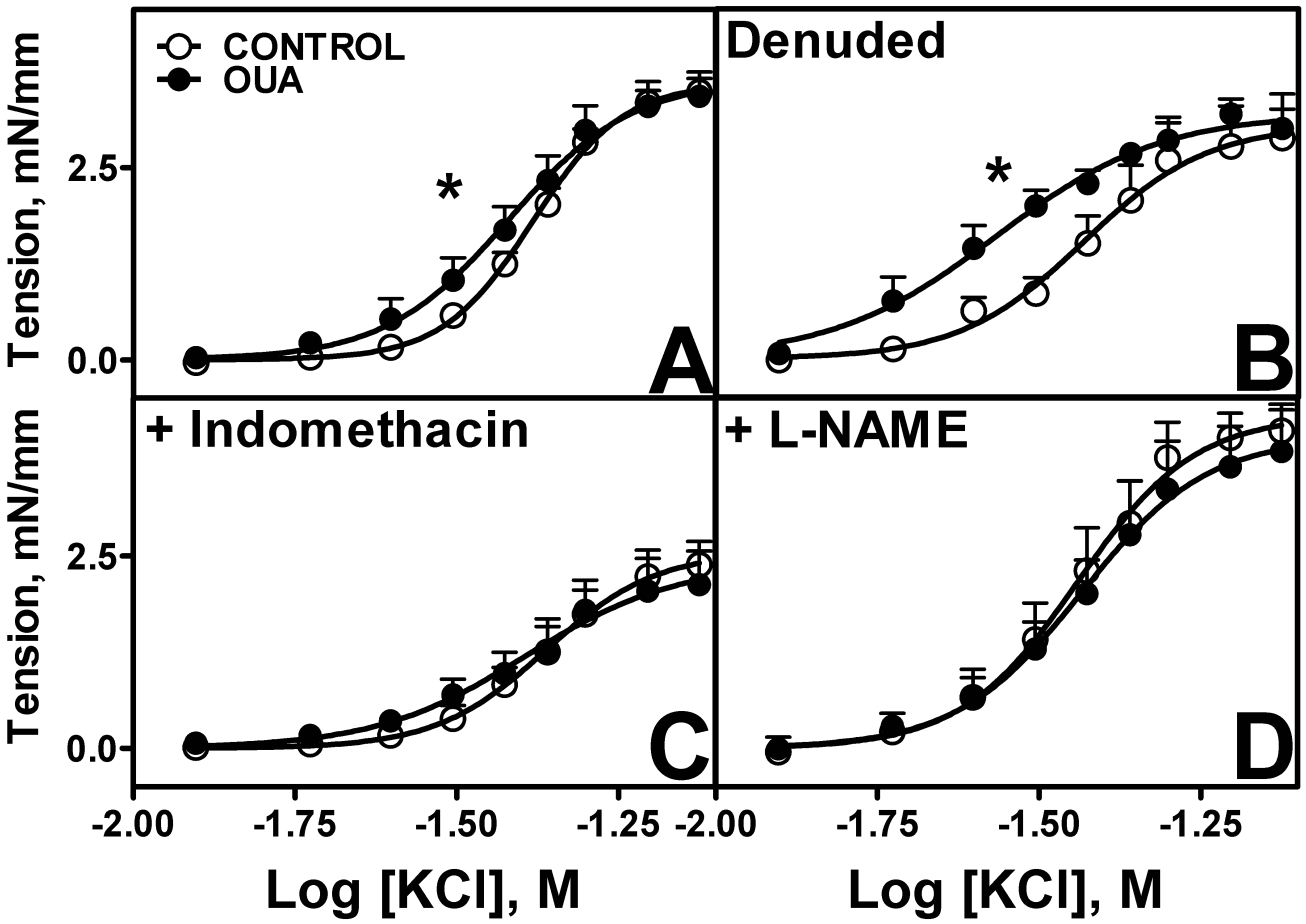

Figure 2. Contractile responses to potassium chloride (KCl) in MRA from Control and Ouabain-treated rats

A. Intact arteries (control ♀, n=13; Oua ●, n=9). B. Denuded arteries (control ♀, n=4; Oua ●, n=4). C. Arteries pre-incubated with indomethacin 10^{-5}M (control ♀, n=10; Oua ●, n=8). D. Arteries pre-incubated with L-NAME 10^{-4}M (control ♀, n=10; Oua ●, n=9). Data are expressed as mean±SEM. * $p<0.05$ in sensitivity *vs* control arteries.

