Increased Na,K,Cl cotransporter and Na, K-ATPase activity of vascular tissue in two-kidney Goldblatt hypertension

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The properties of the Na/K pump and Na,K,Cl cotransporter were studied in vascular tissue of two-kidney Goldblatt hypertensive rats. These transport systems were measured as ouabain-sensitive and bumetanide-sensitive ^{86}Rb/K uptake in aortic rings, left ventricular muscle and soleus skeletal muscle fibers of control and hypertensive Sprague-Dawley rats. A dramatic increment in Na/K pump activity was observed in intact aortic rings from the hypertensive group. The same was true for the Na,K,Cl cotransporter. The transport parameters related to the left ventricular muscle and soleus skeletal muscle were not significantly altered in the hypertensive rats. Measurements of the catalytic isoforms of the Na^+, K^+-ATPase in the aortic rings indicated that both isoforms (α_1 and α_2) were elevated in the same proportion in the hypertensive rats. The results also indicate that the endothelium plays an important role in both transport systems: in the absence of endothelium, a much lower ^{86}Rb/K uptake was observed than in intact aortic rings, either from control or hypertensive vascular tissue. Nevertheless, when the ^{86}Rb/K transport activity was measured in denuded aortic rings, a significantly higher ouabain and bumetanide sensitive ^{86}Rb/K uptake was also observed in the hypertensive rats. These data also show no alteration in the endothelium of the hypertensive rats as compared to control animals. The presence of endothelium had a more striking effect on the α_2 catalytic isoform activity than on the α_1 isoform. We conclude that there is a significant increment in the Na/K pump and Na,K,Cl cotransporter in two kidney-Goldblatt hypertension, that is specific for vascular smooth muscle.

***Key terms**: cotransport, endothelium, hypertension, Na,K,Cl cotransport, sodium pump, vascular ion transporters, vascular smooth muscle*

INTRODUCTION

Hypertensive states are of diverse etiology. Specific causal mechanisms have been difficult to identify. However, a relationship between salt balance and the development of high blood pressure has been known for many years. Na,K-ATPase is responsible for the maintenance of sodium and potassium electrochemical gradients

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across the cell membrane and plays an important role in many fundamental processes, such as the control of contractility, excitability and cell volume regulation. The activity of Na,K-ATPase in various tissues has been principal focus of attention, although its contribution to the etiology and/or maintenance of hypertension is still not clear.

The sodium pump is a heterodimeric molecule consisting of alpha, catalytic, and beta subunits. At least three alpha subunits isoforms and two beta isoforms have been characterized and cloned. The distribution and regulation of the isoforms in different tissues vary (8, 13), suggesting distinct physiological roles for particular isozymes (4). Conflicting data have appeared. Some investigators have reported either decreased, phasic or elevated total activity of the NA,K-ATPase in diverse forms of hypertension (14, 27, 30, 33); few studies have been directed to assess catalytic isoforms. Herrera *et al* (12) reported specific regulation of gene expression in two models of hypertension. Songu-Mize *et al* (34) described an up-regulation of α_1 and α_2 Na,K-ATPase subunits in aortic smooth muscle cells under sustained mechanical strain. Despite this up-regulation of the α-subunit expression, the Na,K-ATPase activity was significantly inhibited, demonstrating that the expression of Na pump isoforms does not necessarily correlate with activity.

Recently, it has been suggested that the Na,K,Cl cotransporter might also play a role in the etiology of hypertension. O'Donnell *et al* (22) found a reduced activity of this transporter in aortic smooth muscle cells from spontaneously hypertensive rats. These findings are in opposition to Smith *et al* (31) and Adragna *et al* (1) studies, in which elevated cotransport was observed in erythrocytes of hypertensive patients. It has been shown that angiotensin II can regulate Na,K,Cl cotransporter activity and it is known that Na,K-ATPase is regulated by mineralocorticoids (20, 44). In cultured vascular smooth muscle cells, Tseng and Berk (39) found an increased Na,K,Cl cotransporter activity, under the hypertrophic effect of angiotensin II.

