

# Effects of ouabain on vascular reactivity

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## Abstract

Ouabain is an endogenous substance occurring in the plasma in the nanomolar range, that has been proposed to increase vascular resistance and induce hypertension. This substance acts on the  $\alpha$ -subunit of  $\text{Na}^+, \text{K}^+$ -ATPase inhibiting the  $\text{Na}^+$ -pump activity. In the vascular smooth muscle this effect leads to intracellular  $\text{Na}^+$  accumulation that reduces the activity of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and to an increased vascular tone. It was also suggested that circulating ouabain, even in the nanomolar range, sensitizes the vascular smooth muscle to vasopressor substances. We tested the latter hypothesis by studying the effects of ouabain in the micromolar and nanomolar range on phenylephrine (PE)-evoked pressor responses. The experiments were performed in normotensive and hypertensive rats *in vivo*, under anesthesia, and in perfused rat tail vascular beds. The results showed that ouabain pretreatment increased the vasopressor responses to PE *in vitro* and *in vivo*. This sensitization after ouabain treatment was also observed in hypertensive animals which presented an enhanced vasopressor response to PE in comparison to normotensive animals. It is suggested that ouabain at nanomolar concentrations can sensitize vascular smooth muscle to vasopressor stimuli possibly contributing to increased tone in hypertension.

## Key words

- Ouabain
- $\text{Na}^+$ -pump
- Hypertension
- Rat tail vascular bed perfusion
- Arterial blood pressure
- Vascular reactivity
- Phenylephrine

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## Introduction

Recently, ouabain has been described as an endogenous substance occurring in the nM range in the plasma of hypertensive subjects (1-3). These findings have strengthened the proposal that ouabain can increase vascular resistance and induce hypertension (4). Since ouabain inhibits the  $\text{Na}^+$ -pump activity in vascular smooth muscle (VSM) cells, two consequences of this inhibition occur resulting from the intracellular  $\text{Na}^+$  increment (5-7). First, the membrane is depolarized (8,9) and second, the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger activity is reduced (6,7,10). Independent of either effect, the final result is the

increase in intracellular  $\text{Ca}^{2+}$  concentration and an increase in vascular tone. This possibility suggests a putative role of ouabain as a pressor agent. Indeed, a direct ouabain vasoconstrictor effect has been reported (4) but only at high concentrations of this compound. However, a direct hypertensive effect produced by endogenous ouabain is unlikely since very low ouabain concentrations occur in plasma (1). Thus, another mechanism must be proposed to explain the pressor action.

The possible explanation for this effect is that ouabain acts on the  $\alpha$ -subunits of  $\text{Na}^+, \text{K}^+$ -ATPase inhibiting  $\text{Na}^+$ -pump activity (11,12).  $\text{Na}^+, \text{K}^+$ -ATPase is an integral protein of the

plasma membrane, a heterodimer composed of two subunits, a larger catalytic  $\alpha$ -subunit and a heavily glycosylated  $\beta$ -subunit (11,12). This enzyme is a receptor for digitalis compounds that specifically inhibit its activity and occurs as multiple isoforms for  $\alpha$ - and  $\beta$ -subunits with both high and low sensitivities to digitalis inhibition (13), according to their catalytic  $\alpha_1$ -,  $\alpha_2$ - or  $\alpha_3$ -subunits. Recent reports have indicated that the VSM displays all 3 types of  $\alpha$ -subunits of  $\text{Na}^+, \text{K}^+$ -ATPase (14,15), including the high-affinity subunits  $\alpha_2$  and  $\alpha_3$  for ouabain. Consequently, differential effects will depend on the plasma concentration of circulating digitalis and also on the percentage of each enzyme isoform present in tissue.

