The development of hypertension is firmly associated with high salt intake, and reducing daily salt intake results in a decrease in both systolic blood pressure (SBP) and diastolic blood pressure of mildly hypertensive patients. It is, therefore, vital to understand the detailed mechanisms relating salt intake and blood pressure regulation.

In this regard, isolation of endogenous cardiotonic steroids such as ouabain and marinobufagenin from human and experimental animals, and findings of their involvement in cardiovascular and renal diseases, have a significant impact on the understanding of complex mechanisms of hypertension. Although there are inconsistent findings about the levels of plasma endogenous ouabain after salt administration, acute NaCl loading, deoxycorticosterone acetate (DOCA)-salt treatment, and high-salt diet have been shown to be associated with an increase in plasma ouabain in rats and humans. Augmented levels of plasma marinobufagenin have been detected in acute plasma volume expansion, high-salt diet, pregnancy-related hypertension, essential hypertension, primary aldosteronism, renal failure, and adrenocorticotropic hormone (ACTH)-induced hypertension.

Furthermore, Huang et al. and Krep et al. reported that central and peripheral administration of Digibind, an antibody Fab fragment to digoxin, significantly lowered the blood pressure of hypertensive high-salt and DOCA-salt treated rats.

Na,K-ATPase consists of three subunits, α, β and FXYD protein family. Four α isoforms of Na,K-ATPase, α1–α4 have been identified and show variable tissue distribution: the α1 isoform is expressed abundantly in most tissues; the α2 isoform is detected in brain, heart, skeletal and vascular smooth muscle, and adipocytes; the α3 isoform is predominant in neurons and ovaries; the α4 isoform is exclusively expressed in sperm. In most mammals, including humans, all four α isoforms are sensitive to ouabain, but in mice and rats, the α1 Na,K-ATPase is remarkably resistant to ouabain, leading investigators to postulate that the sensitive α2 isoform plays an important regulatory role in the cardiovascular system, despite its more restricted expression pattern.

The development of hypertension is firmly associated with high salt intake, and reducing daily salt intake results in a decrease in both systolic blood pressure (SBP) and diastolic blood pressure of mildly hypertensive patients. It is, therefore, vital to understand the detailed mechanisms relating salt intake and blood pressure regulation.

In this regard, isolation of endogenous cardiotonic steroids such as ouabain and marinobufagenin from human and experimental animals, and findings of their involvement in cardiovascular and renal diseases, have a significant impact on the understanding of complex mechanisms of hypertension. Although there are inconsistent findings about the levels of plasma endogenous ouabain after salt administration, acute NaCl loading, deoxycorticosterone acetate (DOCA)-salt treatment, and high-salt diet have been shown to be associated with an increase in plasma ouabain in rats and humans. Augmented levels of plasma marinobufagenin have been detected in acute plasma volume expansion, high-salt diet, pregnancy-related hypertension, essential hypertension, primary aldosteronism, renal failure, and adrenocorticotropic hormone (ACTH)-induced hypertension.

Furthermore, Huang et al. and Krep et al. reported that central and peripheral administration of Digibind, an antibody Fab fragment to digoxin, significantly lowered the blood pressure of hypertensive high-salt and DOCA-salt treated rats.

Na,K-ATPase consists of three subunits, α, β and FXYD protein family. Four α isoforms of Na,K-ATPase, α1–α4 have been identified and show variable tissue distribution: the α1 isoform is expressed abundantly in most tissues; the α2 isoform is detected in brain, heart, skeletal and vascular smooth muscle, and adipocytes; the α3 isoform is predominant in neurons and ovaries; the α4 isoform is exclusively expressed in sperm. In most mammals, including humans, all four α isoforms are sensitive to ouabain, but in mice and rats, the α1 Na,K-ATPase is remarkably resistant to ouabain, leading investigators to postulate that the sensitive α2 isoform plays an important regulatory role in the cardiovascular system, despite its more restricted expression pattern.

The development of hypertension is firmly associated with high salt intake, and reducing daily salt intake results in a decrease in both systolic blood pressure (SBP) and diastolic blood pressure of mildly hypertensive patients. It is, therefore, vital to understand the detailed mechanisms relating salt intake and blood pressure regulation.

