Adrenal-dependent modulation of the catalytic subunit isoforms of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in aorta

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Adrenal-dependent modulation of the catalytic subunit isoforms of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in aorta. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E1072–E1081, 1998.—Na\textsuperscript{+}-K\textsuperscript{+}-ATPase gene expression and activity were studied in aortas from adrenalectomized (ADX) rats and ADX rats with deoxycorticosterone supplement (ADX-Doca). Northern analysis of RNA from ADX rats revealed a significant decrease in \(\alpha_2\)-mRNA levels (38.5 ± 8.3% of control, \(P < 0.01\)) that was prevented by DOCA (\(P < 0.05\)). A decrease to 55.8 ± 7.7% in \(\alpha_2\)-isoform protein was observed 8 days after adrenal removal (\(P < 0.05\)); DOCA reversed this effect (90.8 ± 10.9%). Adrenalectomy induced a decrease of 68.5 ± 4.5% in \(\beta_1\)-mRNA (\(P < 0.01\)) and 52.7 ± 8.3% in ADX-Doca rats (\(P < 0.01\)). Also, a reduction in \(\beta_1\)-isoform protein that was not prevented by DOCA was detected after adrenalectomy (47.1 ± 11%, \(P < 0.01\)). In contrast, no differences in \(\alpha_1\)-mRNA or -protein levels were observed. Vascular sodium pump activity was reduced to 59.8 ± 4.6% of control values after adrenalectomy (\(P < 0.01\)); this reduction was reversed by DOCA. Our data indicate that corticosteroids regulate Na\textsuperscript{+}-K\textsuperscript{+}-ATPase isoform expression and activity in vascular tissue in vivo, suggesting a mineralocorticoid-dependent modulation of \(\alpha_2\)-Na\textsuperscript{+}-K\textsuperscript{+}-ATPase gene expression in aorta, with \(\beta_1\)-isoform expression dependent on the presence of glucocorticoids.

vascular smooth muscle cells; aldosterone; hypertension

Sodium-potassium-adenosinetriphosphatase (Na\textsuperscript{+}-K\textsuperscript{+}-ATPase) of vascular smooth muscle cell (VSMC) membrane maintains the electrochemical Na\textsuperscript{+}-K\textsuperscript{+} gradient across the plasma membrane. The Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is a heterodimeric protein, composed by a large \(\alpha\)-subunit of \(\approx 100\) kDa and a smaller glycosylated \(\beta\)-subunit of \(\approx 55\) kDa. All functional sites involved in catalysis have been delineated to the \(\alpha\)-subunit. Four distinct \(\alpha\)-isoforms have been identified in rat tissues, namely \(\alpha_2\), \(\alpha_3\), and \(\alpha_4\) (44, 45). The \(\alpha\)-isoforms are encoded by separate genes, and their expression has been demonstrated to be tissue specific and developmentally regulated (7, 9, 12, 21, 41). The \(\beta\)-subunit appears to be required for the \(\alpha\)-catalytic function, and it is involved in the membrane integration of the \(\alpha\)-subunits (1, 16). Three \(\beta\)-subunit isoforms (\(\beta_1\), \(\beta_2\), and \(\beta_3\)) have been cloned in rats (17, 31, 32).

The critical contribution of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity to the modulation of intracellular Na\textsuperscript{+} and Ca\textsuperscript{2+} (10, 11, 13), vascular smooth muscle tone (3), and peripheral resistance (37), and its role in the pathogenesis of hypertension have been proposed by several investigators (6, 8, 20, 22). Corticosteroid hormones have been implicated in the regulation of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in mammalian kidney and colon (53, 54), and the hypertensive effect of high levels of corticosteroids with increments in total peripheral resistance is well established. The presence of high-affinity corticosteroid receptors in rat aorta (26, 39) and the assessment of sodium pump activity in rat arteries under corticosteroid treatment suggest molecular regulatory effects of adrenal corticosteroid hormones on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity of rat arteries. For example, dexamethasone has been shown to increase Na\textsuperscript{+} pump activity in rat tail arteries (19, 50). In the DOCA salt-hypertensive rat, increased (46), decreased (42), and variable changes (47) in vascular Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity have been described. In addition, aldosterone infusion in rats induced an increase in blood pressure, accompanied by an increase in ouabainsensitive sodium efflux in tail arteries (15). A recent study of \(\alpha\)-catalytic subunit mRNA abundance in the aorta of DOCA salt-hypertensive rats has shown a marked increase in \(\alpha_1\)- and \(\alpha_2\)-mRNA (48). Finally, in VSMCs, both dexamethasone and aldosterone (38, 40) induced the \(\alpha_1\)- and \(\beta_1\)-gene expression. However, the hormonal effects on the expression of the \(\alpha_2\)-isoform were not evaluated in this model because, contrary to the rat vascular tissue in vivo, the cultured rat VSMCs exclusively express the \(\alpha_1\)-catalytic isoform (40).