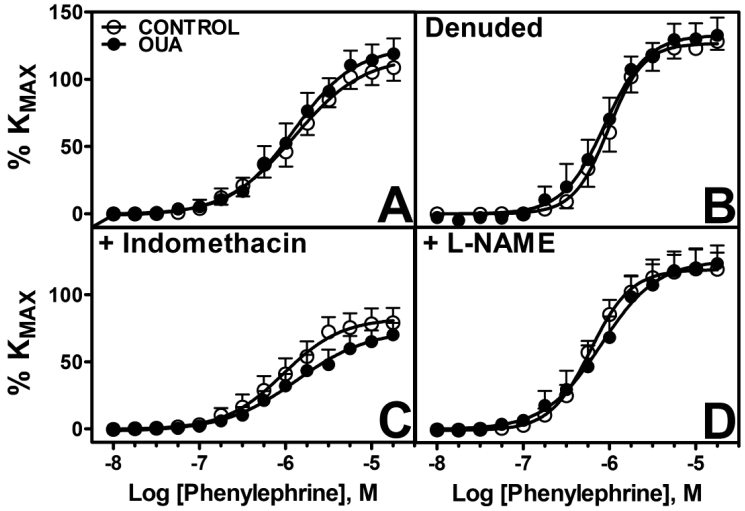

Figure 3. Contractile responses to phenylephrine (PE) in MRA from Control and Ouabain-treated rats

A. Untreated arteries (control ○, n=9; Oua ●, n=8). B. Denuded arteries (control ○, n=4; Oua ●, n=4). C. Arteries pre-incubated with indomethacin 10^{-5}M (control ○, n=9; Oua ●, n=8). D. Arteries pre-incubated with L-NAME 10^{-4}M (control ○, n=8; Oua ●, n=8). Data are expressed as mean±SEM.

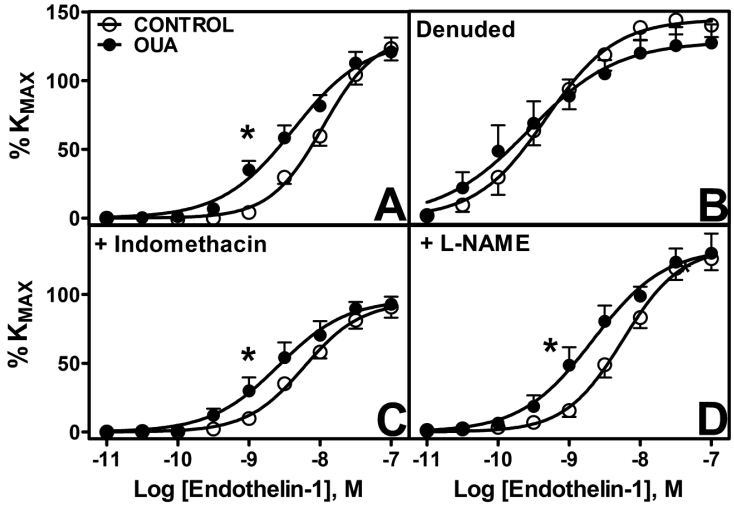

Figure 4. Contractile response to endothelin-1 (ET-1) in mesenteric arteries from control and ouabain-treated rats

A. Untreated arteries (control ♀, n=9; Oua ●, n=8). B. Denuded arteries (control ♀, n=4; Oua ●, n=4). C. Arteries pre-incubated with indomethacin 10^{-5}M (control ♀, n=9; Oua ●, n=8). C. Arteries pre-incubated with L-NAME 10^{-4}M (control ♀, n=8; Oua ●, n=8). Data are expressed as mean±SEM. *, $p<0.05$ in sensitivity *vs* control arteries.

