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# Effects of ouabain on the pressor response to phenylephrine and on the sodium pump activity in diabetic rats

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**Abstract**

The diabetes mellitus insulin-dependent is usually associated with cardiovascular disorders and with changes in the activity of the $Na^+,K^+$-ATPase. The effects of ouabain, a $Na^+,K^+$-ATPase inhibitor, on the pressor response of 7-day streptozotocin-induced diabetes were investigated in anesthetized rats and on the vascular reactivity of the perfused rat tail vascular bed. Diabetes was characterized by hyperglycemia ($86 \pm 7.8$ vs. $471 \pm 18.5$ mg/dl) without changes in arterial blood pressure. Blood pressure increased after the treatment with 18 μg/kg ouabain in controls but not in diabetic rats; acute hyperglycemia, in non-treated rats, did not change these effects. Control tail vascular beds showed increased maximal response to phenylephrine after treatment with 10 nM ouabain for 1 h; this response was abolished in streptozotocin-treated rats. These rats showed an increased sensitivity to phenylephrine without changing the maximal vasoconstrictor response when compared to control rats. The relaxation induced by acetylcholine was reduced in diabetic rats. The functional activity of the $Na^+,K^+$-ATPase was inhibited in vascular beds from diabetic rats, when compared to control rats, and the inhibition of the $Na^+,K^+$-ATPase with 10 nM ouabain was not effective in these rats. Results suggested that in 7-day diabetic rats, the increase of arterial blood pressure or the sensitization of the vascular bed produced by ouabain is lost as a consequence of the reduction of the functional activity of the $Na^+,K^+$-ATPase probably as a result of insulin lack and a deficient endothelial nitric oxide activity. 

*Keywords:* Diabetes; Ouabain; $Na^+,K^+$-ATPase; Phenylephrine; Vascular reactivity; Streptozotocin; Nitric oxide; (Rat)

## 1. Introduction

The diabetes mellitus insulin-dependent is pathology characterized by hyperglycemia resulting from a lack of insulin secretion by the pancreatic B-cells (Öztürk et al., 1996). Also, it has been reported that cardiovascular disorders, such as hypertension, are associated to diabetes (Epstein and Sowers, 1992; Landsberg 1992; Moreau et al., 1995). Moreover, insulin has been shown to modulate vascular reactivity inducing vasodilatation (Zemel et al., 1992; Öztürk et al., 1996). This effect is partially linked to the activation of the $Na^+,K^+$-ATPase which seems to contribute to the vasodilator effect of insulin (Ewart and Klip, 1995; Gupta et al., 1996; Tack et al., 1996).

In diabetic rats, besides lack of insulin, the concentration of plasma ouabain increases (Chen et al., 1993a,b). This endogenous compound is an inhibitor of the $Na^+,K^+$-ATPase (Hamlyn et al., 1982; Blaustein, 1993) and was characterized as an isomer of the digitalis compound ouabain (Mathews et al., 1991).

The cell membrane enzyme $Na^+,K^+$-ATPase is the biochemical expression of the electrogenic $Na^+$-pump, and exchanges 3 $Na^+$ for 2 $K^+$ maintaining the resting potential of excitable cells (Fleming, 1980; Lingrel, 1992; Skou and Esmann, 1992). The lack of insulin reduces the amount of units of $Na^+,K^+$-ATPase in the membrane, reducing the $Na^+$-pump activity (Vér et al., 1997). Moreover, the association with increased plasma levels of ouabain would produce a further reduction in the $Na^+$-pump activity leading to $Na^+$ accumulation in the myoplasm (Blaustein, 1988; Blaustein et al., 1991). This condition reduces the activity of the $Na^+/Ca^{2+}$ exchange mechanism and, consequently, increases the amount of activator $Ca^{2+}$ accessible for contraction in several tissues, such as the vascular smooth muscle (Rembold et al., 1992). Since insulin affects the activity of the $Na^+,K^+$-ATPase in several tissues (Raccah et al., 1996; Vér et al., 1997; Bányász and Kovács,

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1998), including the vascular smooth muscle (Gupta et al., 1996; Tack et al., 1996), its lack, associated with the plasma ouabain increment, would influence mechanisms which modulate vascular reactivity and blood pressure, therefore playing a central role in the development and maintenance of hypertension.

In this study, pressor responses to phenylephrine were investigated, before and after ouabain treatment, together with the evaluation of the functional activity of the $Na^+,K^+$-ATPase, in anesthetized animals and in the perfused rat tail vascular bed from control and streptozotocin-induced diabetic rats.

## 2. Material and methods

Studies were performed on male Wistar normotensive rats, weighing 200–300 g. The care and use of the laboratory animals were in accordance with NIH guidelines. All rats had free access to water and were fed with rat chow ad libitum.

Two main groups of rats were used: controls and 7-day streptozotocin-treated rats (50 mg/kg of rat diluted in 0.1 M citrate buffer solution, pH 4.5, i.v. single injection). Blood glucose was measured by using a hemoglucotest before and after streptozotocin treatment. Animals which did not develop hyperglycemia were excluded. All animals had their blood pressure measured in vivo by using a tail cuff method (IITC Model 29 Pulse Amplifier, IITC, CA).

With the protocols described below, we investigated changes of pressor responses to phenylephrine in anesthetized rats and in tail vascular beds using ouabain. With these doses or concentrations this digitalis compound enhances vascular reactivity, as previously reported (Songu-Mize et al., 1995; Vassallo et al., 1997; Rossoni et al., 1999a,b).

### 2.1. Anesthetized animals

Rats ($N = 18$) were anesthetized with urethane (1.2 g/kg, i.p.) supplemented when necessary. The jugular vein and the femoral artery were dissected for drug infusion and arterial blood pressure measurements, respectively. Arterial blood pressure was measured by using a pressure transducer (Gold P23XL) connected to a MP 100 (FUNBEC, Sao Paulo) amplifier and recorded on a polygraphic recorder (RG 300, FUNBEC, Sao Paulo).