The renin-angiotensin system participates in the pathogenesis of the development of some types of hypertension (30). Therefore, the purpose of the present study was to evaluate the regulation of the Na,K-ATPase specific isoforms and Na,K,Cl cotransport activity, in a model of high renin renal hypertension in rats: two-kidney Goldblatt hypertension. We assessed the α_1 and α_2 activities by measuring high and low ouabain sensitive ^{86}Rb/K uptake in the intact tissue. Experiments were done in two tissues of cardiovascular importance, aorta and left ventricular muscle, and also in skeletal muscle as an example of a tissue which is not under mechanical strain.

There is growing evidence that the endothelium may modulate different transport activities in vascular smooth muscle. Therefore, we studied the effect of endothelium on the sodium-pump and bumetanide-sensitive sodium transport in the two-kidney Goldblatt hypertension. In addition, a possible endothelial dysfunction associated with this hypertensive model was evaluated (7, 36, 41).

METHODS

Animal preparation.

Male Sprague-Dawley rats weighing 120-150 g were divided into two groups. Hypertension was induced to the first group of rats by placing a U-shaped silver clip with an internal gap of 22 mm around the left renal artery. The right kidney was left untouched (two-kidney Goldblatt renal hypertension). The second group of rats was sham operated and was designated as control group.

Blood pressure was measured before the surgery, and then twice a week, by a tail-cuff technique, until the experiments were done. During this time animals had ad libitum access to food and water. After 3-4 weeks, those animals with a blood pressure above 160 mm Hg or higher at two consecutive measurements from the first group of rats, and with pressure of 120 mm Hg or less from the second group, were selected for the experimental procedures.

Tissue preparation and incubation.

Rats were killed by decapitation. The thoracic aorta was quickly excised and placed in cold (4°C) physiological Krebs-Ringer buffer (KRB). Rings (4-6 mm) were prepared after the aorta was dissected free of connective tissue with special care taken to avoid damage to the endothelium. Endothelium from aortic rings was removed by inserting a stainless steel wire into the lumen and gently rolling the ring on a filter paper soaked in KRB. At the beginning of the experiments, normal or hypertensive aortic rings were equilibrated for 1 h in KRB 95% O_2-5% CO_2 at 37°C.

Isolation of heart muscle. The hearts were removed from control and hypertensive rats, and placed in cold KRB. The left ventricular cardiac muscle was isolated. Slices weighing 25 to 35 mg were equilibrated in separated vials in KRB, and bubbled with 95% O_2-5% CO_2.

Immediately after excised, tissues were equilibrated in the standard medium for 15-60 min in the presence or absence of ouabain and/or bumetanide. The standard incubation medium was Krebs-Ringer-bicarbonate-glucose buffer (KRB), pH 7.4 containing (in mM): 119 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$ and 5 D-glucose containing 0 or 50 μM bumetanide and 0 or 10 μM or 10 mM ouabain. Finally, tissues were incubated in 2 ml KRB containing ^{86}Rb (0.1 μCi/ml) in the presence or absence of ouabain and/or bumetanide, as described previously (37).

The reaction was stopped by transferring the slices into iced KRB; the tissue was then quickly washed in cold buffer and gently blotted. Radioactivity of the samples was determined by the Cerenkov radiation in a liquid scintillation counter.

Isolation of skeletal muscle. The soleus muscles were removed from control and hypertensive rats and then groups of fibers weighing 8-15 mg were incubated in separate vials in a similar way than that described for ventricular muscle tissue.

Sodium pump activity.

The Na,K-ATPase activity was measured by ouabain-sensitive ^{86}Rb/K uptake. Total pump activity was calculated by the difference between zero and 10^{-2} M ouabain; α_1 activity was measured at 10^{-5} M ouabain and α_2 by the difference between total pump activity minus α_1 (4). Results are expressed as nmoles ^{86}Rb/K uptake per min/g tissue.

Na,K,2Cl cotransporter.

Experiments that examined the effect of hypertension on the Na,K,2Cl cotransporter were performed in identical manner as those indicated above for the sodium pump activity, except that bumetanide was added a final concentration of 50 μM to the pre-incubation and incubation media, when it was required.

The activity of the Na,K,2Cl cotransporter was calculated by the difference between total ^{86}Rb/K uptake in the presence and absence of bumetanide.

Statistical Analysis.

Values are expressed as means ± SEM's. Statistical comparisons were done by Student's *t*-test or by analysis of variance when appropriate.

RESULTS

Characteristics of the hypertensive rats.

Table I summarizes the blood pressure, renal function and body weight for control and hypertensive groups at the time of the experiments.