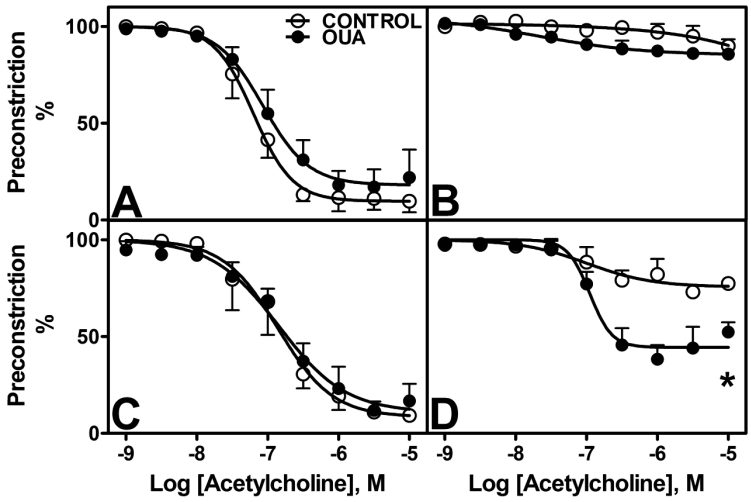

Figure 5. Endothelium-dependent dilatation to acetylcholine (ACh) in MRA from Control and Ouabain-treated rats

A. Untreated arteries (control ♀, n=4; Oua ●, n=4). B. Denuded arteries (control ♀, n=4; Oua ●, n=4). C. Arteries pre-incubated with indomethacin 10^{-5}M (control ♀, n=4; Oua ●, n=4). D. Arteries pre-incubated with L-NAME 10^{-4}M (control ♀, n=4; Oua ●, n=4). Data are expressed as mean±SEM.

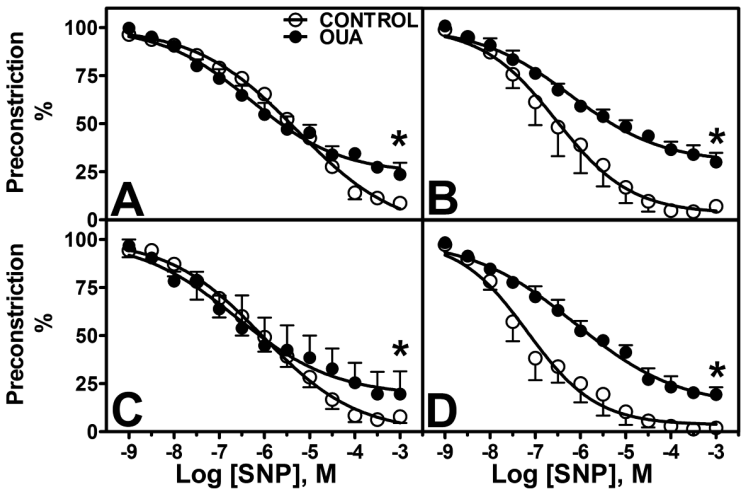

Figure 6. Endothelium-independent dilatation to sodium nitroprusside (SNP in MRA from Control and Ouabain-treated rats

A. Untreated arteries (control ♀, n=4; Oua ●, n=4). B. Denuded arteries (control ♀, n=4; Oua ●, n=4). C. Arteries pre-incubated with indomethacin 10^{-5}M (control ♀, n=4; Oua ●, n=4). D. Arteries pre-incubated with L-NAME 10^{-4}M (control ♀, n=4; Oua ●, n=4). Data are expressed as mean±SEM.