We hypothesized that in vivo endogenous corticoids exert a regulatory effect on sodium pump isoform expression affecting catalytic activity in vascular smooth muscle. Previously reported work has focused on DOCA salt-hypertensive animals, and, surprisingly, no report about the effect of adrenalectomy on sodium pump expression in vascular tissue has been published. Therefore, the purpose of the present work was to identify the \(\alpha\)- and \(\beta\)-subunit isoforms present in the rat aorta and to study in vivo the molecular regulatory effect of adrenal steroids on the isoforms and activity of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. We used adrenalectomized animals (ADX) and ADX animals plus the mineralocorticoid DOCA (ADX-DOCA). Our characterization studies by RT-PCR and Northern blot demonstrated \(\alpha_1\)-, \(\alpha_2\)-, and \(\beta_1\)-isoform expression in rat aorta. The functional studies \([\text{\textsuperscript{38}}\text{Rb/K uptake in aortic rings and K}\textsuperscript{+}-dependent p-nitrophenolphosphatase membrane activity (K}\textsuperscript{-pNPNPase})\] demonstrated a diminished Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in aortas from ADX animals. A recovery of the sodium pump activity was obtained in ADX animals with 8-day DOCA-replacement therapy. These effects

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were associated with changes in gene expression and protein concentration of $\alpha_2$- and $\beta_1$-isoforms, as shown by Northern and Western blot analyses.

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats (130–150 g) were divided into three groups: ADX, ADX-DOCA, and a control group. The ADX animals were laparatomized under anesthesia with ether, and the adrenal glands were removed. The ADX-DOCA group was also adrenalectomized, and immediately after surgery, the animals were subjected to daily intramuscular injections of DOCA (0.5 mg/100 g body wt/day) during 8 days. All animals were fed with standard rat chow, and the ADX group received 0.9% NaCl (wt/vol). Control and ADX-DOCA groups were allowed to have tap water.

**RNA isolation.** The thoracic aorta was immediately processed after all adventitia were removed, and it was washed with sterile ice-cold 0.9% NaCl solution. The tissue of one to three animals was used for total cellular RNA extraction with the guanidine thiocyanate or Trizol (GIBCO Life Sciences) method as per specifications of the manufacturer. RNA concentration was determined by spectrophotometry in triplicate and used in RT-PCR or Northern blot studies.

**RT-PCR studies.** The initial identification of $\alpha_2$- and $\beta_1$-subunit isoforms expressed in aorta of control, ADX, and ADX-DOCA rats was performed by RT-PCR from total cellular RNA. Rat skeletal muscle, kidney, or brain total cellular RNA was used as a positive control. Aliquots of 5–10 µg of RNA were incubated for 5 min at 65°C, transferred to ice, and centrifuged at 4°C for 1 min at 12,000 g (4°C). For RT, 2- to 9-µl aliquots (0.3–2 µg) were incubated in 5 mM MgCl₂, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 1 mM dNTPs, 1 U/µl AMV reverse transcriptase (Promega), 1 U/µl RNasin, and oligo(dT) primer or random hexamers at 42°C for 45 min following instructions tase (Promega), 1 U/µl RNasin, and oligo(dT) primer or random hexamers at 42°C for 45 min following instructions for each primer set. PCR products were analyzed by electrophoresis on 1% ethidium bromide-stained agarose gels. In each case, a single band of the predicted size was obtained.