Since the  $\text{Na}^+$ -pump extrudes  $\text{Na}^+$  from the cells its failure leads to the accumulation of intracellular  $\text{Na}^+$  which is followed by an intracellular  $\text{Ca}^{2+}$  increment (6). Even if the initially induced myoplasmic  $\text{Ca}^{2+}$  increment is small, the amplifier action of the sarcoplasmic reticulum (SR) favors the possibility of an increased vascular tone upon activation (3,16). Recently, it has been shown that even small increments of myoplasmic  $\text{Ca}^{2+}$  may increase vascular tone because of

the amplifier action of the SR (3,16). An indication of this possibility was reported with low ouabain concentrations (10 nM), increasing the constrictor action of caffeine (17) on rat aortic rings and increasing the vasopressor responses to phenylephrine (PE) in the isolated rat tail vascular bed from normotensive and hypertensive rats (18-20). In a previous report we also showed that pretreatment with subtherapeutic doses of ouabain enhances PE vasopressor effects in anesthetized normotensive and hypertensive rats (21,22). If this mechanism is operative small concentrations of ouabain would increase the vascular resistance by smooth muscle sensitization to vasopressor substances. This possibility suggests that circulating ouabain may increase vascular resistance by smooth muscle sensitization to vasopressor substances even at very low concentrations.

## Material and Methods

### *In vitro* experiments

Rat tail vascular beds obtained from normotensive male Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats, weighing 300-400 g, were used in this study. Five to nine rats were used for each experimental group.

The day before the experiments the systolic blood pressure (SBP) of the rats was measured using the tail-cuff method (IITC Model 29 Pulse Amplifier, IITC Inc., CA).

For the perfusion experiments the rats were anesthetized with sodium pentobarbital (35 mg/kg, *ip*) and received 100 units of heparin. The tail artery was then dissected and cannulated near the insertion of the tail using stretched PE 50 tubing. The preparations were perfused (Ismatec Reglo pump) with Krebs-Henseleit (KH) bicarbonate buffer solution, pH 7.4, bubbled with 5%  $\text{CO}_2$ -95%  $\text{O}_2$ , containing 27.2 mM  $\text{NaHCO}_3$ , 119 mM  $\text{NaCl}$ , 1 mM  $\text{NaH}_2\text{PO}_4$ , 1.2 mM

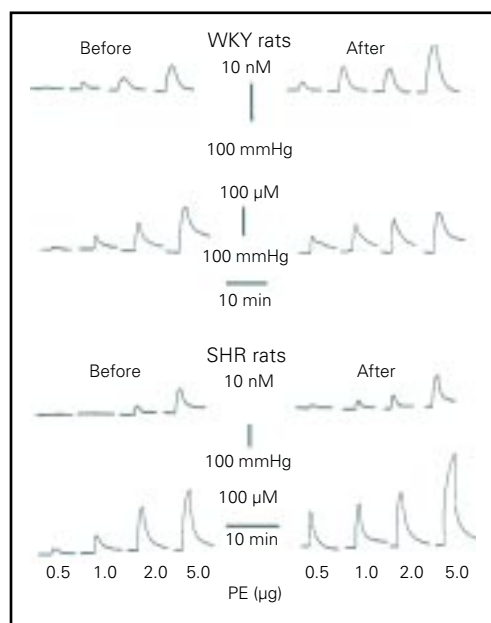


Figure 1 - Typical tracings demonstrating the effects of 100  $\mu\text{M}$  and 10 nM ouabain treatment on the phenylephrine (PE)-induced vasopressor changes in rat tail vascular beds from normotensive Wistar Kyoto (WKY) and hypertensive (SHR) rats. Observe the sensitization to the PE pressor response that occurs after ouabain treatment.

MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 11 mM glucose, 5 mM KCl and 0.03 mM EDTA, at a constant flow of 2.5 ml/min. After a 30- to 45-min stabilization period the experimental protocol was initiated. Randomized bolus doses of phenylephrine (0.5, 1, 2 and 5 µg, in 0.1 ml) were injected and the developed pressure was measured with a COBE CDX pressure transducer connected to a Grass 7P16 preamplifier and recorded with a Grass 7 recorder. Since a constant flow was maintained the changes in pressure were a direct indication of vascular resistance. The preparations were then continuously perfused with KH plus ouabain (100 µM or 10 nM for 30 min and 60 min, respectively) and the same protocol was repeated.

#### ***In vivo* experiments**

Studies were performed in 22 male Wistar normotensive rats (NR), weighing 200 to 380 g, and 24 SHR, weighing 190 to 350 g.

The day before the experiments systolic blood pressure of the rats was measured using the tail-cuff method (IITC Model 29 Pulse Amplifier, IITC Inc.). Rats were anesthetized with urethane (1.2 g/kg, *ip*) supplemented when necessary. The jugular vein and the carotid artery were dissected for drug infusion and arterial blood pressure measurements. ECG was also measured using standard procedures.