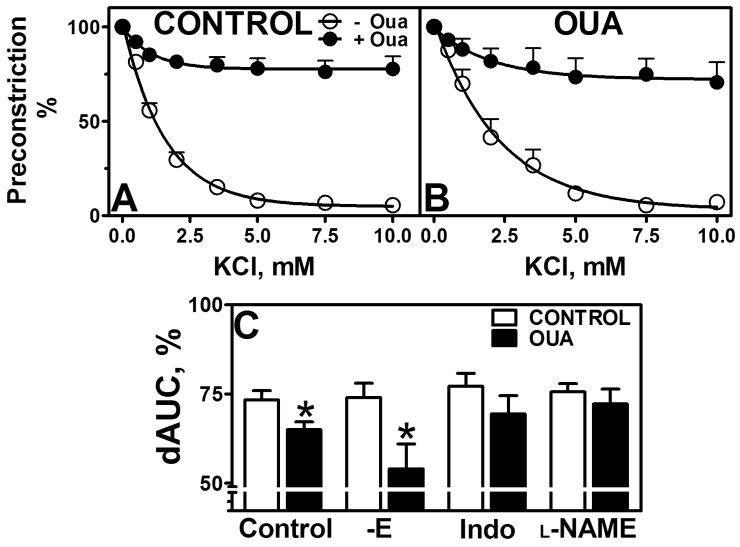

Figure 7. Functional Na^+-K^+ ATPase activity in mesenteric arteries

Functional Na^+-K^+ ATPase activity was measured in MRA from control and ouabain-treated animals as the relaxation to low concentrations of KCl in arteries pre-constricted with 10^{-6}M PE. The specificity of the relaxation was tested by pre-incubation with ouabain 10^{-4}M (−Oua/+Oua). A. Arteries from control animals (n=13, ○ − Oua/● + Oua). B. Arteries from ouabain-treated animals (n=10, ○ −Oua/● + Oua). C. Functional Na^+-K^+ ATPase activity expressed as the difference in area under the curve (dAUC, %) in arteries from control (□) and ouabain-treated animals (■), denuded, and pre-incubated with indomethacin (Indo, 10^{-5}M) or L-NAME (10^{-4}M) as indicated. Data are expressed as mean ±SEM. *, $p<0.05$ *vs* control arteries