**Northern blot analysis.** Equivalent amounts (15–25 µg) of total cellular RNA were electrophoresed on 2.2 M formaldehyde-agarose gels, washed with 50 mM NaOH, blotted, and fixed onto nylon membranes (GeneScreen Plus, Du Pont), as previously described by Bonilla et al. (9). The membranes were hybridized to $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\beta_1$-Subunits of Na⁺-K⁺-ATPase cDNA probes. The cDNA probes were amplified by PCR with rat sequence-specific primers (20–25 nucleotides/primer) as indicated in RT-PCR studies. Rat aorta, skeletal muscle, or brain cDNA was used as a template for the reactions. PCR products were analyzed by electrophoresis on 1% ethidium bromide-stained agarose gels, purified with Wizard PCR preparation (Promega), and labeled with a random primer labeling kit (Promega). Hybridization was performed at 65°C for 24 h with 25 ng 5'-[32P]triphosphate-labeled probes (10⁶ to 2 × 10⁸ counts/min). Before autoradiography, membranes were washed stringently two times for 5 min with a solution of 2× saline-sodium citrate (SSC) plus 0.1% SDS (1× SSC is 0.15 M NaCl and 0.15 M sodium citrate, pH = 7.0) and then two times for 15 minutes with a solution of 0.1× SSC plus 0.1% SDS at 65°C. Films (Fuji RX) were placed in contact with the membranes in cassettes containing intensifying screens and were left exposed at −45°C for 24–72 h, and three to four plates were used to ensure that we did not saturate the film. The amount of mRNA present in each lane was quantified by computer scanning densitometry analysis, comparing the intensity of the experimental sample with the control rat mRNA sample. Standard curves were run to work in the linear range. The values obtained were corrected by the total cellular RNA loaded in each lane, as quantified by computer scanning densitometry of 28S and 18S ribosomal RNA subunits in ethidium bromide-stained pictures of gels before transfer (21).

**Membrane preparation and Western blot analysis.** To minimize the potential selective enrichment of different pump isoforms during the purification procedure, we prepared a crude membrane fraction. Cleaned and washed thoracic aortas from three animals were homogenized by a motor-driven Potter-Elvehjem Teflon homogenizer in ice-cold buffer containing 50 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µM leupeptin, 2 µM pepstatin, and 50 mM β-mercaptoethanol, pH = 7.4. The homogenate was centrifuged at 3,000 g for 10 min (4°C), and the supernatant was centrifuged at 100,000 g for 45 min (4°C). The membranes were suspended in 300 µl of 10 mM Tris-HCl, 10 mM EDTA, 0.5 mM PMSF, 10% glycerol (vol/vol), and 50 mM β-mercaptoethanol (pH = 7.4) and stored at −20°C.

**SDS-polyacrylamide gels were prepared according to the method of Laemmli (27).** The blotting procedures were according to Towbin et al. (52). After blotting, the polyvinylidene difluoride membranes were blocked with 5% nonfat milk in Tris-buffered saline (20 mM Tris-HCl, 137 mM NaCl) plus 0.1% Tween 20. After washing, separate membranes were incubated with mouse monoclonal anti-$\alpha_1$ kindly provided to us by Dr. M. Caplan; mouse monoclonal anti-$\alpha_2$-M2 kindly provided to us by Dr Kathleen Sweadner; rabbit polyclonal anti-$\beta_1$ (Upstate Biotechnology); or rabbit polyclonal anti-$\alpha_3$ (Upstate Biotechnology). Blots were developed by enhanced chemiluminescence (ECL; Amersham) with horse-radish peroxidase-linked secondary antibodies. Films (Hyperfilm MP, Amersham) were placed in contact with the membranes in cassettes containing intensifying screens, and four to five plates were used to avoid film saturation. Standard curves were run to work in the linear range. The signal intensity present in each lane was quantified by computer scanning densitometry analysis, comparing the intensity of the experimental sample with the control rat sample lane.

**Quantification dots on Western and Northern blots.** Samples were quantified with a ScanMaker II HR (MICROTEK) interfaced to an IBM Aptiva computer with a Gel-Perfect C Program (9).
Sodium pump activity: $^{86}$Rb/K uptake in aortic rings. The Na$^+$-K$^+$-ATPase activity was measured by ouabain-sensitive $^{86}$Rb/K uptake in aortic rings according to Bofill et al. (7). Briefly, after the rats were decapitated, the thoracic aorta was quickly removed and washed with ice-cold Krebs-Ringer buffer (KRB) containing (in mM) 4.2 KCl, 1.19 KH$_2$PO$_4$, 120 NaCl, 25 NaHCO$_3$, 1.2 MgSO$_4$, 1.3 CaCl$_2$, and 5 D-glucose (pH = 7.4). The aorta was dissected free of connective tissue, and special care was taken to avoid damage to the endothelium and rings (4-6 mm). At the beginning of the experiments, aortic rings were equilibrated for 1 h in 2 ml of KRB at 37°C in a water-saturated atmosphere containing 95% O$_2$-5% CO$_2$ in a Dubnoff incubator. Thereafter, the aortic rings were equilibrated for 15 min in KRB (37°C) in the presence of ouabain (10$^{-3}$ or 10$^{-2}$ M), when indicated. Finally, the tissues were incubated in 2 ml of KRB containing $^{86}$Rb (0.1 mCi/ml) in the presence or absence of ouabain for 15 min, as described previously (18). Transferring the aortic rings into ice-cold KRB stopped the reaction; the tissue was then quickly washed in cold buffer and gently blotted. Radioactivity of the samples was determined in triplicate by Cerenkov radiation in a liquid scintillation counter in the presence of 0.1% Tween 20 (2 ml).