Resting mean arterial pressure was increased in hypertensive rats as compared with controls. Body weight was similar between the one clip two-kidney rats and the control animals (sham-operated). There were no differences between the two groups in renal function, as measured by plasma creatinine.

Na,K-ATPase activity in different tissues from hypertensive animals.

We have investigated the Na-pump activity, measured as ouabain sensitive ^{86}Rb/K uptake, in three different muscle tissues: cardiac, vascular smooth muscle, and skeletal muscle

Table I

Characteristics of control and hypertensive rats

Group	Control	Hypertensive
Body weight (g)	250.0 ± 10.1	236.0 ±14.5
Mean arterial pressure (mm Hg)	118.0 ± 5.4	175.0 ± 6.3*
Plasmatic creatinine (mg/100 ml)	0.62 ± 0.30	0.65 ±0.40

Means ± SEM's. * $p < 0.001$ (n = 30)

of hypertensive rats, as compared to control animals. Ouabain-sensitive ^{86}Rb/K uptake was linear for at least 20 minutes in these tissues. Na,K-ATPase activity was significantly increased in vascular smooth muscle from hypertensive compared to control rats ($p < 0.05$). In contrast, in ventricular and skeletal muscle the Na pump activity from hypertensive animals was slightly, but not significantly lower than controls, as shown in Figure 1.

Isoform specific activity.

It has been established by other groups and by us (4), that -in the range of 10^{-6}-10^{-5} M ouabain- the α_2 isoform is ~90% saturated with ouabain, whereas α_1 has ~10% bound. This is also true for aortic rings which have a two component inhibition ouabain curve, with K_i for both isoforms similar to those described in other tissues (data not shown). Thus, in all three muscle tissues, we used a concentration of 10^{-5} M ouabain to inhibit the α_2 isoform almost exclusively. Increasing the concentration of ouabain to 10^{-2} M led to a complete inhibition of both α_1 and α_2 isoforms.

Figure 2 shows the percentage of α_1 catalytic isoform activity as compared to total pump activity. As shown in the graph, the percentage of α_1 isoform in all three muscle tissues studied did not change in

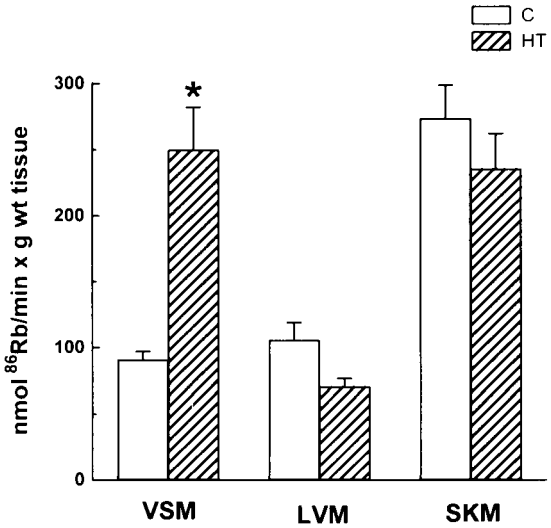

Fig 1. Ouabain-^{86}Rb/K uptake in intact aortic rings and ventricular and skeletal muscles of control (C) and hypertensive (HT) rats (2 kidney-Goldblatt rats). Aortic rings (VSM) with endothelium, left ventricular muscle (LVM) slices and soleus skeletal muscle (SKM) incubated in KRB. Ouabain-sensitive ^{86}Rb-uptake determined in presence of 10 mM ouabain. Means ± SEM's of 8 experiments done in triplicate samples. * $p < 0.05$