Vasopressor reactivity was tested by injecting increasing concentrations of PE (0.1, 0.25 and 0.5 µg) administered in small volumes (5 µl or less), before and 1 h after ouabain treatment. The rats were divided into 3 groups and each group was submitted to one ouabain treatment, 18, 1.8 and 0.18 µg/kg, respectively, for 1 h. SBP, diastolic blood pressure (DBP) and ECG were monitored continuously throughout the experiments.

Data were analyzed statistically by ANOVA and the Student *t*-test for small samples. A significant ANOVA was fol-

lowed by Tukey's *post hoc* test. The results are reported as means ± SEM and were considered to be significant at the 5% level.

## **Results**

#### ***In vitro* experiments**

Figure 1 shows the sensitizing effect of 100 µM ouabain on PE-evoked vasoconstrictor responses in the tail vascular bed from normotensive and hypertensive rats. Before ouabain pretreatment the developed perfusion pressure increased with PE concentration. The continuous perfusion of 100 µM ouabain sensitized the vessels increasing the vasoconstrictor responses (20). However, since circulating ouabain concentration in the plasma of hypertensive subjects is approximately 10 nM (1,2) the same protocol was repeated after 60 min pretreatment with 10 nM ouabain in other tail vascular beds isolated from normotensive and hypertensive rats. A similar result is shown in Figure 1 where it is seen that the sensitization process occurred at a lower level after 10 nM ouabain. It is interesting to note that normalizing the results obtained before and after 10 nM ouabain the percentage increase in pressure was higher in SHR (Figure 2).

#### ***In vivo* experiments**

Normotensive rats (SBP = 126 ± 1.98 mmHg) and SHR (SBP = 177 ± 3.76 mmHg) were used for these studies. Figure 3 shows the vascular reactivity represented by PE-evoked pressor responses from NR and SHR pooled together before ouabain treatments. SHR displayed a higher systolic and diastolic blood pressure than NR and systolic PE-evoked pressor responses were higher in the SHR group.

#### **18 µg/kg ouabain treatment**

SBP increased in both NR and SHR

groups after ouabain treatment (18  $\mu\text{g}/\text{kg}$ ) and SBP values were higher in the SHR group. PE pressor responses occurred for both groups but were higher in the SHR group. DBP values were also higher in the SHR group after ouabain treatment but similar for NR (Figure 4).

Figure 2 - Effect of 10 nM ouabain percent changes above baseline perfusion pressure on phenylephrine-induced pressor responses in isolated perfused rat tail vascular beds from hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats. Points represent means  $\pm$  SEM. \* $P < 0.05$  for comparison between before (triangles) and after (circles) 10 nM ouabain treatment (ANOVA).

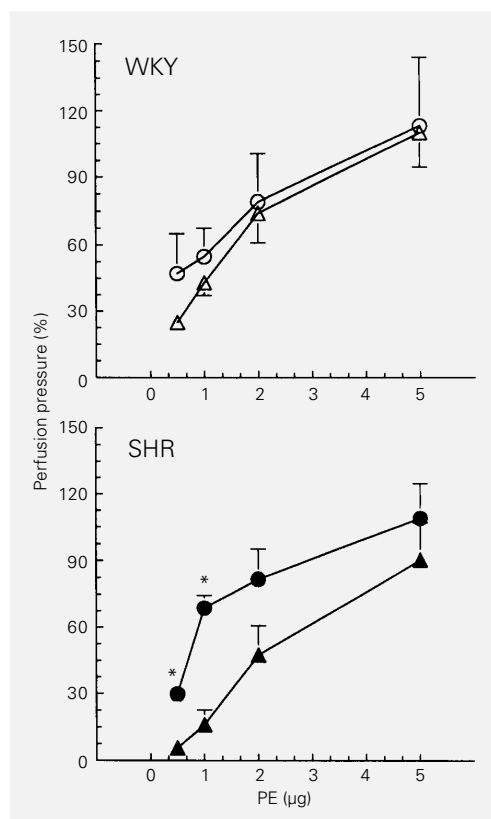
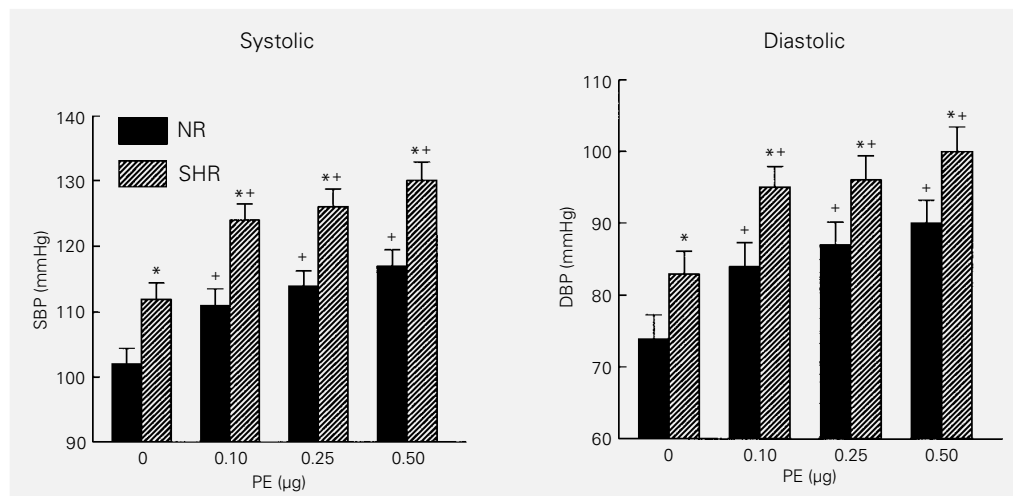