In this regard, isolation of endogenous cardiotonic steroids such as ouabain and marinobufagenin from human and experimental animals, and findings of their involvement in cardiovascular and renal diseases, have a significant impact on the understanding of complex mechanisms of hypertension. Although there are inconsistent findings about the levels of plasma endogenous ouabain after salt administration, acute NaCl loading, deoxycorticosterone acetate (DOCA)-salt treatment, and high-salt diet have been shown to be associated with an increase in plasma ouabain in rats and humans. Augmented levels of plasma marinobufagenin have been detected in acute plasma volume expansion, high-salt diet, pregnancy-related hypertension, essential hypertension, primary aldosteronism, renal failure, and adrenocorticotropic hormone (ACTH)-induced hypertension.

Furthermore, Huang et al. and Krep et al. reported that central and peripheral administration of Digibind, an antibody Fab fragment to digoxin, significantly lowered the blood pressure of hypertensive high-salt and DOCA-salt treated rats.

Na,K-ATPase consists of three subunits, α, β and FXYD protein family. Four α isoforms of Na,K-ATPase, α1–α4 have been identified and show variable tissue distribution: the α1 isoform is expressed abundantly in most tissues; the α2 isoform is detected in brain, heart, skeletal and vascular smooth muscle, and adipocytes; the α3 isoform is predominant in neurons and ovaries; the α4 isoform is exclusively expressed in sperm. In most mammals, including humans, all four α isoforms are sensitive to ouabain, but in mice and rats, the α1 Na,K-ATPase is remarkably resistant to ouabain, leading investigators to postulate that the sensitive α2 isoform plays an important regulatory role in the cardiovascular system, despite its more restricted expression pattern.

The development of hypertension is firmly associated with high salt intake, and reducing daily salt intake results in a decrease in both systolic blood pressure (SBP) and diastolic blood pressure of mildly hypertensive patients. It is, therefore, vital to understand the detailed mechanisms relating salt intake and blood pressure regulation.

In this regard, isolation of endogenous cardiotonic steroids such as ouabain and marinobufagenin from human and experimental animals, and findings of their involvement in cardiovascular and renal diseases, have a significant impact on the understanding of complex mechanisms of hypertension. Although there are inconsistent findings about the levels of plasma endogenous ouabain after salt administration, acute NaCl loading, deoxycorticosterone acetate (DOCA)-salt treatment, and high-salt diet have been shown to be associated with an increase in plasma ouabain in rats and humans. Augmented levels of plasma marinobufagenin have been detected in acute plasma volume expansion, high-salt diet, pregnancy-related hypertension, essential hypertension, primary aldosteronism, renal failure, and adrenocorticotropic hormone (ACTH)-induced hypertension.

Furthermore, Huang et al. and Krep et al. reported that central and peripheral administration of Digibind, an antibody Fab fragment to digoxin, significantly lowered the blood pressure of hypertensive high-salt and DOCA-salt treated rats.

Na,K-ATPase consists of three subunits, α, β and FXYD protein family. Four α isoforms of Na,K-ATPase, α1–α4 have been identified and show variable tissue distribution: the α1 isoform is expressed abundantly in most tissues; the α2 isoform is detected in brain, heart, skeletal and vascular smooth muscle, and adipocytes; the α3 isoform is predominant in neurons and ovaries; the α4 isoform is exclusively expressed in sperm. In most mammals, including humans, all four α isoforms are sensitive to ouabain, but in mice and rats, the α1 Na,K-ATPase is remarkably resistant to ouabain, leading investigators to postulate that the sensitive α2 isoform plays an important regulatory role in the cardiovascular system, despite its more restricted expression pattern.

The development of hypertension is firmly associated with high salt intake, and reducing daily salt intake results in a decrease in both systolic blood pressure (SBP) and diastolic blood pressure of mildly hypertensive patients. It is, therefore, vital to understand the detailed mechanisms relating salt intake and blood pressure regulation.