#### 2.1.1. Protocol 1: effects of ouabain on the arterial blood pressure and phenylephrine induced pressor response in anesthetized rats

The following protocol was used in two groups; controls ($N = 6$) and streptozotocin-treated rats ($N = 6$). Systolic and diastolic blood pressures were monitored continuously. After a 30-min stabilization period, the pressor reactivity was tested by injecting increasing concentrations of phenylephrine (0.1, 0.4, 0.8 and 1.6 μg) administered in small volumes (5 μl or less). To investigate if the effects of ouabain in diabetic rats affect the arterial blood pressure and the pressor response to phenylephrine, 18 μg/kg ouabain was administered. According to previous reports (Vassallo et al., 1997), this dose is enough to increase arterial blood pressure in normotensive rats. The same increasing concentrations of phenylephrine were readministered in controls or in streptozotocin-treated rats 1 h after the treatment with ouabain.

#### 2.1.2. Protocol 2: effects of hyperglycemia on the pressure response of ouabain and phenylephrine-induced pressor response in anesthetized rats

To verify if hyperglycemia could have a direct effect on the arterial blood pressure or on the phenylephrine pressor reactivity, a similar protocol was performed in control, non-treated rats before and after ouabain treatment. Acute hyperglycemia was produced by the administration of glucose (30%), in a volume equivalent to 10% of the blood volume (considered as 1 ml/100 g of body weight) ($N = 6$). Blood glucose was assayed by using hemoglucotest and hyperglycemia was maintained for more than 3 h.

### 2.2. Pressor response of the rat tail vascular bed

Tail vascular beds obtained from 22 male albino Wistar rats were used in this study. For the perfusion experiments, the rats were anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and received heparine (500 UI, i.p.). After 10 min, a 1-cm strip of the tail artery was dissected free and cannulated near the base of the tail using a safelet cath 24 $G \times 3/4$ in. (Nipro). The rat tail vascular bed was perfused with Krebs–Henseleit solution (120 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, 2 mM $NaH_2PO_4$, 27 mM $NaHCO_3$, and 11 mM glucose) plus EDTA — 0.03 mM, bubbled with 5% $CO_2$–95% $O_2$, at $36 \pm 0.5$°C, using a peristaltic pump (Milan, Colombo) at a constant flow of 2.5 ml/min. The tail was then severed from the body and placed in a tissue bath containing the same Krebs buffer. After a 30- to 45-min equilibration period, the experimental protocol was initiated. The baseline perfusion pressure was measured by using a pressure tranducer (TP-200T, Nihon-Kohden) and recorded on a polygraphic recorder (ANAMED, AM-820). Since a constant flow was maintained, the changes in the mean perfusion pressure represented changes in vascular resistance.

#### 2.2.1. Protocol 1: effects of ouabain on the phenylephrine-induced pressor response

The experimental protocol consisted of a dose–response curve to phenylephrine (0.1, 0.3, 1, 3, 10, 30 and 100 μg), administered as bolus injections of 100 μl in control conditions and repeated after 1 h under a continuous infusion of Krebs plus 10 nM ouabain. At the end of the experiments, preparations were pre-contracted with a con-

Table 1
Values of blood pressure, blood glucose and body weight in control and 7-day streptozotocin-induced diabetic rats

| | Control | Diabetic |
|---|---|---|
| Blood pressure (mm Hg) | 128 ± 2.40 | 133 ± 2.73 |
| Blood glucose (mg/dl) | 85.7 ± 7.82 | 471 ± 18.5[a] |
| Body weight (g) | 273 ± 7.82 | 247 ± 8.62[b] |

Numbers in each cell represent the mean ± SEM.
[a] $P < 0.01$ ($t$-test, control vs. diabetic rats).
[b] $P < 0.05$ ($t$-test, control vs. diabetic rats).

tinuous infusion of phenylephrine ($10^{-7}$ M) and endothelial integrity was tested by using a bolus injection of acetylcholine (5 μg in 100 μl). This protocol was performed in both controls ($N = 6$) and streptozotocin-treated ($N = 6$) rats.

*2.2.2. Protocol 2: measurement of the functional activity of $Na^+$,$K^+$-ATPase before and after ouabain treatment*

To assess the functional activity of the $Na^+$,$K^+$-ATPase, the technique described by Webb and Bohr (1978) was used. The functional activity of the $Na^+$,$K^+$-ATPase was assayed in two groups of tail vascular beds obtained from controls ($N = 6$) and streptozotocin-treated ($N = 6$) rats. Tails were obtained and perfused as described before. After a 30-min equilibration period, under perfusion with 5.4 mM $K^+$, the nutrient solution was changed to 0 $K^+$. The tails were perfused for 30 min in this condition and then the preparations were precontracted with $10^{-7}$ M phenylephrine. Once a plateau of mean perfusion pressure was attained, the extracellular $K^+$ concentration was increased in steps (1, 2, 4 and 6 mM) each one of 5-min duration. This protocol was repeated after a 1-h treatment with 10 nM ouabain.

*2.3. Drugs and reagents used*

Streptozotocin, ouabain, phenylephrine, acetylcholine and urethane were purchased from Sigma; heparin was purchased from Roche and sodium pentobarbital from Fontoveter.

*2.4. Statistical analysis*

Results are presented as means ± S.E.M. Regarding perfusion pressure measurements, results are presented as changes in the perfusion pressure, subtracting peak pressure from baseline pressure. Non-linear regression analysis determination of the maximum change in pressure ($E_{max}$) and the logarithm of the dose producing one-half of $E_{max}$