Figure 3 - Effect of phenylephrine (PE) on the systolic blood pressure (SBP) and diastolic blood pressure (DBP) of normotensive (NR) and hypertensive (SHR) rats. \* $P < 0.01$  for comparison between NR and SHR; + $P < 0.01$  for comparison between PE and controls (ANOVA).



### 1.8 $\mu\text{g}/\text{kg}$ ouabain treatment

Figure 5 shows the changes of SBP and DBP in NR and SHR groups before and after ouabain treatment. In the NR group PE evoked similar pressor responses for both SBP and DBP before and after ouabain treatment. In contrast, SHR showed an increase in SBP and DBP after ouabain treatment and PE pressor effects were enhanced.

### 0.18 $\mu\text{g}/\text{kg}$ ouabain treatment

With this dose nanomolar levels of circulating ouabain might be attained and still produce similar effects as those observed with the 1.8  $\mu\text{g}/\text{kg}$  ouabain dose (Figure 6). Again, very low doses of ouabain enhanced the PE-evoked pressor response of SHR, showing that these animals are more sensitive to ouabain.

## Discussion

The existence of a plasma natriuretic factor that inhibits  $\text{Na}^+, \text{K}^+$ -ATPase has been suspected for many years. A few years ago ouabain, a steroid compound that has been identified in plasma and tissue extracts and in the adrenal cortex, has been suggested as the source of this compound (1-3).