In this regard, isolation of endogenous cardiotonic steroids such as ouabain and marinobufagenin from human and experimental animals, and findings of their involvement in cardiovascular and renal diseases, have a significant impact on the understanding of complex mechanisms of hypertension. Although there are inconsistent findings about the levels of plasma endogenous ouabain after salt administration, acute NaCl loading, deoxycorticosterone acetate (DOCA)-salt treatment, and high-salt diet have been shown to be associated with an increase in plasma ouabain in rats and humans. Augmented levels of plasma marinobufagenin have been detected in acute plasma volume expansion, high-salt diet, pregnancy-related hypertension, essential hypertension, primary aldosteronism, renal failure, and adrenocorticotropic hormone (ACTH)-induced hypertension.

Furthermore, Huang et al. and Krep et al. reported that central and peripheral administration of Digibind, an antibody Fab fragment to digoxin, significantly lowered the blood pressure of hypertensive high-salt and DOCA-salt treated rats.

Na,K-ATPase consists of three subunits, α, β and FXYD protein family. Four α isoforms of Na,K-ATPase, α1–α4 have been identified and show variable tissue distribution: the α1 isoform is expressed abundantly in most tissues; the α2 isoform is detected in brain, heart, skeletal and vascular smooth muscle, and adipocytes; the α3 isoform is predominant in neurons and ovaries; the α4 isoform is exclusively expressed in sperm. In most mammals, including humans, all four α isoforms are sensitive to ouabain, but in mice and rats, the α1 Na,K-ATPase is remarkably resistant to ouabain, leading investigators to postulate that the sensitive α2 isoform plays an important regulatory role in the cardiovascular system, despite its more restricted expression pattern.
attempt to explore the specific role of these various isoforms, we have generated genetically modified mice with specifically altered sensitivities to ouabain: α2 Na,K-ATPase (α1R/Rα2R/R) and a ouabain-sensitive (“humanized”) α1 Na,K-ATPase (α1S/Sα2R/R). We have shown that mice expressing either a ouabain-sensitive α1 or α2 isoform (α1R/Rα2S/S or α1S/Sα2R/R) can develop ACTH-dependent hypertension, whereas the mice with two resistant isoforms (α1R/Rα2R/R) remain normotensive during ACTH treatment. α1R/Rα2R/R mice also show lower blood pressure during pregnancy. By contrast, we found that 2-kidney, 1-clip renovascular hypertension is equivalent in all three genotypes, and therefore does not depend on a ouabain-sensitive α1 or α2 subunit. Since previous studies have specifically reported a link between DOCA-salt hypertension and endogenous cardiac responses to graded doses of ouabain in separate groups of untreated mice and mice pre-treated with Digibind (300 ng/gBW).

DOCA-salt and telemetric blood pressure measurement. Using the PA-C10 transmitter (Data Science International, St Paul, MN), continuous ambulatory blood pressure was recorded as described previously, using left carotid placement of the catheter tip, and subcutaneous implantation of the transmitter body. Adult female α1R/Rα2S/S, α1R/Rα2R/R, and α1S/Sα2R/R mice were uninephrectomized at least 2 weeks prior to transmitter placement, and at least 7 days of further recovery was permitted after transmitter surgery. After recording baseline blood pressure for 4–5 days, DOCA pellets (100 mg/21-day release) were implanted subcutaneously. After 5 additional days, tap water was replaced with 1% NaCl. This protocol was followed to provide information regarding the relative contributions of DOCA and high salt in each of the two genotypes. Blood pressure was recorded in 1-min episodes at 5-min intervals every other day using a PowerLab system and Chart software (ADInstruments, Colorado Springs, CO).

Digibind treatment during DOCA-salt hypertension. In separate groups of α1S/Sα2R/R and α1R/Rα2R/R mice, we examined the acute blood pressure response to the infusion of Digibind, a Fab antibody fragment that binds digoxin-like molecules. Mice were anesthetized with isoflurane and the left carotid artery was cannulated with catheters prepared from Micro-Renathane tubing (MRE-025; Braintree Scientific Tubing, Braintree, MA). The catheter was tunneled subcutaneously over the shoulder and through an incision in the scruff, then passed through a stainless steel spring attached to a swivel (model 375/25; Instech Laboratories, Plymouth Meeting, PA) secured at the top of the cage. The base of the spring was anchored with Kwik-Cast (World Precision Instruments, Sarasota, FL), and upon recovery, the system allowed for unrestrained movement around the cage. The catheter was connected to a pressure transducer (COBÉ Cardiovascular, Arvada, CO) for measurement of arterial pressure throughout the experiment and heparinized saline (40 U/ml) was infused intra-arterially at 0.1 μl/min. Mice were allowed to recover from surgery for 2–3 h prior to the beginning of a 30-min baseline recording period. At that time, mice were administered 300 ng/g of body weight (BW) Digibind (GlaxoSmithKline Philadelphia, PA) through the carotid catheter, and blood pressure was continuously measured thereafter until the termination of the experiment. Data were collected and analyzed using a PowerLab system, and average values for mean arterial pressure were measured at 5-min intervals.