Total pump activity was calculated by the difference between zero and 10$^{-3}$ M ouabain. Low ouabain-affinity activity was the $^{86}$Rb/K uptake in the presence of 10$^{-5}$ M activity minus that in 10$^{-3}$ M ouabain. The high ouabain-sensitive activity was calculated as the difference in $^{86}$Rb/K uptake activity in the absence and presence of 10$^{-5}$ M ouabain (18).

Sodium pump activity-enzyme activity in crude membranes. K$^+$-pNPPase activity was measured according to the method described by Bers (4), with slight modifications. Crude membrane preparations from aorta were treated with 0.05% Triton X-100 for 20 min at 37°C and then diluted eightfold with cold buffer (1 mM EDTA, 10 mM Tris·HCl, pH 7.4). The reaction mixture (0.7 ml) contained (in mM) 20 NaCl plus 1 ouabain or 20 KCl, 20 MgCl$_2$, 1 EDTA, 50 Tris·HCl (pH 7.4), and 30 µg of membrane protein. After a 5-min incubation at 37°C for temperature equilibration, the reaction was started by the addition of 100 µl p-nitrophenolphosphate (final concentration 5 mM). The reaction was terminated 35 min later by adding 50 µl 50% TCA and cooling on ice. After the samples were centrifuged to remove precipitated protein, 100 µl of 10 N NaOH were added to the supernatant. Absorbance at 410 nm was recorded, and K$^+$-pNPPase activity was calculated as the difference in values observed in the presence of K$^+$ minus the values observed in the presence of Na$^+$ and ouabain. A standard curve from 5 to 60 nM p-nitrophenolphosphate was routinely run.

Statistical analyses. Values are expressed as means ± SE. Differences between mean values of densitometry data, K$^+$-pNPPase, and $^{86}$Rb/K uptake were assessed by Student's t-test (unpaired) or by one-way ANOVA when appropriate. Differences were accepted as significant at the P < 0.05 level.

RESULTS

Expression of catalytic subunit isoforms of Na$^+$-K$^+$-ATPase. To elucidate the α-subunit isoforms that are present in the rat aorta, we conducted Northern, West-
ern, and PCR studies in isolated tissue from control, ADX, and ADX-DOCA rats. As shown below, we confirmed the presence of α1- and α2-isoforms by Northern and Western measurements.

As a first approach to assess α3-expression in rat aorta, we performed Northern blot studies on aortic total RNA and brain total RNA that serve as a positive control (Fig. 1A). No hybridization signal was observed with the α3-specific cDNA probe in aortic RNA isolated from control, ADX, and ADX-DOCA animals. As expected, an intense hybridization to a 3.8-kb band in brain RNA was observed, showing the specificity of the probe. Second, the presence of α3-mRNA in rat aorta was also checked by the more sensitive RT-PCR method. Figure 1B shows the results obtained with two different sets of specific α3-subunit primers. No mRNA transcripts were detected in four experiments with variable amounts of aortic RNA used in the RT assay (0.1–2.0 µg) or with 30–40 amplification cycles. Also, no product was detected when studying total RNA obtained from aorta of ADX or ADX-DOCA animals. As a positive control, we used brain total RNA, obtaining a strong band of PCR product of the expected size after 30 cycles of amplification. A third independent approach to detect α3-isofrom expression was to identify the protein in rat aorta. Western blot and immunocytochemical studies performed with specific monoclonal antibodies (Upstate Biotechnology) did not detect α3-subunit protein in crude aortic membranes (100 µg) or in aortic sections from control animals (data not shown). All of these results indicate no α3-isofrom expression in rat aorta, or at least α3-expression at very low levels, under the detection limits of our assays. As far as we know, no α3-isofrom expression has been detected in rat vascular endothelial cells (57). Only one paper (43) was found that showed the presence of α3-isofrom in rat tail artery. Considering that the tail artery is a thermoregulator organ in rat, this could reflect a functional difference in regional α-catalytic expression of rodent vascular tissue. However, our control experiments also failed to detect α3-expression in rat tail artery at the mRNA level, suggesting that this isoform is not significantly expressed in rat arteries.