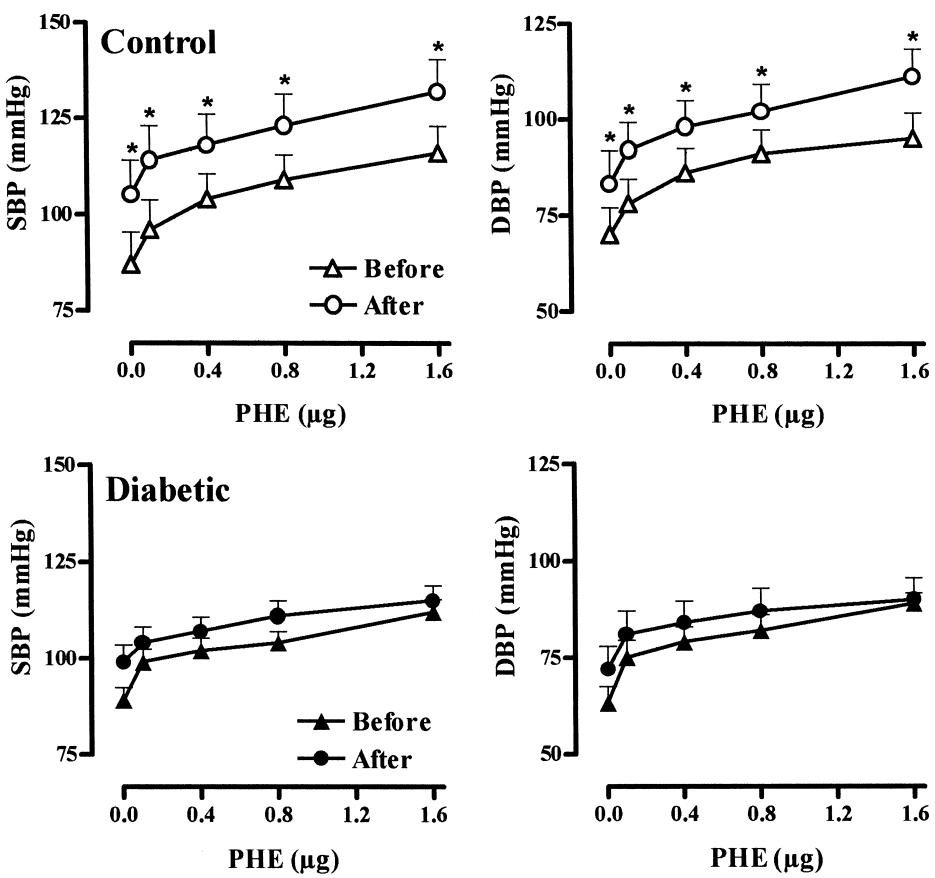


Fig. 1. Effects of increasing doses of phenylephrine (PHE) on the systolic (SBP) and diastolic (DBP) blood pressure before (triangles) and 60 min after (circles) ouabain (18 μg/kg) administration in control (open symbols) and streptozotocin-induced (filled symbols) diabetic rats. Each point represents the mean ± S.E.M. * $P < 0.05$, two-way ANOVA, before vs. after ouabain.

Table 2
Effects of hyperglycemia on the pressor effects of phenylephrine before and after treatment with 18 μg/kg ouabain

| Phenylephrine (μg) | | Systolic pressure (mm Hg) | | Diastolic pressure (mm Hg) | |
|---|---|---|---|---|---|
| | | Control | HG | Control | HG |
| 0 | Before | 87±8.4 | 91±4.8 | 70±7.1 | 66±5.2 |
| | After | 105±9[a] | 111±4.6[a] | 83±7.2[a] | 84±4.5[a] |
| 0.2 | Before | 96±7.7 | 97±4.2 | 78±6.5 | 72±5.4 |
| | After | 114±9[a] | 116±5.3[a] | 92±7.2[a] | 89±4.4[a] |
| 0.4 | Before | 104±6.6 | 102±4 | 86±6.4 | 76±5.2 |
| | After | 118±8[a] | 120±5.1[a] | 98±6.9[a] | 91±4.7[a] |
| 0.8 | Before | 109±6.5 | 105±3.5 | 91±6.3 | 79±5 |
| | After | 123±8.3[a] | 122±5.6[a] | 102±7.4[a] | 93±4.5[a] |
| 1.6 | Before | 116±7 | 113±4.3 | 95±6.6 | 86±4.8 |
| | After | 132±8.4[a] | 129±4.8[a] | 111±7.4[a] | 99±4.8[a] |

Before — control condition before ouabain; after — condition after ouabain treatments; control — normoglycemic rats; HG — hyperglycemic rats. Observe that there are no differences in the systolic or diastolic blood pressure among means of control and hyperglycemic rats. In each cell, the number represents the mean±S.E.M.

[a] $P < 0.01$ (two-way ANOVA, before vs. after ouabain).

(log $EC_{50}$) was employed to obtain fitting curves using the GraphPad Prism Software (San Diego, CA). Data were analyzed using the Student's *t*-test and a repeated measures analyses of variance (ANOVA) followed by a post-hoc protected *t*-test (Tukey test). $P < 0.05$ was considered significant.

## 3. Results

All rats used in these experiments were normotensive (Table 1). Streptozotocin-induced diabetes was characterized by the presence of hyperglycemia obtained by treating rats for 7 days with streptozotocin, and the body weight of diabetic rats was smaller than controls (Table 1).

### 3.1. Anesthetized animals

Fig. 1 shows the pressor responses to phenylephrine obtained in control and streptozotocin-treated rats before and after 1 h treatment with 18 μg/kg ouabain. Systolic and diastolic blood pressures increased after ouabain treatment in control rats and phenylephrine pressor responses were maintained at these higher blood pressures. In streptozotocin-treated rats, no changes in pressor responses to phenylephrine were observed in the pre-ouabain condition