To test the efficacy of Digibind in blocking responses to cardiotonic steroids, cardiac contractile responses to graded doses of ouabain were measured in the presence or absence of Digibind on α1S/Sα2R/R mice, since the hearts of these mice are exquisitely sensitive to cardiotonic steroids. Following ketamine/thiobutabarbital anesthesia, the right femoral artery and vein were cannulated, and a 1.4-French Millar Mikro-Tip transducer (Model SPR-671; Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the left ventricle. Pressure measurements were made for escalating doses of ouabain in separate groups of untreated mice and mice pre-treated with Digibind (300 ng/gBW).
Measurement of endogenous cardiotoxic steroids. The concentrations of plasma ouabain and marinobufagenin were measured by competitive fluoroimmunoassay as described previously. To determine plasma marinobufagenin contents, 0.05 μg of marinobufagenin-thyroglobulin conjugate, anti-marinobufagenin antibody (1:2000, both kindly provided by Dr Bagrov, NIH), and europium-labeled anti-mouse IgG (1:100, PerkinElmer, Waltham, MA) were used. Plasma was collected from mice after 21 days of DOCA treatment with either tap water or 1% NaCl, and 2–4 plasma samples from animals in the same experimental group were pooled to provide sufficient sample volume for the assay.

Whole tissue preparation and western blot analysis. Brain, heart, and kidney were harvested at day 21 of DOCA treatment and immediately frozen in liquid nitrogen. The frozen tissues were homogenized, and western blot analysis was performed and GAPDH as a loading control.

Data analysis. Data are shown as means ± s.e.m. For tail-cuff experiments, post-baseline data were analyzed using a mixed-effects regression model (SAS v9.2; SAS, Cary, NC). Fixed effects of diet, genotype, and day relative to start of DOCA treatment were included in the model along with all interactions, and a linear effect of time was assumed based inspection of the data. Other analyses were performed by one-way or two-way analysis of variance with repeated measures where appropriate. Post hoc comparisons of individual means were performed where appropriate using the Holm–Sidak test, and differences were considered to be statistically significant at P < 0.05.

RESULTS

Tail-cuff measurements of SBP in α1R/α2S/S and α1R/α2R/R mice

Consistent with our earlier studies, baseline blood pressure was not different between α1R/α2S/S and α1R/α2R/R mice (Figure 1, top). Unlike our previous findings in ACTH-induced hypertension, there were no differences between α1R/α2S/S and α1R/α2R/R mice in the response to DOCA, with or without NaCl, regardless of diet (Group × Day interaction, P = 0.75). In both groups of mice, there was a greater change in blood pressure in response to DOCA + NaCl than in response to DOCA + water (Diet × Day interaction, P < 0.0001), and this difference was comparable in both groups (Group × Diet × Day three-way interaction, P = 0.67). No other model terms were found to be significant. Post hoc analysis indicated no differences in blood pressure in response to DOCA + water in either group of mice, and that there was a marked pressure increase in response to DOCA + salt (P < 0.001), suggesting a salt-dependent model of hypertension under these conditions. These findings indicate that the ouabain-sensitive binding site of the α2 Na,K-ATPase does not play a critical role in the development of DOCA-salt hypertension.