Ouabain inhibits the  $\text{Na}^+$ -pump by bind-

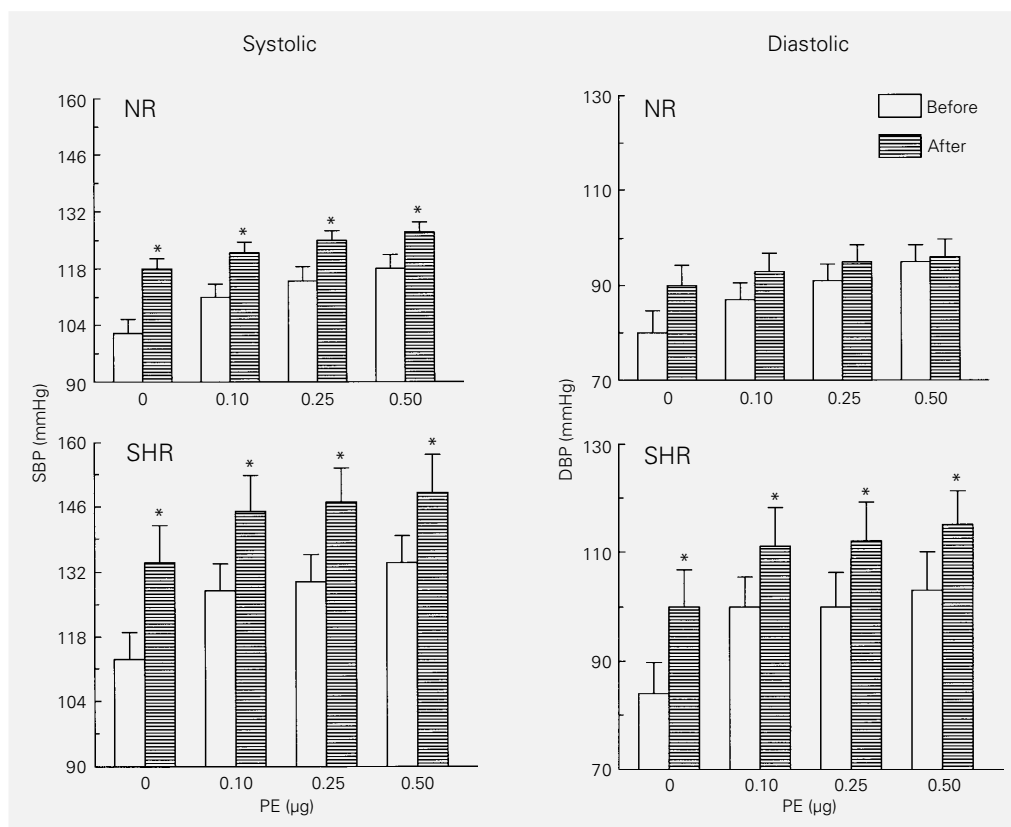


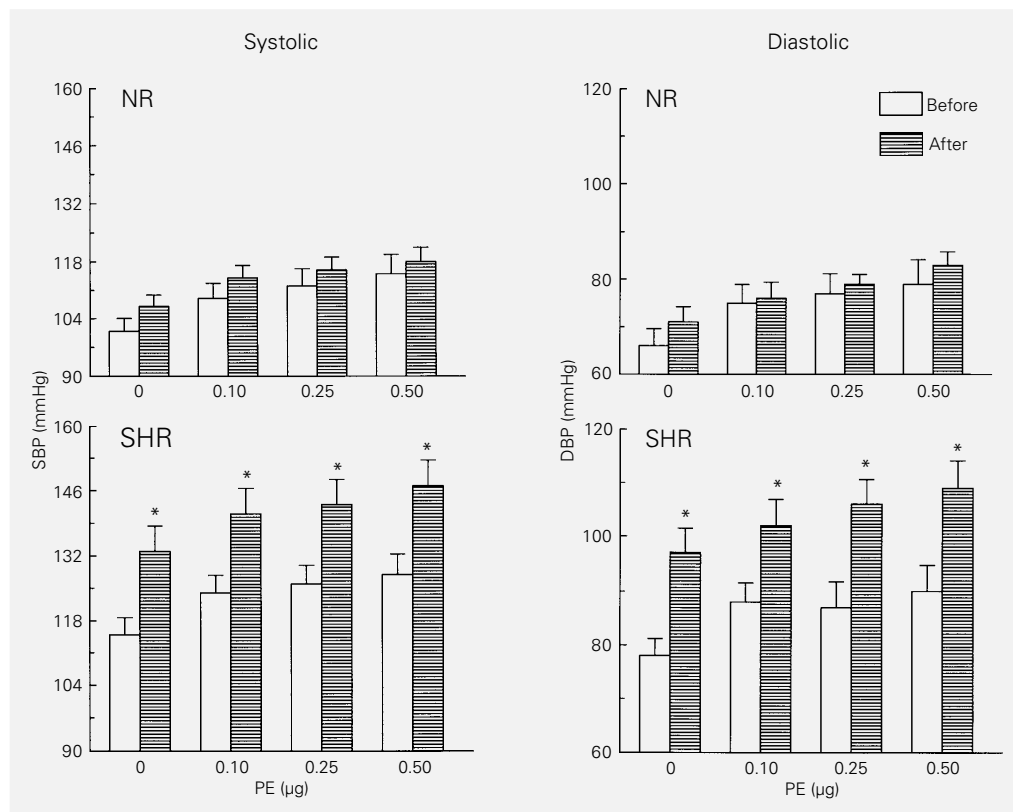
Figure 4 - Effects of increasing doses of phenylephrine (PE) on the systolic blood pressure (SBP) and diastolic blood pressure (DBP) of normotensive (NR) and hypertensive (SHR) rats before and after 1-h treatment with 18 µg/kg ouabain. Column height indicates the mean and the bar the SEM. \*P<0.05 for comparison between before and after ouabain treatment (ANOVA).