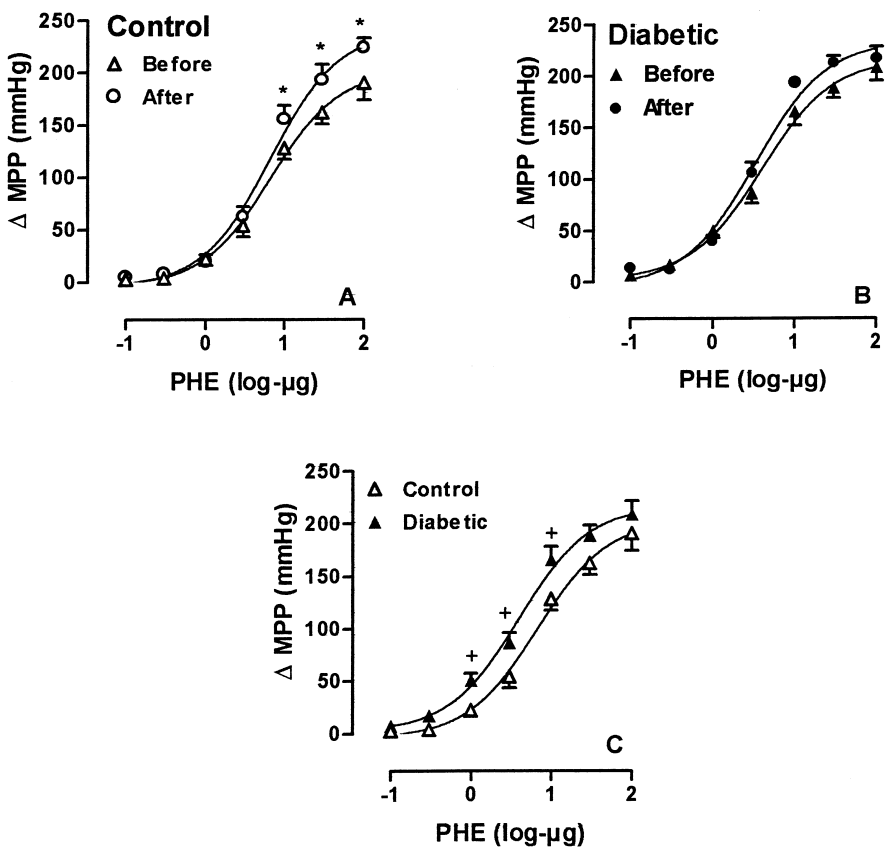


Fig. 2. Dose–response curves to phenylephrine (PHE) in the tail vascular bed from control and streptozotocin-induced diabetic rats. Upper panels (A and B) show effects of increasing doses of PHE on the change in the mean perfusion pressure (Δ MPP) before (triangles) and 60 min after (circles) the infusion with 10 nM ouabain in control (open symbols) and streptozotocin-induced (filled symbols) diabetic rats. Lower panel (C) shows the comparison between dose–response curves to PHE from control and diabetic rats before ouabain treatment. Each point represents the mean ± S.E.M. Two-way ANOVA, * $P < 0.05$, before vs. after ouabain and + $P < 0.05$, control vs. diabetic rats. Observe that ouabain sensitizes the tail vascular bed to pressor responses of PHE in control but not in diabetic rat tail vascular beds.

Table 3
Effects of 7-day streptozotocin-induced diabetes on the pressor effects of acetylcholine (Ach) and on the parameters of the dose–response curve ($E_{max}$ — maximal response and log $EC_{50}$ — sensitivity) of phenylephrine, before and 60 min after treatment with 10 nM ouabain

| | Control | Control + ouabain | Diabetic | Diabetic + ouabain |
|---|---|---|---|---|
| Relaxation to Ach (%) | 71.3 ± 5.6 | | 49.8 ± 5.6[a] | |
| $E_{max}$ of phenylephrine (mm Hg) | 193 ± 14.3 | 227 ± 9.4[b] | 215 ± 11.2 | 220 ± 10.3 |
| log $EC_{50}$ of phenylephrine | 0.806 ± 0.1 | 0.762 ± 0.79 | 0.566 ± 0.57[a] | 0.467 ± 0.05 |

Numbers in each cell represent the mean ± S.E.M.
[a] $P < 0.01$ (*t*-test, control vs. diabetic).
[b] $P < 0.01$ (*t*-test, control vs. control + oubain).

compared to controls. However, in this group, the treatment with ouabain did not increase systolic nor diastolic blood pressure and phenylephrine pressor responses were similar to the pre-ouabain condition.

To verify if this behavior of streptozotocin-treated rats was dependent on the increased plasma glucose concentration, hyperglycemia was induced by acute administration of glucose (30% solution), in a volume equivalent to 10% of the estimated blood volume of the rats. In this situation, the effects of ouabain on the blood pressure and pressor responses to phenylephrine did not differ from those observed in control rats (Table 2). The basal blood glucose of the anesthetized rats was 114 ± 2.44 mg/dl and increased significantly to 580 ± 91 mg/dl after glucose administra-

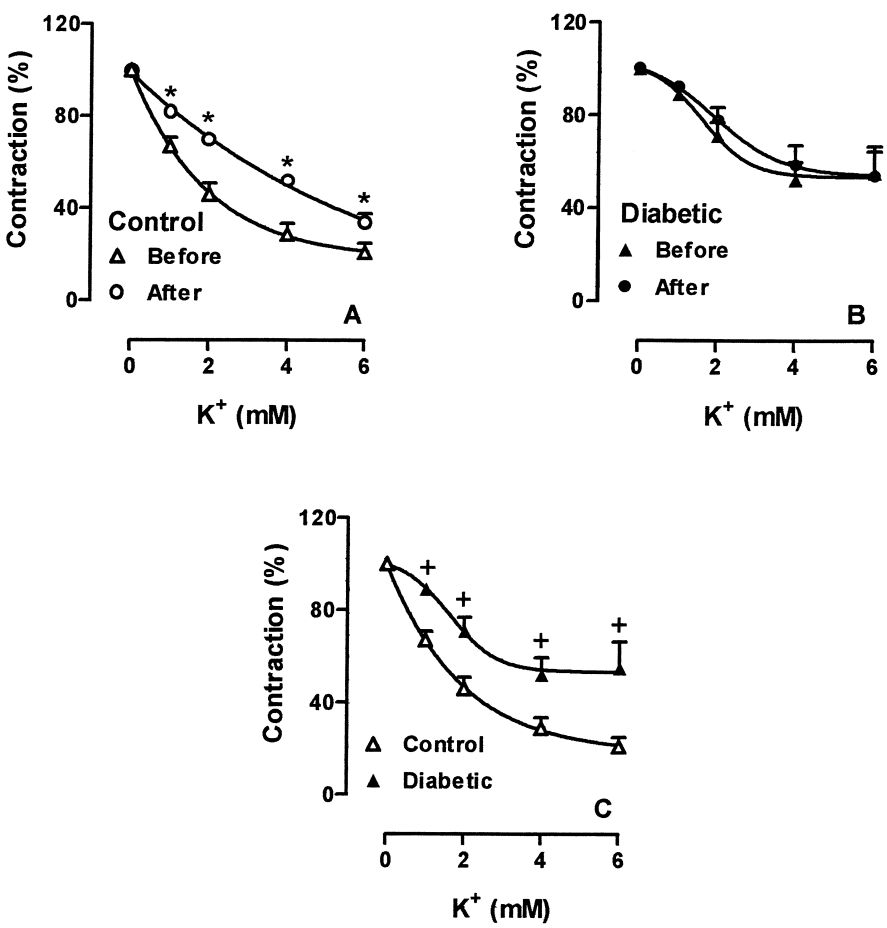


Fig. 3. Functional activity of $Na^+,K^+$-ATPase from rat tail vascular beds evaluated by the percentage reduction of mean perfusion pressure, obtained in preparations pre-contracted with continuous infusion of $10^{-7}$ M PHE, following increasing concentrations of extracellular $K^+$. Upper panels (A and B) show curves obtained before (triangles) and after (circles) 10 nM ouabain for 60 min in control (open symbols) and streptozotocin-induced diabetic rats (filled symbols). Lower panel (C) shows the comparison between the functional activity of $Na^+,K^+$-ATPase from control and diabetic rats before ouabain treatment. Each point represents the mean ± S.E.M. Two-way ANOVA, $^*P < 0.05$, before vs. after ouabain and $+P < 0.05$, control vs. diabetic rats. Observe that diabetic rats presented a reduction of the functional activity of the $Na^+,K^+$-ATPase and ouabain did not inhibit the tone reduction produced by the increase of the extracellular $K^+$.