We also measured body weight of mice throughout the experiment (Figure 1, bottom). After DOCA pellets were implanted, both α1R/α2S/S and α1R/α2R/R mice given water showed a steady and significant increase in body weight, 2.7 ± 0.3 and 2.1 ± 0.6 g at day 20, respectively. Mice given NaCl increased body weight by 1.5 ± 0.6 and 1.0 ± 0.3 g at day 20, respectively. Mice given NaCl showed a significant and comparable in all four groups (Day main effect, P < 0.0001; no significant interactions). Since the weight gain in NaCl fed mice appeared to be slightly delayed, and could be related to reduced drinking in these mice, we performed balance studies in a subset. Though sodium intake appeared to be slightly lower in α1R/α2R/R vs. α1R/α2S/S mice, a similar change was
a2 Na,K-ATPase does not mediate DOCA-salt hypertension

seen in sodium excretion such that overall balance was not different (data not shown). Heart and kidney weights (HW and KW) were also determined at day 21, since cardiotonic steroids and their binding site in Na,K-ATPase have been implicated in the process of organ hypertrophy.

There were no differences in the HW/BW or KW/BW ratio between the genotypes, whether drinking water or NaCl (Table 1). In addition, NaCl feeding during DOCA treatment resulted in larger normalized heart and kidney weights in both α1R/Rα2S/S and α1R/Rα2R/R mice, and the differences were comparable. These data indicate that the ouabain-binding site of α2 Na,K-ATPase does not contribute critically to DOCA-salt triggered cardiac and renal remodeling. Interestingly, while there were no genotype differences in normalized heart weight, DOCA-salt treatment increased absolute heart rate in α1R/Rα2S/S but not α1R/Rα2R/R mice. The possibility that the hearts of α1R/Rα2R/R mice respond to DOCA-salt with a slightly greater degree of hypertrophy cannot be ruled out.

Table 1 | Body weight, heart weight and kidney weight at day 21 of DOCA-salt treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>BW (g)</th>
<th>HW (mg)</th>
<th>KW (mg)</th>
<th>HW/BW</th>
<th>KW/BW</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1R/Rα2S/S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>25.1 ± 1.1</td>
<td>130 ± 13</td>
<td>274 ± 21</td>
<td>5.1 ± 0.3</td>
<td>10.9 ± 0.6</td>
<td>5</td>
</tr>
<tr>
<td>NaCl</td>
<td>23.1 ± 0.7</td>
<td>130 ± 6</td>
<td>352 ± 40**</td>
<td>5.6 ± 0.2**</td>
<td>15.2 ± 1.5**</td>
<td>6</td>
</tr>
<tr>
<td>α1R/Rα2R/R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>24.2 ± 1.2</td>
<td>111 ± 5</td>
<td>217 ± 7</td>
<td>4.6 ± 0.1</td>
<td>9.1 ± 0.3</td>
<td>6</td>
</tr>
<tr>
<td>NaCl</td>
<td>25.9 ± 0.3</td>
<td>145 ± 2**</td>
<td>381 ± 30**</td>
<td>5.6 ± 0.1**</td>
<td>14.7 ± 1.1**</td>
<td>5</td>
</tr>
</tbody>
</table>

Two-way ANOVA

| Grp Effect | 0.09 | 0.96 | 0.71 | 0.16 | 0.28 |
| Trt Effect | 0.45 | 0.05 | <0.001 | <0.001 | <0.001 |
| Grp × Trt  | 0.10 | 0.05 | 0.18 | 0.21 | 0.51 |

The data shown are mean ± s.e.

ANOVA: analysis of variance; BW, body weight; Grp, Group; HW, heart weight; KW, kidney weight; Trt, Treatment.

*P < 0.05 compared to α1R/Rα2S/S group fed the same diet (water or NaCl) corresponding water group, and **P < 0.05, compared to the water-treated group in the same genotype.

Figure 2 | Telemetric measurements of mean arterial pressure (MAP) in α1R/Rα2S/S (wild type), α1R/Rα2R/R, and α1R/Rα2R/R mice during deoxycorticosterone acetate (DOCA)-salt hypertension. All mice received the DOCA pellet on day zero and were given 1% NaCl to drink at day 4, as indicated. Two-way analysis of variance (ANOVA) results are shown, and indicate that all three groups responded to the DOCA-salt treatment with a similar increase in blood pressure (day effect, P < 0.001; Group × day interaction, P = 0.16). ANOVA, analysis of variance.