ing to the  $\alpha$ -subunits of the enzyme (11,12). Since the heart, vessels, kidneys and nervous tissue carry at least two isoforms of the enzyme (12-14), profound effects on homeostatic mechanisms may be produced by ouabain. One of these mechanisms is the regulation of blood pressure. By inhibiting the  $\text{Na}^+$ -pump of VSM ouabain increases the intracellular  $\text{Na}^+$  concentration leading to two main effects, membrane depolarization and reduced  $\text{Na}^+/\text{Ca}^{2+}$ -exchanger activity (6-9). Both effects result in an increase in intracellular  $\text{Ca}^{2+}$  concentration and vascular tone. Having such actions ouabain could play a central role in the development of hypertension (23). Many reports suggested such a role since ouabain can produce hypertension during long-term administration in rats (4) and potentiate serotonin-evoked contractions of aortic rings (17). However, a direct pressor effect is unlikely since plasma ouabain concentrations occur at nanomolar levels

(1). Despite these considerations, ouabain could also increase vascular tone by other mechanisms. Recently, it has been shown that small increments of myoplasmic  $\text{Ca}^{2+}$  may produce increased vascular tone because of the amplifier action of the SR (3,16). The possibility of such occurrence has been shown recently with low ouabain concentrations (10 nM) that increased caffeine-evoked contractions in rat aortic rings (17).

We tested the hypothesis that ouabain increases vascular resistance by smooth muscle sensitization to vasopressor substances using a perfused rat tail vascular bed preparation from normotensive and hypertensive rats. This preparation displayed an intrinsic basal tone and a high sensitivity to vasopressor substances. Also, since many *in vitro* experiments, both biochemical or physiopharmacological, have been performed with the rat tail artery, the present preparation permits a correlation between

Figure 5 - Effects of increasing doses of phenylephrine (PE) on the systolic blood pressure (SBP) and diastolic blood pressure (DBP) of normotensive (NR) and hypertensive (SHR) rats before and after 1-h treatment with 1.8  $\mu\text{g}/\text{kg}$  ouabain. Column height indicates the mean and the bar the SEM. \* $P < 0.05$  for comparison between before and after ouabain treatment (ANOVA).



these studies and functional experiments (14,24,25).

We also tested this hypothesis *in vivo* using anesthetized normotensive and hypertensive rats. The results showed that in both rat strains ouabain pretreatment affected the pressor responses to PE. Without any treatment SHR displayed higher values of SBP and DBP both before and after PE-evoked pressor responses.

All ouabain treatments increased SBP and DBP and enhanced PE-evoked pressor responses in SHR. However, NR only showed this response after 18  $\mu\text{g}/\text{kg}$  ouabain treatment. The lower doses (1.8 and 0.18  $\mu\text{g}/\text{kg}$ , subtherapeutic doses) did not change the arterial blood pressure and had no effects on the PE-evoked pressor responses. These results suggest that a) SHR display higher arterial blood pressure levels than NR even during PE-evoked pressor responses, b) ouabain sensitizes SHR and NR to PE-evoked pressor responses when therapeutic doses

are used, and c) subtherapeutic doses of ouabain only show effects in the SHR strain.

The expression of  $\text{Na}^+, \text{K}^+$ -ATPase and its  $\alpha$ -isoform is known to be regulated by multiple factors such as developmental stages (26), hormones (27) and the occurrence of hypertension (15). Also, it has been shown that  $\alpha$ -subunits of  $\text{Na}^+, \text{K}^+$ -ATPase consist of different  $\alpha_1$ -,  $\alpha_2$ - and  $\alpha_3$ -subunits with different affinities for digitalis (13). Since the  $\text{Na}^+, \text{K}^+$ -ATPase isoforms may change in several situations, the maintenance of the sensitizing mechanism could be of clinical relevance. In the present study we investigated if the sensitization mechanism is operative in normotensive and hypertensive rats. The results presented here suggest that the sensitization mechanism is operative in both normotensive and hypertensive animals.