tion ($P < 0.01$, *t*-test). The blood glucose was maintained after 30 min until the end of the experiments at $400 \pm 31$ mg/dl.

### 3.2. Pressor responses of the rat tail vascular bed

Previous experiments from our laboratory, which suggested that the treatment of the rat tail vascular bed for 1 h with 10 nM ouabain enhanced the vasopressor responses to bolus doses of phenylephrine (Rossoni et al., 1999a,b), were reproduced here. This ouabain sensitization was used to investigate the effects of diabetes on the pressor reactivity of the rat tail vascular bed.

Preparations from control animals showed the typical increase in $E_{max}$ to phenylephrine produced by ouabain, without changing the sensitivity (Fig. 2A; Table 3). Using a similar protocol in preparations from streptozotocin-treated animals, no increment of $E_{max}$ or sensitivity (log $EC_{50}$) to phenylephrine was obtained (Fig. 2B; Table 3). No differences in baseline perfusion pressure were observed among the groups, both before and after the treatment with ouabain (results not shown).

Also, comparing the phenylephrine dose–response curves from control and streptozotocin-treated rats obtained before ouabain treatment, it was seen that tail vascular beds from diabetic rats behaved differently (Fig. 2C). Although showing similar $E_{max}$ for phenylephrine stimulation, the log $EC_{50}$ for phenylephrine was reduced in diabetic preparations (Table 3).

The endothelial function of these preparations was investigated by using the relaxation obtained in response to bolus injections of acetylcholine. The preparations from diabetic rats showed a significant reduction of the relaxation induced by acetylcholine ($71.3 \pm 5.6\%$ in controls vs. $49.8 \pm 5.6\%$ of relaxation to acetylcholine in diabetic rats, $P < 0.05$, student's *t*-test, Table 3).

To explain those previous findings, we tested the functional activity of the $Na^+,K^+$-ATPase using the changes of the vascular tone obtained in pre-contracted preparations produced by increasing extracellular $K^+$ concentrations. Since we knew from previous studies (Rossoni et al., 1999a) that ouabain reduces the $Na^+,K^+$-ATPase activity (Fig. 3A), if the amount of enzyme is reduced by diabetes the response to ouabain treatment would be affected. In Fig. 3B, it can be seen that in the diabetic rats no further inhibition of the $Na^+,K^+$-ATPase is produced by ouabain. However, it is interesting to stress that the functional activity curve of the $Na^+,K^+$-ATPase, obtained from diabetic animals, is depressed compared to controls (Fig. 3C).

## 4. Discussion

Results in this paper show that 7-day streptozotocin-induced diabetes did not present the sensitization of the phenylephrine pressor effect, which is normally observed in anesthetized rats, after ouabain treatment. Moreover, no changes of $E_{max}$ to phenylephrine, usually enhanced after ouabain treatment, were observed in the tail vascular bed of streptozotocin-treated rats. The results also suggested that those effects are probably related to the reduction of the activity of the $Na^+,K^+$-ATPase and of the sensitivity to ouabain.

Insulin is one of the hormones that regulates the synthesis and activity of the $Na^+,K^+$-ATPase (Gupta et al., 1996; Vér et al., 1997). The activity and the amount of $Na^+,K^+$-ATPase units in the plasma membrane are reduced in diabetic animals and insulin administration partially restores normal conditions (Gupta et al., 1996). Moreover, the increased plasma levels of endogenous ouabain in those animals may be inhibiting the already small number of $Na^+,K^+$-ATPase unities. Assuming that these changes are occurring in the 7-day streptozotocin-induced diabetic rats, alterations on the normal behavior of pressure and vascular reactivity should be expected. To address this question, we used ouabain to enhance arterial blood pressure responses and the pressor action of phenylephrine in the tail vascular bed, as previously reported (Songu-Mize et al., 1995; Vassallo et al., 1997; Rossoni et al., 1999a,b). Ouabain has the ability to inhibit the $Na^+$-pump in several tissues including the vascular smooth muscle (Bova et al., 1991; Blaustein, 1993; Doris, 1994). Inhibiting the $Na^+$-pump internal $Na^+$ concentration increases and the activity of the $Na^+/Ca^{2+}$ exchanger is reduced. This produces an increase of intracellular $Ca^{2+}$ and, consequently, increases myocardial contraction or vascular smooth muscle tone (Blaustein, 1988; Blaustein et al., 1991; Bova et al., 1991; Rembold et al., 1992), enhancing the reactivity of the vascular smooth muscle to pressor agents (Blaustein, 1993; Songu-Mize et al., 1995; Vassallo et al., 1997; Rossoni et al., 1999a).

Ouabain treatment was not able to increase arterial blood pressure in diabetic anesthetized rats, as it was seen in controls. However, arteries incubated in vitro with elevated concentrations of glucose for a few hours presented impairment of endothelium-dependent relaxation (Tesfamariam, 1993; Félétou et al., 1994). We then search for a putative effect of acute hyperglycemia in vivo that is responsible for these changes. This was discarded since the elevation of blood glucose in control animals did not change the pressor actions of ouabain on the arterial blood pressure nor the phenylephrine pressor responses. In addition, chronic hyperglycemia has been reported to reduce nitric oxide bioavailability blunting the endothelial-dependent vasodilatation produced by this compound (Tesfamariam, 1993). Therefore, some of the effects saw in streptozotocin-treated rats might be due to chronic hyperglycemia.