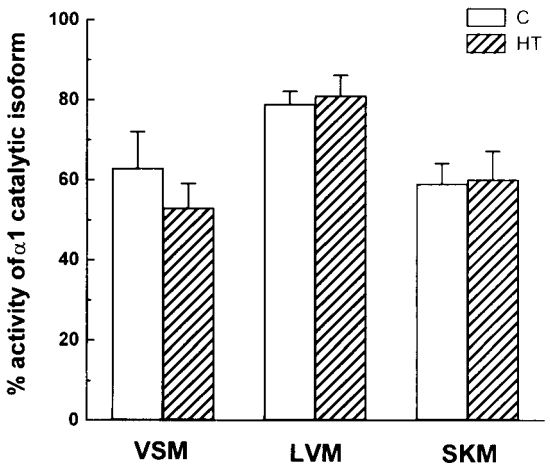

Fig 2. Catalytic activity of α_1-isoform of Na,K-ATPase in control (C) and hypertensive (HT) rats. Aortic rings (VSM), left ventricular muscle (LVM) slices and skeletal muscle (SKM) incubated with KRB. ^{86}Rb/K uptake determined in presence or absence of 10^{-5} M or 10^{-2} M ouabain. Difference in activity in absence and presence of 10 μM and 10 mM ouabain taken as α_1 isoform activity and expressed as percentage of total sodium pump activity. Means ± SEM's of 7 separate experiments, done in triplicate samples.

hypertensive rats despite the elevated Na,K-ATPase activity of the vascular smooth muscle. These results suggest that the larger aortic Na pump activity of hypertensive rats is due to an up-regulation in the activity of both α_1 and α_2 isoforms of Na,K-ATPase, in a similar proportion.

Na,K,Cl cotransporter in hypertensive rats.

Na,K,Cl cotransporter was assessed in aortic and left ventricular muscles from hypertensive and control animals as bumetanide-sensitive ^{86}Rb/K uptake. The cotransporter activity was markedly increased in aortic muscle from hypertensive rats (193.2 ± 29.3 nmoles ^{86}Rb / min x g wt tissue) as compared with control animals (69.3 ± 15.0 nmoles ^{86}Rb / min x g wt tissue) ($p < 0.001$).

By the contrary, the activity of the cotransporter in ventricular muscles from hypertensive and control rats was similar for both groups (64.5 ± 13.4 nmoles ^{86}Rb / min x g wt tissue and 41.7 ± 8.8 nmoles ^{86}Rb / min x g wt tissue, respectively) (see Fig 3).

Role of endothelium.

Since a previous study had suggested that endothelium affected Na pump and Na,K,Cl cotransporter activity, we performed studies to determine whether the effect of endothelium on those transporters could be altered in hypertension. We measured ouabain and bumetanide sensitive ^{86}Rb/K uptake in aortic rings, from hypertensive and control animals, with intact endothelium compared to rings from which endothelium had been previously removed. As shown in Table II, the absence of endothelium reduced the ouabain sensitive ^{86}Rb/K uptake in ~ 30% in hypertensive and control rats. Therefore, a higher Na,K-ATPase activity was observed in hypertensive aortic rings, whether measured in the absence or presence of endothelium. It is important to point out that, in the absence of endothelium, the pumping activity of α_1 isoform was slightly increased as compared to α_2-mediated transport. This difference was significant only for the hypertensive group of rats. The data would suggest a greater stimulatory effect of endothelium on Na,K-ATPase α_2-isoform activity. The higher values observed in the intact aortic rings versus denuded rings were not due to the uptake of ^{86}Rb/K in the endothelial cells, since this is a minimal part of the total ^{86}Rb/K uptake, as shown by us and others.

The removal of endothelium had dramatic effects on Na,K,Cl cotransporter activity. In both groups of rats, hypertensive and normotensive, the cotransporter activity measured after the endothelium had been removed was reduced to 52.3% and 45.8%, respectively, as compared to the endothelium-intact rings (Table II). Nevertheless, in both conditions, with or without endothelium the Na,K,Cl cotransporter activity was significantly higher in aortic rings from hypertensive animals.

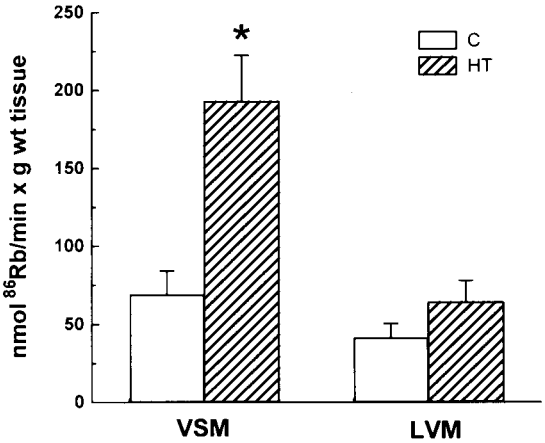

Fig 3. Bumetanide-sensitive ^{86}Rb/K uptake in aortic rings and ventricular muscle of control (C) and hypertensive (HT) rats. Aortic rings (VSM) and left ventricular muscle (LVM) slices incubated in KRB with 50 μM bumetanide, where applicable. Bumetanide-sensitive ^{86}Rb/K calculated by subtracting uptake values obtained in presence of bumetanide, from uptake obtained in its absence. Means ± SEM's of 7 to 9 separate experiments, each point being assayed in triplicate. * $p < 0.05$.