To assess α-expression, RT-PCR studies were carried out. No α4-mRNA was detected in four different experiments with 0.3–2.0 µg of total RNA from aorta of control, ADX, and ADX-DOCA animals. Also, no α4-product was obtained with 30 to 40 amplification cycles (Fig. 1C). As a positive control, we used testis total cellular RNA (0.3 µg), obtaining a strong band of product of the predicted size. The RT reaction efficiency was determined by amplification of glyceraldehyde-3-phosphate-dehydrogenase cDNA, demonstrating that the absence of α4-product in rat aorta did not result from RT failure.

Effect of adrenalectomy on catalytic Na\(^{+}\)-K\(^{+}\)-ATPase isoform gene expression. We studied α1- and α2-isoforms from thoracic aorta of control, ADX, and ADX-DOCA rats. Each specific cDNA probe was hybridized to separate Northern blots containing equivalent amounts of total RNA in each lane. The relative amount of each isoform was determined and normalized to the control value.

The effect of adrenalectomy on rat aortic gene expression of α1-Na\(^{+}\)-K\(^{+}\)-ATPase isoform at 8 days is shown in Fig. 2. Also, the effect of DOCA-replacement therapy was studied. As expected, the α1-cDNA probe hybridized with a 3.7-kb mRNA transcript. The densitometric analysis of each blot revealed that no significant changes in α1-mRNA relative amounts were found in tissues from ADX animals or in ADX-DOCA rats when compared with controls (Fig. 2B).

Figure 3A shows the effect of adrenalectomy in α2-catalytic isoform expression at 8 days. As previously described, the probe recognized two transcripts of 5.3 and 3.4 kb corresponding to mRNAs encoding the α2-subunit (7, 41). The densitometric analysis demonstrated that the adrenalectomy induced a decrement in α2-isoform gene expression levels (Fig. 3B). At 8 days,
the α2-mRNA density was 38.5 ± 8.3% of control level (P < 0.001). The DOCA administration to the ADX animals almost prevented the reduction in α2-mRNA levels (84.7 ± 5.6% of control value). A significant difference was observed between ADX rats without or with DOCA, P < 0.001, ADX vs. ADX-DOCA.

Effect of adrenalectomy on β1-isoform gene expression. A dramatic effect of adrenalectomy was observed in β1-gene expression in the vascular tissue. As demonstrated by Northern blot studies in the ADX group, these transcripts diminished to 31.5 ± 4.5% of the control level at 8 days (P < 0.001, control vs. ADX). The group that received DOCA replacement had 47.3 ± 8.3% of the basal β1-mRNA levels [P < 0.001, control vs. ADX-DOCA; nonsignificant (NS), ADX vs. ADX-DOCA; see Fig. 4, A and B].

Finally, the expression of β2-subunits was also analyzed in total RNA extracted from aorta of control, ADX, and ADX-DOCA animals by RT-PCR. For β2-expression studies, rat brain RNA and soleus total RNA were used as positive and negative controls, respectively (51). A strong product band of the expected size was obtained when 0.6 μg of brain RNA were used (Fig. 4C).

Fig. 4. Gene expression of β-Na+/K+-ATPase mRNA isoforms in control, ADX, and ADX-DOCA rats. A: representative ethidium bromide-stained gel (20 μg each lane) and blot (from 4 separate experiments). Size of ribosomal RNAs are noted at left. Arrowheads, location of β2-mRNA transcripts. B: bars are means ± SE of control (open bar), ADX (filled bar), and ADX-DOCA (hatched bar) rats. *P < 0.001, ADX vs. control and ADX-DOCA vs. control. Nonsignificant, ADX vs. ADX-DOCA. C: representative RT-PCR experiment for β2-detection. Total RNA from aorta of control (2 and 1 μg, lanes 2 and 3, respectively), ADX (1 μg, lane 4), and ADX-DOCA rats (1 μg, lane 5) was used in RT-PCR experiments as described. Rat skeletal muscle (soleus, lane 1) and brain total RNA (lane 6) were used as negative and positive controls, respectively.
translational and posttranslational levels. Therefore, we decided to measure and compare the relative levels of the α₁-, α₂-, and β₁-isofoms present in rat aortic membranes by Western blot.