A similar response was observed in vitro, when the tail vascular bed was used. The enhancement of $E_{max}$ to phenylephrine, which is normally observed after 10 nM ouabain treatment in control rats, did not alter in streptozo-

tocin-treated rats. One possible explanation for the reduction of ouabain effects in the diabetic rats could be the decreased activity of the $Na^+,K^+$-ATPase resulting from the lack of insulin. Ohara et al. (1991) presented findings consistent with the hypothesis that the $Na^+,K^+$-ATPase activity and gene expression were reduced using aortic cells isolated from 7- and 14-day diabetic rats . These findings were accompanied by a rise in intracellular $Ca^{2+}$ concentration, a fact that occurs when the $Na^+,K^+$-ATPase activity is reduced. Previous reports (Smith et al., 1997), however, obtained from aorta and superior mesenteric arteries of 12 weeks streptozotocin-induced diabetic rats, also suggested that the intrinsic $Na^+,K^+$-ATPase activity could be modulated by endothelial factors.

Nitric oxide is one endothelial factor, which stimulates the $Na^+,K^+$-ATPase (Gupta et al., 1996). The reduction of nitric oxide release would reduce the activity of the $Na^+,K^+$-ATPase. Previous results from our laboratory (Rossoni et al., 1999b), obtained with tail vascular beds from hypertensive $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) treated rats, showed a reduced $Na^+,K^+$-ATPase activity, which suggests the existence of a modulator effect of NO on the activity of the $Na^+,K^+$-ATPase activity. Since the relaxation of the tail vascular bed produced by acetylcholine is nitric oxide-dependent and nitric oxide is known to stimulate the $Na^+,K^+$-ATPase (Gupta et al., 1996), the reduction of the relaxation produced by acetylcholine, observed in our results, is another finding which might contribute to the idea of a reduced activity of the $Na^+,K^+$-ATPase in the tail vascular bed from diabetic rats.

There are reports which support the idea that the bioavailability of nitric oxide might be reduced in the diabetic rats. According to them (Tesfamariam and Cohen, 1992; Tesfamariam, 1993; Diederich et al., 1994; Cosentino and Luscher, 1998), the impaired endothelium-dependent relaxation, observed in vessels from diabetic animals, is caused by elevated glucose concentration and enhanced inactivation of nitric oxide by free radicals. However, Pieper (1999) reported that endothelium-dependent relaxation to acetylcholine was normal after 1 week being altered only after 8 weeks of diabetes. This apparent contradiction might be explained by the different preparations used. Pieper (1999) used aortic rings and, in our experiments, the rat tail vascular bed was used. The reduction of the effects of acetylcholine, which we observed, was 20% (see Table 3) and this result might suggest that some vascular beds, such as the tail, may present an endothelial dysfunction prior to other vessels like the aorta.

We then tested the functional activity of the $Na^+,K^+$-ATPase by changing the extracellular $K^+$ concentrations in preparations pre-contracted with phenylephrine and zero $K^+$. Previous reports showed that this protocol correlates with the activity of the $Na^+,K^+$-ATPase (Webb and Bohr, 1978; Ponte et al., 1996). Results showed that diabetic animals, as expected, displayed a reduced functional activity of the $Na^+,K^+$-ATPase when compared to controls. Also, the inhibitory action produced by ouabain on the activity of the $Na^+,K^+$-ATPase, which is normally observed in control preparations (Rossoni et al., 1999a), was abolished in tail vascular beds from diabetic rats. These results suggest a reduction of the functional activity of the $Na^+,K^+$-ATPase in diabetic rats which is in accordance to previous findings (Blaustein et al., 1991; Vér et al., 1997). Since the effect of ouabain, increase of intracellular calcium resulting from the increment of intracellular $Na^+$, is dependent on the inhibition of the $Na^+,K^+$-ATPase, the lack of this enzyme would reduce this effect.

Other ouabain actions, such as membrane depolarization, deserve comments. In vascular and other nonvascular smooth muscle membrane depolarization and reduction of the activity of the $Na^+,K^+$-ATPase occur simultaneously (Urquilla et al., 1978; Abel et al., 1981; Hershman et al., 1995). However, in our experiments membrane depolarization seems to be unlikely. The main reason is because the amount of ouabain we used (10 nM ouabain) was, at least, 300 times less then the amount used by other authors, 3 μM ouabain (Urquilla et al., 1978; Abel et al., 1981; Hershman et al., 1995). Such high concentrations inhibit all isoforms of the $Na^+,K^+$-ATPase. The concentration we used (10 nM ouabain) is the range for inhibition of the high affinity $\alpha_2$ isoform while the μM range is for the affinity of the $\alpha_1$ isoform (O'Brein et al., 1994; Blanco and Mercer, 1998). According to Blaustein et al. (1998), the high affinity $\alpha_2$ isoform has a specific distribution in vascular myocytes, a microregion adjacent to the $Na^+/Ca^{2+}$ exchanger and an underlying sarcoplasmic reticulum. This structure was named plasmerosome. This region might explain the putative mechanism of the enhanced reactivity of the rat tail vascular bed to phenylephrine, observed in control animals after the treatment with low concentrations of ouabain and also, the increment of systolic and diastolic blood pressures (Blaustein, 1988; Blaustein et al., 1991, 1998; Vassallo et al., 1997; Rossoni et al., 1999a).

Then, low concentrations of ouabain can augment $Ca^{2+}$ transients in vascular smooth muscle without increasing the cytosolic sodium (Arnon et al., 2000). Moreover, the baseline perfusion pressure in perfused rat tail arteries does not change after 10 nM ouabain treatment (Rossoni et al., 1999a). Since this mechanism only raises cytosolic calcium concentration upon activation, phenylephrine might increase $E_{max}$. Since changes in receptor activation does not occur changes in log $EC_{50}$ are unlikely. Also, because the $\alpha_1$ isoform of $Na^+,K^+$-ATPase is not affected depolarization might not occur. Considering these aspects our results and the ones from Urquilla et al. (1978), Abel et al. (1981) and Hershman et al. (1995) are not incompatible. Moreover, the results obtained with streptozotocin-treated rats, in which the $Na^+,K^+$-ATPase is reduced, are exactly as predicted by their results, a smaller pressor response to ouabain of anesthetized diabetic animals and the absence of pressor response of their tail vascular beds.