Plasma levels of ouabain and marinobufagenin in α1R/Rα2S/S and α1R/Rα2R/R mice

Elevated levels of cardiotonic steroids have been reported in a variety of hypertension-prone states. We therefore analyzed plasma concentrations of ouabain and marinobufagenin. Because the samples from several (2–4) animals were pooled to provide sufficient volume for assay, the number of values for each mean was low (n = 2–4). For this reason,
we have presented the results in the form of a box plot in Figure 3 to represent individual values, but it is important to note that the mean values represent integrated data from a larger number of mice (6–8, in each group). Ouabain levels differed slightly but significantly in water vs. NaCl fed mice. In the α1R/α2S/S mice, DOCA-salt resulted in a slightly higher plasma concentration of ouabain, whereas in α1R/α2R/R mice, ouabain concentration was slightly lower in the DOCA-salt mice (two-way analysis of variance: interaction \( P = 0.003 \)). Accordingly, ouabain concentration in DOCA-salt α1R/α2S/S mice was significantly higher than in DOCA-salt α1R/α2R/R mice. Plasma marinobufagenin levels were modestly higher following DOCA-salt treatment in both α1R/α2S/S or α1R/α2R/R mice, and the differences were similar (two-way analysis of variance: treatment effect \( P = 0.048 \), interaction \( P = 0.58 \)).

Blood pressure responses to Digibind in hypertensive α1R/α2S/S and α1R/α2R/R mice
Although DOCA-salt produced comparable increases in blood pressure in α1R/α2S/S and α1R/α2R/R mice, the observed differences in circulating steroid levels allows for the possibility of differing mechanisms of hypertension in the two groups (i.e., steroid-dependent vs. steroid-independent). To test this, we administered intravenous Digibind to awake mice that had been treated with DOCA-salt for either 14 or 21 days. As shown in Figure 4a, at baseline in awake, instrumented mice, blood pressure was elevated to an equivalent extent in both α1R/α2S/S and α1R/α2R/R mice (day 14: 148 ± 6 vs. 148 ± 4; day 21: 149 ± 11 vs 141 ± 10). Importantly, Digibind had no significant effect on blood pressure over the 80-min time period in either group of mice on either day 14 or 21.

To verify that our dose of Digibind could effectively block the functional responses to circulating cardiotonic steroids in this setting, we performed an acute experiment to examine responsiveness to administered ouabain, at escalating doses, in the
presence and absence of Digibind. We measured the maximal rate of left ventricular pressure development (first derivative of left ventricular pressure, LV dP/dt\textsubscript{max}) as a sensitive index of the effect of ouabain, since the cardiac inotropic response to ouabain is well-established. As shown in Figure 4b, ouabain-induced changes in LV dP/dt were completely blocked by 300 ng/gBW Digibind up to a ouabain dose of at least 13 pmol/gBW. If one assumes a volume of distribution equivalent to extracellular fluid volume, this dose of ouabain would be expected to produce acute plasma levels of around 65 nmol/l, which far in excess of those expected for endogenous steroids.\textsuperscript{6,32} These data indicate that effective doses of Digibind are unable to lower blood pressure in this model of DOCA-salt hypertension.

Protein expression of α1, α2, and α3 Na,K-ATPase during DOCA-salt hypertension

The expression of α1, α2, and α3 Na,K-ATPase subunit in brain, heart, and kidney were examined by western blot in α1\textsuperscript{R/R}α2\textsuperscript{S/S} and α1\textsuperscript{R/R}α2\textsuperscript{R/R} mice treated with DOCA + water versus DOCA + NaCl. As shown in Figure 5, the abundance of α1 subunit protein did not change in response to DOCA or DOCA-salt in any tissue tested from either of the two genotypes. There was also no effect of DOCA or DOCA + salt on the expression pattern of the α2 subunit in the brain, as shown in Figure 6a,d. By contrast, in the heart, α2 subunit expression was substantially elevated in α1\textsuperscript{R/R}α2\textsuperscript{R/R} mice treated with DOCA, with or without NaCl (Figure 6b,e), compared to α1\textsuperscript{R/R}α2\textsuperscript{S/S} mice. There was no detectable band corresponding to α2 Na,K-ATPase in the kidney by western blot (data not shown). Figure 6c,f illustrate the expression pattern of the α3 subunit in the brain, and show that the expression pattern was not different between α1\textsuperscript{R/R}α2\textsuperscript{S/S} and α1\textsuperscript{R/R}α2\textsuperscript{R/R} mice, and that DOCA-salt treatment had no effect relative to DOCA without salt. The α3 subunit was not detected in the heart or kidney by western blot (data not shown).