As expected, a band that migrates with an apparent molecular weight of 100,000 was recognized by the anti-α₁-specific monoclonal antibody (Fig. 5A). No differences in α₁-protein subunit amounts were found at 8 days among groups (Fig 5B). However, as shown in Fig. 6, the α₂-catalytic subunit declined to 55.8 ± 7.7% of the control levels 8 days after adrenalecetomy (P < 0.01). The DOCA-replacement therapy in ADX animals prevented the reduction in α₂-protein amount (90.8 ± 10.5% of control level, P < 0.01, ADX vs. ADX-DOCA).

The β₁-subunit protein levels were assessed in crude aortic membranes at 8 days. The results are shown in Fig. 7. Compared with control membranes, a marked decrease in ADX group was observed (41.6 ± 10%, P < 0.001). The administration of DOCA to ADX rats was not able to restore protein levels that were similar to ADX group (47.1 ± 11%; P < 0.001, control vs. ADX-DOCA; NS, ADX vs. ADX-DOCA).

Regulatory effect of adrenal steroids in Na⁺-K⁺-ATPase activity. The regulatory effect of adrenal steroids and DOCA on the sodium pump activity of rat aorta was evaluated by two different approaches: K⁺-pNPPase in isolated membranes and ouabain-sensitive ⁸⁶Rb/K uptake on intact aortic rings.

The K⁺-pNPPase activity in aortic membranes of control rats was 2.8 ± 0.22 nmol·mg protein⁻¹·min⁻¹. The ADX group showed a significant reduction to 57 ± 14% (1.6 ± 0.39 nmol·mg protein⁻¹·min⁻¹; P < 0.05, control vs. ADX). The DOCA-replacement therapy prevented this decrement (2.6 ± 0.22 nmol·mg protein⁻¹·min⁻¹; NS, control vs. ADX-DOCA; P < 0.05, ADX vs. ADX-DOCA).

To evaluate the Na⁺-K⁺-ATPase activity on intact aortic rings, we measured the ouabain-sensitive ⁸⁶Rb/K uptake. As shown in Table 1, a significant decrease in the ouabain-sensitive ⁸⁶Rb/K uptake was observed in ADX rats (59.8 ± 4.6% of control rats, P < 0.01, control vs. ADX); this uptake reflects the total Na⁺-K⁺-ATPase activity on intact tissue. DOCA replacement reversed the diminished Na⁺-K⁺-ATPase-mediated uptake observed in aortic rings from ADX rats (92.6 ± 9.1% of the...
control group), confirming the results obtained when measuring K\textsuperscript{+}-pNPPase activity in membranes (P < 0.01, ADX vs. ADX-DOCA; NS, control vs. ADX-DOCA).

In rats, the α isoforms differ in ouabain sensitivity: α\textsubscript{1} is ouabain resistant, whereas α\textsubscript{2} and α\textsubscript{3} are highly sensitive to the drug; consequently, the α\textsubscript{2}-catalytic isoforms of vascular tissue could be functionally distinguished with high (10\textsuperscript{-3} M) or low (10\textsuperscript{-5} M) ouabain concentrations (31). The results of such experiments are included in Table 1. As shown, in control tissue, the \textsuperscript{86}Rb/K\textsuperscript{+} uptake mediated by the ouabain-sensitive component was ~30% of the total pump activity, whereas in aortic rings from ADX rats there is a disappearance of the high ouabain-affinity component (P < 0.001, ADX vs. control). Interestingly, the disappearance of this activity after adrenalectomy was prevented by DOCA treatment. In fact, in the ADX-DOCA groups, the ouabain-sensitive activity was similar to control rats. On the other hand, no differences in the low ouabain-affinity-mediated \textsuperscript{86}Rb/K\textsuperscript{+} uptake were found between groups. These results in the \textsuperscript{86}Rb/K\textsuperscript{+} uptake mediated by the high and low ouabain-affinity components are in agreement with the pattern of expression of α\textsubscript{1}- and α\textsubscript{2}-catalytic isoforms of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase.

DOCA treatment to control rats produced no statistically significant change in total Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity (control rats, 167.1 ± 15.2 nmol \textsuperscript{86}Rb/K\textsuperscript{+}·g·wt\textsuperscript{-1}·min\textsuperscript{-1}; control + DOCA, 183.2 ± 9.6 nmol \textsuperscript{86}Rb/K\textsuperscript{+}·g·wt\textsuperscript{-1}·min\textsuperscript{-1}; n = 8 rats).