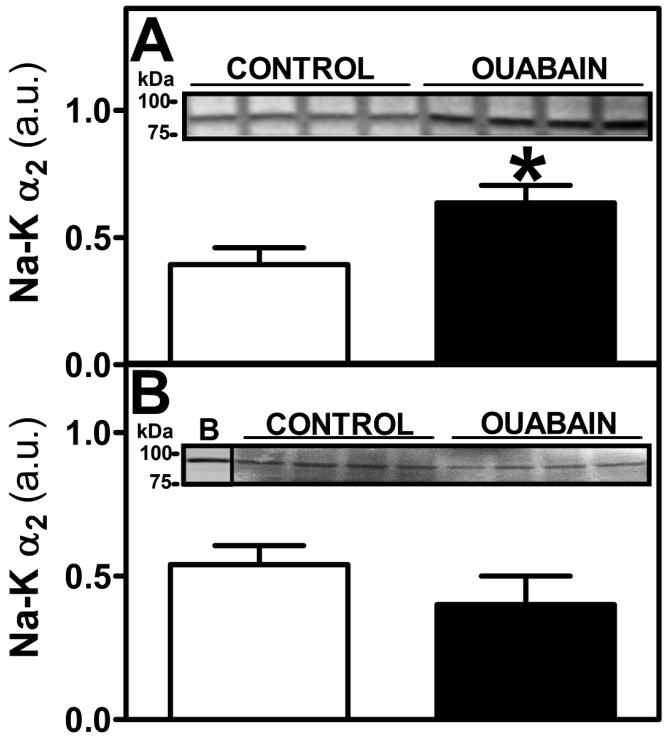

Figure 8. Na^+-K^+ α_2 isoform expression in heart tissue and mesenteric artery

A. Na^+-K^+ α_2 isoform expression was measured by western blot in microsomes from heart tissue obtained from control (□, n=5) and ouabain-treated (**.**, n=5) rats. B. Na^+-K^+ α_2 isoform expression was measured by western blot in total protein extracts of main mesenteric artery obtained from control (□, n=4) and ouabain-treated (**.**, n=4) rats B: Rat brain. Inset: A representative gel is shown. Results from the densitometric analysis are expressed in arbitrary units (a.u.) as described in Materials & Methods. Data are expressed as mean±SEM. *, $p<0.05$ *vs* control.

Table 1

Maximal responses (R_{MAX}) and sensitivity (pD_2) to contractile agonists (potassium chloride (KCl), phenylephrine (PE), and endothelin-1 (ET-1)) in mesenteric arteries from Control and Ouabain-treated (OUA) rats. Results are shown for intact, and denuded arteries and arteries pre-incubated with indomethacin or L-NAME as indicated.

	Intact		Denuded	
	CONTROL	**OUA**	**CONTROL**	**OUA**
K_{MAX}, mN/mm	3.69±0.18	3.51±0.27	3.11±0.6	3.2±0.3
pD_2	1.38±0.01	1.43±0.03*	1.43±0.03†	1.58±0.05†*
PE_{MAX}, % K_{MAX}	107.6±9	116.8±11	127.4±5†	129.4±4†
pD_2	5.96±0.1	6.02±0.16	6.0±0.07	6.11±0.11
ET-1_{MAX}, % K_{MAX}	137±8	126±11	146±10	129±15
pD_2	7.98±0.1	8.48±0.17*	9.3±0.2†	9.6±0.4†
	+ Indomethacin		+ L-NAME	
	CONTROL	**OUA**	**CONTROL**	**OUA**
K_{MAX}, mN/mm	2.51±0.29†	2.3±0.44†	4.23±0.33†	4.1±0.31†
pD_2	1.36±0.03	1.41±0.03	1.44±0.02†	1.44±0.03
PE_{MAX}, % K_{MAX}	78.5±10.3†	68.5±8.5†	117±12.5	119±13.7
pD_2	6.1±0.15	6.02±0.2	6.25±0.06†	6.18±0.17
ET-1_{MAX}, % K_{MAX}	98±9†	100±6†	135±9	136±18
pD_2	8.22±0.06†	8.54±0.2*	8.23±0.08†	8.76±0.2*

*p<0.05 vs control;

†p<0.05 vs intact arteries.

Table 2

Maximal responses (R_{MAX}) and sensitivity (pD_2) to vasodilatory agonists (acetylcholine (Ach) and sodium nitroprusside (SNP)) in mesenteric arteries from Control and Ouabain-treated (OUA) rats. Results are shown for intact and denuded arteries, and arteries pre-incubated with indomethacin or L-NAME as indicated.

	Intact		Denuded	
	CONTROL	**OUA**	**CONTROL**	**OUA**
ACh_{MAX}, %	87±3	84±3	5.4±2.4†	8.7±2.3†
pD_2	7.3±0.1	7.1±0.1	nd	nd
SNP_{MAX}, %	91.4±2	74.8±7*	97.3±2.2	73.6±8*
pD_2	5.5±0.1	5.93±0.4	6.51±0.3†	6.13±0.4
	+ Indomethacin		+ L-NAME	
	CONTROL	**OUA**	**CONTROL**	**OUA**
ACh_{MAX}, %	88±2	92±8	26.3±0.1†	55.6±6.9†*
pD_2	6.93±0.2	6.81±0.1	6.85±0.5	6.87±0.1
SNP_{MAX}, %	93.6±2	69.1±7*	96.2±3	88.2±7
pD_2	6±0.4	6.2±0.2	6.8±0.5†	6.14±0.4

*p<0.05 vs control;

†p<0.05 vs intact arteries.

nd: not determined.