**DISCUSSION**

The primary objective of the study was to explore the physiological significance of the ouabain-binding site of α2 Na,K-ATPase in DOCA-salt hypertension. We have previously shown in several studies that ACTH-induced hypertension is blocked or blunted in mice in which the α2 subunit of the Na,K-ATPase has been rendered insensitive to ouabain.\textsuperscript{23,27} By contrast, we more recently demonstrated that 2-kidney, 1-clip renovascular hypertension develops normally in these α2 ouabain-resistant mice. Our present findings add a third model of experimental hypertension to this line of investigation, and our results show that DOCA-salt hypertension also does not require a ouabain-sensitive α1 or α2 subunit to fully develop. Compared to both α1\textsuperscript{R/R}α2\textsuperscript{S/S} (wild type) and α1\textsuperscript{S/S}α2\textsuperscript{R/R} mutant mice, the α1\textsuperscript{R/R}α2\textsuperscript{R/R} mice developed comparable levels of DOCA-salt hypertension. We must therefore conclude that in this model of DOCA-salt hypertension is not
dependent on elevated levels of endogenous circulating cardiotonic steroids interacting with the α2 Na,K-ATPase subunit. It is important to note, however, that all of the mice tested in this study retained a ouabain-sensitive α3 subunit, which leaves open the possibility that circulating steroids are involved in the development of high blood pressure, perhaps working through the central and sympathetic nervous system.

There is compelling evidence in the literature suggesting a role for endogenous cardiotonic steroids in blood pressure regulation, and some of the earliest and most convincing data were derived using the DOCA-salt rat model. As early as 1982, Kojima et al. reported that administration of digoxin antibody resulted in an immediate fall (within 10–20 min) in blood pressure in ACTH-hypertensive rats, a similar effect was observed with both preimmune IgG and IgG (Fab)_2 fragment, and the investigators suggested caution in the interpretation of studies using Digibind.33 Although our present data (Figure 4) are consistent with the hypothesis that salt loading may increase plasma levels of marinobufagenin, the differences we observed were subtle. Since we saw no effect of Digibind on blood pressure in any of animals, we think it unlikely that these changes reflect the mechanism that produces the increase in blood pressure. It is important to note, however, that Digibind is a polyclonal Fab that has the potential to change considerably with different pools over the years, and this may limit the reliability of inferences made between the present data and that from earlier studies. In light of these uncertainties, our data from α2 ouabain-resistant mutant mice adds an important finding to the body of literature regarding the role of endogenous cardiotonic steroids in the pathogenesis of hypertension.

As these earlier data show, Digibind can be an effective tool if used cautiously, and in our studies we endeavored to optimize the conditions under which we performed our measurements. Our initial inclination was to measure the blood pressure response by telemetry by administering the Digibind intraperitoneally. We realized though, that this would not permit any degree of confidence regarding the time course of absorption and volume of distribution of the antibody fragment, and we were lead to conclude that vascular access would be necessary. We attempted our first experiments, therefore in anesthetized mice but it became clear that the effects of anesthesia on the established level of hypertension was too unpredictable to render useful results. We therefore decided to perform these experiments in acutely instrumented mice that...
were allowed to recover from a anesthesia for several hours. Mice treated in this manner exhibited sustained and stable levels of blood pressure, and could be treated with Digibind without the added stress of handling and injection. Though we cannot rule out possible confounding effects of acute postoperative stress, our previous experience using this approach has shown that untreated mice exhibit a normal blood pressure, display few signs of perioperative stress, and respond normally to a wide range of vasoactive and cardiotonic substances.