A possible explanation for this behavior may be the existence of a similar or increased abundance of the  $\alpha_3$ -isoform in both normotensive and hypertensive rat tail vas-

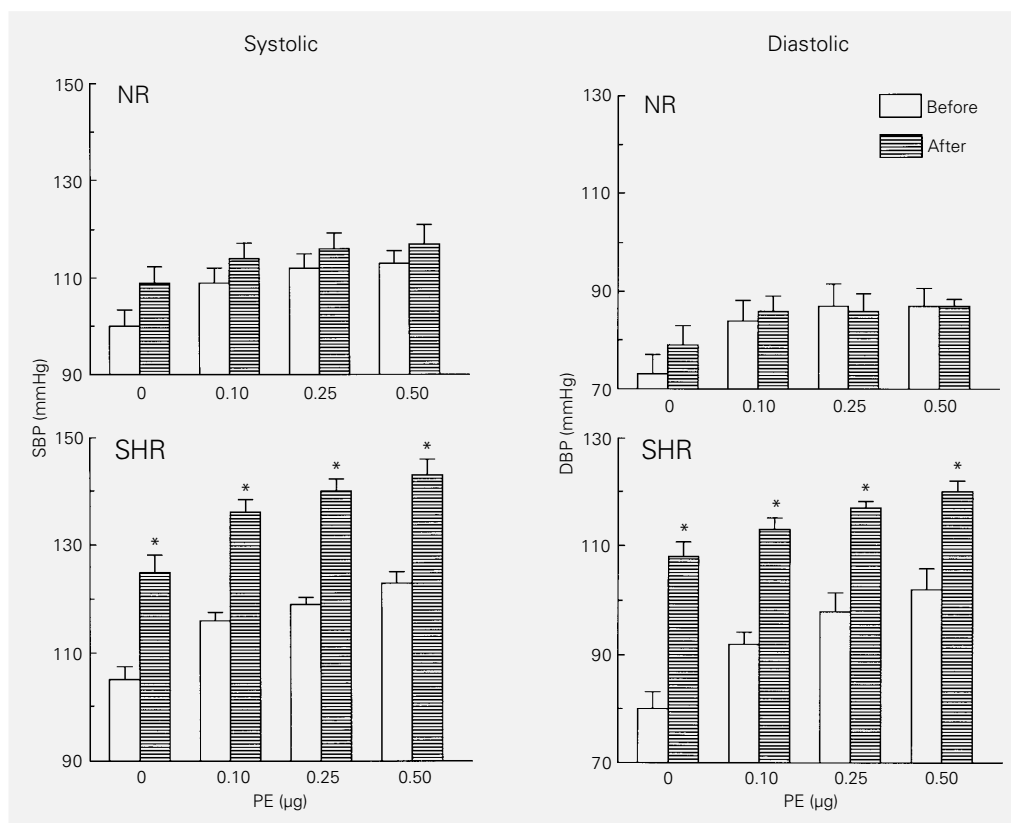


Figure 6 - Effects of increasing doses of phenylephrine (PE) on the systolic blood pressure (SBP) and diastolic blood pressure (DBP) of normotensive (NR) and hypertensive (SHR) rats before and after 1-h treatment with 0.18 µg/kg ouabain. Column height indicates the mean and the bar the SEM. \*P<0.05 for comparison between before and after ouabain treatment (ANOVA).

cular beds since ouabain binds preferentially to this isoform. However, to our knowledge only Songu-Mize et al. (28) and Herrera et al. (15) presented evidence showing upregulation of the Na<sup>+</sup>-pump activity and of α<sub>1</sub>-mRNA in the rat tail artery and aorta, respectively, together with a downregulation of the α<sub>2</sub>-mRNA as a result of DOCA-salt hypertension. An increase in the activity of the Na<sup>+</sup>-pump would favor relaxation during hypertension. This increased activity even resulting from the α<sub>1</sub>-isoform may be inhibited

by ouabain at levels sufficient to produce or maintain the sensitization mechanism. Our results, however, cannot discriminate which mechanism is responsible for this sensitization effect.

We conclude that the sensitization process to vasopressor substances that occurs at nanomolar ouabain concentrations in plasma is operative even in hypertensive animals and may contribute to the increase and maintenance of vascular tone in hypertension.

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