**DISCUSSION**

The results of the present study indicate that endogenous adrenal hormones participate in the molecular regulation of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity in vascular tissue. We found that α\textsubscript{1}, α\textsubscript{2}, and β\textsubscript{1}-subunit isoforms are expressed in rat aorta and that the adrenalectomy reduced the sodium pump activity, differentially affecting sodium pump isoform gene expression. ADX rats were provided with drinking water containing 0.9% NaCl to achieve volume balance and the survival of the ADX animals. Soszynski et al. (48) have shown that varying the sodium intake from a low to a high salt intake produced no statistically significant change in the mRNA abundance for any of isoforms in different tissues, including rat aorta.

Protein and RNA analyses did not detect α\textsubscript{3} and α\textsubscript{4}-isoform expression in aorta, suggesting, in principle, the existence of at least two different Na\textsuperscript{+}/K\textsuperscript{+}-ATPase isoforms in rat aorta, α\textsubscript{1}/β\textsubscript{1} and α\textsubscript{2}/β\textsubscript{1}. The existence of these isoforms in vivo is supported by the fact that in several experimental systems it has been possible to form active combinations of α\textsubscript{1} and α\textsubscript{2}-isoforms with β\textsubscript{1}-subunits (5, 12). Furthermore, it has been shown that nanomolar concentrations of ouabain augment caffeine-evoked contractions in rat arteries (55), and previous studies have shown the existence of low- and high-affinity inhibition components of ouabain-sensitive \textsuperscript{86}Rb/K\textsuperscript{-}uptake in rat aorta (31). Altogether, the present experimental evidence is consistent with the functional expression of α\textsubscript{1}- and α\textsubscript{2}-isoforms in vascular tissue.

The physiological role for multiple sodium pump isoforms in different tissues is actually unknown. It is possible that in rat aorta in vivo the different isoforms

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**Table 1.** Na\textsuperscript{+}/K\textsuperscript{-}-ATPase activity on intact aortic rings from control, ADX, and ADX-DOCA rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Low Ouabain Affinity</th>
<th>High Ouabain Affinity</th>
<th>Total Pump Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>112.9 ± 10.8</td>
<td>50.5 ± 8.3</td>
<td>163.1 ± 18</td>
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<tr>
<td>ADX</td>
<td>97.6 ± 7.6</td>
<td>0.5 ± 0.6</td>
<td>97.6 ± 7.6</td>
</tr>
<tr>
<td>ADX-DOCA</td>
<td>100.7 ± 10.4</td>
<td>50.3 ± 6.2</td>
<td>151.1 ± 14.8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 animals/group. Na\textsuperscript{+}/K\textsuperscript{-}-ATPase activity was expressed as nmol \textsuperscript{86}Rb/K\textsuperscript{-} uptake·g·tissue·wt\textsuperscript{-1}·min\textsuperscript{-1}. Total pump activity was measured as difference of total \textsuperscript{86}Rb/K\textsuperscript{-} uptake minus uptake with 10\textsuperscript{-5} M ouabain. Difference in activity in absence and presence of 10 µM ouabain is taken as high ouabain-affinity component and that in presence of 10 mM 1 and 1 mM ouabain is taken as low-affinity component. \textsuperscript{86}Rb/K\textsuperscript{-} uptake was measured in triplicate. ADX, adrenalectomized rats; ADX-DOCA, ADX rats with DOCA supplement. *P < 0.01, control vs. ADX; †P < 0.01, ADX vs. ADX-DOCA; ‡P < 0.001, control vs. ADX; §P < 0.001, ADX vs. ADX-DOCA.
could be targeted to defined cellular domains, allowing the cell to respond to different demands. In fact, Juhaszova and Blaustein (23) evidenced that the α1-isoform was ubiquitously distributed all over the plasma membrane in astrocytes, neurons, and arterial myocytes in culture, and the ouabain-sensitive isoforms (α2 and α3) were confined to a reticular distribution within the plasma membrane that paralleled underlying endoplasmic or sarcoplasmic reticulum. The above authors suggest that α1 could regulate bulk cytosolic Na⁺ concentration, whereas α2 and α3 might regulate Na⁺ and indirectly Ca²⁺ in a restricted cytosolic space between the plasma membrane and the reticulum. It is well established that rat α1-subunit shows a slightly lower Michaelis constant (Kₘ) for Na⁺ and lower affinity for cardiac glycosides than α2, making this hypothesis very attractive. However, the characterization of isozyme compartmentalization in vascular tissue in vivo remains to be determined.