One important aspect of our results that should not be discounted was that α1S/α2R mice did not exhibit an exaggerated hypertensive response to DOCA-salt. Since the α1 Na,K-ATPase is the primary isoform in the heart, vasculature and kidney, alteration ouabain affinity of the α1 isoform from resistant to sensitive might be expected to greatly enhance the response to a hypertensive challenge if endogenous cardiotonic steroids were indeed involved in that response. Such an effect was in fact observed in these mice with ACTH-induced hypertension. Nonetheless this phenomenon was not observed in the present studies of DOCA-salt hypertension, and strengthens the notion that endogenous cardiotonic steroids acting directly upon the cardiovascular system are not an important contributor to DOCA-salt hypertension in this setting. Again, it is important to stress that a neuronal contribution of α3 Na,K-ATPase to DOCA-salt hypertension cannot be ruled out in the present study since all of the mice retain the ouabain-sensitive α3 subunit.

There are several reports that support the hypothesis that the ouabain-sensitive α3 isoform participates in the central control of blood pressure and pathogenesis of hypertension. It has been shown, for example, that ablation of the AV3V region in the hypothalamus, a proposed storage site of endogenous Na,K-ATPase inhibitors, prevents the onset of DOCA-salt induced hypertension. This work suggests that an endogenous Na,K-ATPase inhibitor is released in the brain and acts locally to affect the sympathetic nervous system and the regulation of vascular tone in the periphery to contribute to the development of this hypertension. Furthermore, Huang and Leenen have presented evidence that endogenous Na,K-ATPase inhibitors are released in the central nervous system of rats prone to developing hypertension during high-salt conditions.

We examined tissue expression of α2 Na,K-ATPase in the α1R/α2R mice and the α1R/α2R mice, and found elevated levels in the hearts of α1R/α2R during DOCA treatment, with or without NaCl. Although we did not evaluate expression of the α2 subunit under basal conditions in this study, we have previously reported cardiac α2 subunit expression is elevated under baseline conditions in α1R/α2R mice compared to wild type, by a nearly 2-to-1 ratio. We can surmise therefore, that the relationship of α2 subunit expression in α1R/α2R mice compared to α1R/α2R mice is relatively preserved during DOCA treatment, with or without salt. This finding is relevant with respect to a potential signaling function of the Na,K-ATPase. In this paradigm, binding of ouabain triggers a signaling cascade involved in numerous cellular events, and it is reasonable to speculate that the disruption of this ouabain-evoked signaling may affect cellular homeostasis. In vitro studies have revealed that binding of ouabain to Na,K-ATPase can induce c-Src mediated endocytosis of the ouabain-bound Na,K-ATPase, leading to lysosomal protein degradation. Failure of this process, as would be expected in the α1R/α2R mice, would favor a build-up of the α2 subunit.

In conclusion, these results indicate that the ouabain-binding sites of α1 or α2 Na,K-ATPase do not play an essential role in increasing blood pressure in this model of DOCA-salt hypertension. These results are similar to our previous findings using the 2-kidney, 1-clip model of hypertension, but are contrary to our findings using ACTH-induced hypertension, where the α2 subunit was found to participate in the blood pressure increase. Though our current mouse model may differ substantially from other, well-established models of hypertension in the rat, our overall findings indicate that the role of endogenous cardiotonic steroids may be more important in stress-related forms of hypertension. Furthermore, since the α1R/α2R, α1R/α2R, and α1S/α2R mice contain an ouabain-sensitive α3 subunit, which is a dominant isoform in neurons, a potential involvement of α3S in DOCA-salt hypertension remains a distinct possibility.

Acknowledgments: The authors are grateful to Michelle L. Nieman and Valerie M. Lasko for their excellent technical assistance and to Maureen L. Bender for animal husbandry. We would also like to express our gratitude to Dr Bagrov for providing reagents used in the assay. This research was supported by National Institutes of Health Grant R01 HL28573 (J.B.L.), R01 HL66062 (J.B.L.), and R01 DK57552 (J.N.L.), and an Institutional Clinical and Translational Science Award (NIH/NCCR SUL 1R00263 14). Its contents are solely the responsibility of the authors and do not represent the official views of the NIH.

Disclosure: The authors declared no conflict of interest.

a2 Na,K-ATPase does not mediate DOCA-salt hypertension