However, regarding the behavior of the tail vascular beds, before ouabain treatment, it is interesting to stress that the log $EC_{50}$ for phenylephrine dose–responses curves was smaller in streptozotocin-treated preparations. This finding represents an increased sensitivity to phenylephrine of streptozotocin-treated preparations, without change in $E_{max}$. This fact might be explained by the endothelial dysfunction characterized by the reduction of the action of acetylcholine in these preparations, as reported for other vascular beds (Tesfamariam, 1993; Diederich et al., 1994; Pieper et al., 1996). Karasu and Altan (1993), who suggested the existence of a relationship, reported a similar conclusion between vascular reactivity and endothelial dysfunction. In addition, other results showed an increased intracellular $Ca^{2+}$ concentration in aortic smooth muscle cells (Ohara et al., 1991) from diabetic rats. The fact that, in diabetic rats, there is a reduction of the functional activity of the $Na^+,K^+$-ATPase, renders relaxation difficult. This might occur because the $Na^+/Ca^{2+}$ exchange activity is reduced by the increment of the intracellular sodium. Taken together, these results might explain why, after 7 days of diabetes, the increased vascular sensitivity, a necessary background for hypertension, was already present, although rats were not hypertensive yet.

However, although streptozotocin treatment shifts the concentration–response curve to phenylephrine, in the tail vascular bed, it does not shift it in blood pressure. Similar findings were observed previously (Vassallo et al., 1997) and the existence of intact reflexes in these anesthetized rats might be the reason for masking this response.

In summary, results suggested that 7-day diabetic rats, although not hypertensive yet, showed signs of enhanced vascular sensitivity. The increase of arterial blood pressure or the sensitization of the tail vascular bed, produced by ouabain, is lost in 7-day streptozotocin-induced diabetic rats. This occurs as a consequence of the reduction of the functional activity of the $Na^+,K^+$-ATPase probably because of insulin lack and a deficient endothelial nitric oxide bioavailability.

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## References

Abel, P.W., Urquilla, P.R., Goto, K., Westfall, D.P., Robinson, R.L., Fleming, W.W., 1981. Chronic reserpine treatment alters sensitivity and membrane potential of the rabbit saphenous artery. J. Pharmacol. Exp. Ther. 217, 430–439.

Arnon, A., Hamlyn, J.M., Blaustein, M.P., 2000. Ouabain augments $Ca^{2+}$ transients in arterial smooth muscle without rising cytosolic $Na^+$. Am. J. Physiol. 279, H679–H691.

Bányász, T., Kovács, T., 1998. Altered [$^3$H] ouabain binding sites to cardiac muscle in insulin-dependent and non-insulin-dependent diabetic rats. Exp. Physiol. 83, 65–76.

Blanco, G., Mercer, R.W., 1998. Isoenzymes of the Na–K-ATPase: heterogeneity in structure, diversity in function. Am. J. Physiol. 275, F633–F650.

Blaustein, M.P., 1988. Sodium/calcium exchange and the control of contractility in cardiac muscle and vascular smooth muscle. J. Cardiovasc. Pharmacol. 12, S56–S68, (suppl.).

Blaustein, M.P., 1993. Physiological effects of endogenous ouabain: control of intracellular $Ca^{2+}$ stores and cell responsiveness. Am. J. Physiol. 264, C1367–C1387.

Blaustein, M.P., Goldman, W.F., Fontana, G., Krueger, B.K., Santiago, E.M., Steele, T.D., Weiss, D.N., Yarowsky, P.J., 1991. Physiological roles of the sodium–calcium exchanger in nerve and muscle. Ann. N. Y. Acad. Sci. 639, 254–274.

Blaustein, M.P., Juhaszova, M., Golovina, V.A., 1998. The cellular mechanism of action of cardiotonic steroids: a new hypothesis. Clin. Exp. Hypertens. 20 (5–6), 691–703.

Bova, S., Blaustein, M.P., Ludens, J.H., Harris, D.W., Du Charme, D.W., Hamlyn, J.M., 1991. Effects of an endogenous ouabainlike compound on heart and aorta. Hypertension 17, 944–950.

Chen, S., Yuan, C., Clough, D., Schooley, J., Haddy, F.J., Pamnani, M.B., 1993a. Role of digitalis-like substance in the hypertension of streptozotocin-induced diabetes in reduced renal mass rats. Hypertension 6, 397–406.

Chen, S., Yuan, C., Clough, D., Haddy, F.J., Pamnani, M.B., 1993b. Role of digitalis-like substance in experimental insulin-dependent diabetes mellitus hypertension. J. Cardiovasc. Pharmacol. 22 (Suppl. 2), S20–S21.

Cosentino, F., Luscher, T.F., 1998. Endothelial dysfunction in diabetes mellitus. J. Cardiovasc. Pharmacol. 32 (Suppl. 3), S54–61.

Diederich, D., Skopec, J., Diederich, A., Daí, F.X., 1994. Endothelial dysfunction in mesenteric resistance arteries of diabetic rats: role of free radicals. Am. J. Physiol. 266, H1153–H1161.

Doris, P.E., 1994. Regulation of Na,K-ATPase by endogenous ouabain-like materials. Proc. Soc. Exp. Biol. Med. 205, 200–212.

Epstein, M., Sowers, J.R., 1992. Diabetes mellitus and hypertension. Hypertension 19, 403–418.

Ewart, H.S., Klip, A., 1995. Hormonal regulation of the $Na^+,K^+$-ATPase: mechanism underlying rapid and sustained changes in pump activity. Am. J. Physiol. 269, C295–C311.

Félétou, M., Rasetti, C., Duhault, J., 1994. Magnesium modulates endothelial dysfunction produced by elevated glucose incubation. J. Cardiovasc. Pharmacol. 24, 470–478.

Fleming, W.W., 1980. The electrogenic $Na^+,K^+$-pump in smooth muscle: physiologic and pharmacologic significance. Ann. Rev. Pharmacol. Toxicol. 20, 129–149.

Gupta, S., Phipps, K., Ruderman, N.B., 1996. Differential stimulation of $Na^+$ pump activity by insulin and nitric oxide in rabbit aorta. Am. J. Physiol. 270, H1287–H1293.

Hamlyn, J.M., Ringel, R., Schaeffer, J., Levison, P.D., Hamilton, B.P., Kowarski, A.A., Blaustein, M.P., 1982. A circulating inhibitor of ($Na^+ + K^+$)ATPase associated with essential hypertension. Nature 300, 650–652.