It is well established that adrenal steroids control the synthesis of Na⁺-K⁺-ATPase in renal tubules and colon-stimulating α₁-gene expression (14, 54). Previous studies on the effect of corticosteroids in VSMCs showed a significant increment in α₁-expression by aldosterone (40) and dexamethasone (38). In contrast, our present study shows that adrenalectomy did not induce changes of α₁-Na⁺-K⁺-ATPase expression in rat aorta. Moreover, DOCA-replacement therapy did not affect α₁-mRNA or -protein levels in aorta. These results indicate that factors other than adrenal hormones also participate in α₁-pump regulation in vivo, eliciting the upregulation of α₁-catalytic subunit in ADX rats.

Different from α₁-gene expression, we found a dramatic downregulation of α₂-mRNA and -protein levels in ADX rats. This was prevented by DOCA-replacement therapy, suggesting a stimulatory role of mineralocorticoids in α₂-gene expression. The effect of DOCA was maximal at 8 days, but it was also present after 4 days of treatment in the ADX rats (data not shown).

The ⁸⁶Rb/K uptake studies showed a marked decrease of Na⁺-K⁺-ATPase activity in aorta from ADX animals, and this activity was recovered by DOCA treatment. A similar result was obtained when measuring Na⁺ pump activity in crude membranes from ADX and ADX-DOCA animals. These results are consistent with the observation presented above on mRNA and protein levels and consistent with earlier observations showing that corticosteroids produce an upregulation of the enzyme activity in cardiac tissue (25) and that adrenalectomy decreased the total Na⁺-K⁺-ATPase activity (49). Altogether, our data suggest that α₂-gene expression in aorta is adrenal (corticosteroid)-dependent and that the sustained presence of endogenous corticoids is required for normal aortic α₂-expression.

On the other hand, adrenalectomy induced a marked downregulation of β₁-mRNA and -protein, suggesting that endogenous levels of adrenocortical hormones are required for normal β₁-subunit expression. Previous reports indicate a substantial increment in β₁-mRNA levels in VSMC cultures on the addition of corticosteroids (38, 40). However, no significant changes in β₁-mRNA and protein amounts were observed in the ADX-DOCA rats compared with ADX animals. These results indicate that the in vivo modulation of β₁-mRNA and protein levels in aorta could be mediated by glucocorticoids or coordinated stimulation of gluco- and mineralocorticoids, because DOCA alone was unable to restore either β₁-mRNA or protein abundance.

Interestingly, the recovery of Na⁺-K⁺-ATPase activity observed in ADX-DOCA animals was correlated with α₂-mRNA and -protein restoration, but no significant effect over β₁-subunit expression was found. Assuming that the assembly of α₁β₂-heterodimer is a prerequisite for Na⁺-K⁺-ATPase function, these results suggest that under normal conditions in rat aorta the pool of β₁-subunits is larger than the α-isofoms pool. Thus only a profound diminution in β₁ could affect α₁/β₁- and α₂/β₁-isozyme function. This hypothesis is supported by observations that in mammalian renal cells in culture, β₁ can mature in excess of α₁-complements, forming an unassociated pool of β₂-subunits (35, 36). Similarly, a disproportionate abundance of β₂ compared with α₁ mRNA subunit content in canine vascular smooth muscle has been observed (2). Also, in rat skeletal muscle, an overall excess of β₂ over α₂-subunit proteins has been reported (28). Another possibility is that DOCA treatment could have a significant effect in the β₂-subunit, which might compensate for the lack of effect of DOCA treatment on β₁-subunit.

In summary, we found α₁-, α₂-, and β₁-isoform expression in rat aorta. Adrenalectomy induced a marked decrease of Na⁺-K⁺-ATPase activity in vascular tissue, with a concomitant decrease in α₁- and β₁-gene expression. In contrast, α₂-isoform expression was unchanged. The DOCA-replacement therapy in ADX rats did not modify α₁-levels but elicited an upregulation of α₂-catalytic isoform expression and recovery of the sodium pump activity, suggesting that mineralocorticoids increase the Na⁺-K⁺-ATPase activity through the upregulation of α₂-pump expression. The DOCA replacement did not produce a significant effect over the reduced β₁-subunit abundance in ADX rats, and the participation of glucocorticoids in the regulation of β-isoform gene expression in vascular tissue needs to be characterized further.

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