DISCUSSION

The idea that hypertension might be associated with alterations in the small intracellular pool of sodium has been known for many years (38). Because Na,K-ATPase maintains the Na gradient, this enzyme has been hypothesized to be involved in the pathogenesis of hypertension through its

Table II

Effect of endothelium on aortic Na,K,Cl cotransport, Na,K-ATPase activity and relative abundance of α_1 catalytic isoform, in control and hypertensive rats.

Rats	Control		Hypertensive	
Aortic rings	E^+	E^-	E^+	E^-
Na,K,Cl nmoles ^{86}Rb/min/g wt tissue	68.3 ± 15.0	31.3 ± 6.4*	193.2 ± 29.3#	101.0 ± 16.4*#
Na,K-ATPase nmoles ^{86}Rb/min/g wt tissue	91.4 ± 7.3	62.6 ± 7.9*	251.0 ± 31.6#	177.8 ± 7.8*#
% of Na-pump due to α_1 activity	62.6 ± 8.4	74.5 ± 11.2	52.0 ± 6.2	74.5 ± 2.3*

Na,K,Cl cotransporter activity measured as bumetanide sensitive ^{86}Rb/K uptake. Total Na,K-AT Pase activity measured as total ^{86}Rb uptake minus uptake with 10^{-2} ouabain. Difference in activity in absence and presence of 10 µM and 10 mM ouabain taken as α_1-activity, and expressed as percentage of total Na-pump activity. ^{86}Rb/K uptake measured in triplicate samples (n = 6). E^+, intact aortic rings; E^-, endothelium denuded aortic rings.
Means ± SEM's. * $p < 0.05$ denuded *vs* intact aortic rings; # $p < 0.05$ hypertensive vs respective control tissue.

effects on kidney sodium handling (2, 21), vascular smooth muscle reactivity (18, 30), and myocardial contractility (12, 19, 23). Alterations in Na-pump activity in animals with experimental hypertension and human subjects with essential hypertension have been shown; nevertheless, disagreement exists regarding the direction of these changes (11, 13, 19, 27, 33).

Na,K-ATPase consists of a transmembrane α-subunit (mol wt = 112,000) and a ß-subunit (mol wt = 35,000) that is glycosilated. At least three isoforms of the catalytic α-subunit, namely α_1, α_2 and α_3, and three isoforms of the ß-subunit have been cloned and sequenced. These isozymes are different in terms of affinity for sodium, hormonal regulation and sensitivity to cardiac glycosides (4, 8, 43). The α_1 isoform is expressed in all tissues. Adult rat aortic, cardiac and skeletal muscles express both the α_1 and α_2-isoforms. Regulation of the abundance and activity of sodium pump isoforms in cardiac and vascular tissue may potentially have important consequences, in terms of cellular Na and K homeostasis, which would affect resting potential, contractility and inotropy.

In the present study, we investigated the activity of this enzyme in three muscle tissues (vascular smooth muscle, ventricular, and skeletal muscle) in two-kidney Goldblatt renal hypertension, in rats. Our results indicate that there is a significantly elevated Na pump activity in aortic rings from hypertensive rats, as compared to control animals (Fig 1). These results are in agreement with previous works that described an increased activity of vascular Na,K-ATPase in the spontaneously hypertensive rats (SHR) (19, 38), and during the intermediate stage of Doca-salt hypertension (17, 33). However, other studies described a significant reduction in Na,K-ATPase activity in hypertensive animals (5, 27). Our data demonstrate that the increment in Na-pump activity was specific for vascular smooth muscle and was neither observed in ventricular nor in skeletal muscles (Fig 1). Therefore, some of the discrepancies could reflect distinct behavior of Na,K-ATPase activity in different cells or tissues, as erythrocytes, leukocytes or skeletal muscle. Ponte *et al* (28) describe a reduced ouabain sensitive ^{86}Rb uptake in SHR vessels. They suggest that former authors could have not taken into account the role of vascular endothelium on Na-pump activity which could have mask the lower Na,K-ATPase activity in hypertension. Table II shows that the removal of endothelium induced a reduction in ouabain sensitive ^{86}Rb uptake in the same range in hypertensive and normotensive animals, approximately 30%. Therefore, even in the absence of endothelium, there was a significantly higher Na pump activity in hypertensive aortic rings than in controls.