Hershman, K.M., Taylor, D.A., Fleming, W.W., 1995. Adaptive supersensitivity and the $Na^+/K^+$ pump in the guinea pig vas deferens: time course of the decline in the alpha 2 subunit. Mol. Pharmacol. 47, 726–729.

Karasu, C., Altan, V.M., 1993. The role of endothelial cells on the alterations in vascular reactivity induced by insulin-dependent diabetes mellitus: effects of insulin treatment. Gen. Pharmacol. 24, 743–755.

Landsberg, L., 1992. Hyperinsulinemia: possible role in obesity-induced hypertension. Hypertension 19, 161–166.

Lingrel, J.B., 1992. Na,K-ATPase: isoform structure, function, and expression. J. Bioenerg. Biomembr. 24, 263–270.

Mathews, W.R., Du Charme, D.W., Hamlyn, J.M., Harris, D.W., Mandel, F., Clark, M.A., Ludens, J.H., 1991. Mass spectral characterization of an endogenous digitalis like factor from human plasma. Hypertension 17, 930–935.

Moreau, P., Lamarche, L.K., Laflamme, A., Calderone, A., Yamaguchi, N., Champlain, J., 1995. Chronic hyperinsulinaemia and hypertension: the role of the sympathetic nervous system. J. Hypertens. 13, 333–340.

O'Brein, W.J., Lingred, J.B., Wallick, E.T., 1994. Ouabain binding kinetics of the rat alpha two and alpha three isoforms of the sodium–potassium adenosine triphosphatase. Arch. Biochem. Biophys. 310, 32–39.

Ohara, T., Sussman, K.E., Draznin, B., 1991. Effect of diabetes on cytosolic free $Ca^{2+}$ and $Na^{+}$–$K^{+}$-ATPase in rat aorta. Diabetes 40, 1560–1563.

Öztürk, Y., Melih Altan, V., Yildizoglu-Ari, N., 1996. Effects of experimental diabetes and insulin on smooth muscle functions. Pharmacol. Ver. 48, 69–112.

Pieper, G.M., 1999. Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: importance of disease duration. Diabetologia 42, 204–313.

Pieper, G.M., Moore-Hilton, G., Roza, A.M., 1996. Evaluation of the mechanism of endothelial dysfunction in the genetically diabetic BB rat. Pharmacol. Lett. 58, 147–152.

Ponte, A., Marin, J., Arribas, S., Gonzáles, S., Barrus, M.T., Salaices, M., Sánches-Ferrer, C.F., 1996. Endothelial modulation of ouabain-induced contractions and sodium-pump activity in aortas of normotensive Wystar–Kyoto and spontaneous hypertensive rats. J. Vasc. Res. 33, 164–174.

Raccah, D., Dadoun, F., Coste, T., Vague, P., 1996. Decreased Na/K ATPase ouabain binding sites in red blood cells of patients with insulin-dependent diabetes and healthy north African control subjects: relationship with diabetic neuropathy. Horm. Metab. Res. 28, 128–132.

Rembold, C.M., Richard, H., Chen, X.L., 1992. $Na^{+}$–$Ca^{2+}$ exchange, myoplasmic $Ca^{2+}$ concentration, and contraction of arterial smooth muscle. Hypertension 19, 308–313.

Rossoni, L.V., Cunha, V., França, A., Vassallo, D.V., 1999a. The influence of nanomolar ouabain on vascular pressor responses is modulated by the endothelium. J. Cardiovasc. Pharmacol. 34, 887–892.

Rossoni, L.V., Cunha, V., Vassallo, D.V., 1999b. Effects of ouabain on the vascular reactivity to phenylephrine in L-NAME induced hypertension. Hypertension 33, 1089, abst. 232.

Skou, J.C., Esmann, M., 1992. The Na,K-ATPase. J. Bioenerg. Biomembr. 24, 249–261.

Smith, J.M., Paulson, D.J., Solar, S.M., 1997. $Na^{+}/K(^{+})$-ATPase activity in vascular smooth muscle from streptozotocin diabetic rat. Cardiovasc. Res. 34, 137–144.

Songu-Mize, E., Vassallo, D.V., Rashed, S.M., Varner, K.J., 1995. Ouabain amplifies contractile responses to phenylephrine in rat tail arteries in hypertension. J. Basic Clin. Physiol. Pharmacol. 6, 309–319.

Tack, C.J.J., Lutterman, J.A., Vervoot, G., Thien, T., Smits, P., 1996. Activation of the sodium–potassium pump contributes to insulin-induced vasodilation in humans. Hypertension 28, 426–432.

Tesfamariam, B., 1993. Free radicals in diabetic endothelial cell dysfunction. Free Radical Biol. Med. 16, 383–391.

Tesfamariam, B., Cohen, R.A., 1992. Free radicals mediate endothelial cell dysfunction caused by elevated glucose. Am. J. Physiol. 263 (2 Pt 2), H321–H326.

Urquilla, P.R., Westfall, D.P., Goto, K., Fleming, W.W., 1978. The effects of ouabain and alterations in potassium concentration on the sensitivity to drugs and the membrane potential of the smooth muscle of the guinea-pig and rat vas deferens. J. Pharmacol. Exp. Ther. 207, 347–355.

Vassallo, D.V., Songu-Mize, E., Rossoni, L.V., Amaral, S.M.C., 1997. Effects of ouabain on the vascular reactivity. Braz. J. Med. Biol. Res. 30, 545–552.

Vér, A., Szánto, I., Bányász, T., Csermely, P., Végh, E., Somogyi, J., 1997. Changes in the expression of $Na^{+}/K^{+}$-ATPase isoenzymes in the left ventricle of diabetic rat hearts: effect on insulin treatment. Diabetologia 40, 1255–1256.

Webb, R.C., Bohr, D.F., 1978. Potassium-induced relaxation as an indicator of $Na^{+}$–$K^{+}$ATPase activity in vascular smooth muscle. Blood Vessels 15, 198–207.

Zemel, M.B., Johnson, B.A., Ambrozy, S.A., 1992. Insulin-stimulated vascular relaxation: role of $Ca^{2+}$-ATPase. Am. J. Hypertens. 5, 637–641.