The cause of the elevation of the Na-pump activity in this model of hypertension

is still not clear. Overbeck *et al* (26) have suggested that the increased pumping ability of the vascular tissue, results from increased number of pump molecules in the vascular smooth muscle induced by a prior exposure to circulating digitalis-like pump inhibitor. This digitalis-like substances are characteristic of volume expanded model of hypertension as Doca-salt hypertension and SHR. However, our results were obtained in the two-kidney Goldblatt renal hypertension where sodium and water retention does not play an important pathogenetic role (25). Alternative explanations could be that the enhanced Na,K-ATPase activity is the result of increased membrane permeability to sodium, or finally mineralocorticoids may induce synthesis of new Na,K-ATPase molecules.

So far, it has been described an up-regulation of aortic α_1 subunit mRNA during hypertension in uninephrectomized DOCA-salt rats, but not in rats infused with angiotensin II for short periods (12). The α_2 mRNA, by contrast, was decreased 3 to 15 times in aortic and ventricular samples from both models of hypertension (12). However, the total or the particular isoform activity of the Na,K-ATPase was not measured in the above study. On the other hand, Songu-Mize *et al* (34) have shown that an up-regulation in the α-subunit expression does not necessarily correlate with an increased Na-pump activity. The present studies have examined whether there is a change in the Na,K-ATPase isoforms activity in two-kidney one clip Goldblatt hypertension, measured as ouabain-sensitive ^{86}Rb/K uptake at two different concentrations of ouabain. As shown in Figure 2, the increase in total Na-pump activity in aortic tissue, from hypertensive rats, is due to a higher activity of both catalytic isoforms of the Na,K-ATPase. In fact, the proportional activity of the isoforms was not different in hypertensive rats as compared to controls. It is interesting to note that in ventricular and skeletal muscles there were no changes in total or specific Na,K-ATPase isozymes activity.

Another Na transport system that has been recently suggested to be abnormal in essential hypertension is Na,K,Cl cotransport (13). Also, an increment of this cotransporter has been related to hypertrophy of vascular smooth muscle cells (39).

We have studied the effect of hypertension on Na,K,Cl cotransporter activity in aortic and ventricular muscle tissue, measured as bumetanide-sensitive ^{86}Rb/K uptake. Our results indicate that Na,K,Cl cotransporter is increased almost three times in aortic tissue from hypertensive rats as compared to control animals. By contrast, in ventricular muscle the cotransporter activity was not different between both groups of rats. So far, there is no data about Na,K,Cl cotransport activity in different experimental models of hypertension. In essential hypertension, there are conflicting results. Adragma *et al* (1) and Smith *et al* (31) have demonstrated elevated cotransport in erythrocytes of essential hypertensive patients. Conversely, Garay *et al* (10) have reported reduced Na,K,Cl cotransport. However, it is important to point out that these works have been done in erythrocytes, and the behaviour of transport proteins on these cells is not necessarily the same of other tissues, such as vascular smooth muscle or ventricular muscle.

The possible involvement of vascular endothelium in the pathogenesis of hypertension has not yet been elucidated although an important endothelial dysfunction associated with hypertensive disease has been reported (7, 28, 40, 41). It is also known that endothelium influences Na-pump activity (28). In this study we have evaluated the effect of endothelium on aortic Na,K,Cl cotransporter and Na,K-ATPase in hypertension. Table II shows that endothelium maintains an stimulatory effect on both transporter activities in hypertensive and control animals. Therefore, the greater ouabain and bumetanide-sensitive ^{86}Rb/K uptake was seen in the presence or absence of endothelium.

As far as we know, the differential effect of endothelium on particular isoforms of the Na-pump has not been previously evaluated. Our results demonstrate that in the presence of endothelium the activity of both isozymes increases, but this increment was higher for α_2 subunit.

This finding suggests that α_2 could be more sensitive to endothelium stimulation, but the verification of this hypothesis will, of course, require further experimental support.

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