Reduction in Maternal Circulating Ouabain Impairs Offspring Growth and Kidney Development

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ABSTRACT

Ouabain, a steroid present in the circulation and in various tissues, was shown to affect the growth and viability of various cells in culture. To test for the possible influence of this steroid on growth and viability in vivo, we investigated the involvement of maternal circulating ouabain in the regulation of fetal growth and organ development. We show that intraperitoneal administration of anti-ouabain antibodies to pregnant mice resulted in a >80% decline in the circulating ouabain level. This reduction caused a significant decrease in offspring body weight, accompanied by enlargement of the offspring heart and inhibition of kidney and liver growth. Kidney growth inhibition was manifested by a decrease in the size and number of nephrons. After the reduction in maternal circulating ouabain, kidney expression of cyclin D1 was reduced and the expression of the α1 isoform of the Na⁺, K⁺-ATPase was increased. In addition, the elevation of proliferation signals including ERK1/2, p-90RSK, Akt, PCNA, and Ki-67, and a reduction in apoptotic factors such as Bax, caspase-3, and TUNEL were detected. During human pregnancy, the circulating maternal ouabain level increased and the highest concentration of the steroid was found in the placenta. Furthermore, circulating ouabain levels in women with small-for-gestational age neonates were significantly lower than the levels in women with normal-for-gestational age newborns. These results support the notion that ouabain is a growth factor and suggest that a reduction in the concentration of this hormone during pregnancy may increase the risk of impaired growth and kidney development.


Ouabain has been recognized as an endogenous steroid hormone involved in the regulation of BP and heart muscle contractility.1,2 The only established receptor for this hormone is the Na⁺, K⁺-ATPase, a major plasma membrane transporter. The Na⁺, K⁺-ATPase utilizes the energy from ATP hydrolysis to catalyze the exchange of intracellular Na⁺ for extracellular K⁺. This activity is essential for the regulation of cell volume and osmolarity, pH and calcium concentrations, maintenance of the plasma membrane electric potential, and the cotransport of ions, glucose, and amino acids across the plasma membrane.3,4

Ouabain has been shown to be synthesized in and released from the adrenal gland.5,6 The binding of the steroid to the Na⁺, K⁺-ATPase inhibits its hydrolytic and ion transporting activities. In addition, the ouabain–Na⁺, K⁺-ATPase interaction was found to induce the assembly of multiple protein complexes into functional microdomains that activate diverse signaling pathways. Among the various cascades activated are Src, 1,4,5-triphosphate receptor that regulates the mitogen-activated protein kinase pathway, reactive oxygen species, and intracellular Ca²⁺ oscillations.7–9 Some of these pathways are tightly involved in the regulation of cell growth.10 Indeed, ouabain has been shown to...
induce stimulatory and inhibitory effects on cell viability and proliferation. The addition of relatively low concentrations of ouabain induces proliferation of several cell types, whereas higher concentrations lead to apoptosis.\textsuperscript{11,12}

We previously found that lowering the endogenous ouabain present in serum-supplemented tissue culture media, by treating the media with specific anti-ouabain antibodies, reduced the viability and growth of several cultured cell lines.\textsuperscript{13} This suggested that ouabain serves as a growth factor, a notion that was recently addressed in relation to kidney growth and development. Studies by Li \textit{et al.} showed that the addition of exogenous ouabain to pregnant rats rescues the development of embryonic kidneys in animals given a low-protein diet, suggesting the steroid’s role in kidney development.\textsuperscript{14} In view of these studies, we hypothesized that circulating ouabain in normal pregnant animals may have a protective effect on the offsprings’ development and growth, particularly of the kidney. To test this hypothesis, we examined the effect of a reduction in circulating maternal ouabain on the weight of the offspring and their tissues. We show that reducing the maternal steroid level, by the administration of specific anti-ouabain antibodies to pregnant mice, had a marked effect on fetal body weight, organ development, and the expression of different proteins and signaling molecules.

Our results strongly support the hypothesis that endogenous ouabain functions as a growth hormone involved in the regulation of fetal growth and kidney development.

**RESULTS**

**Reduction of Endogenous Ouabain in the Culture Medium Inhibits Primary Cell Viability**

In a recent study, we demonstrated that a reduction in the ouabain present in the tissue culture media caused a marked decrease in the viability of various cell lines.\textsuperscript{13} This effect was now tested on primary human fibroblasts, bovine aortic endothelial cells, and rat smooth muscle cells. The endogenous ouabain present at 0.5 nM in the media was reduced by 87\% by treating the culture media with specific anti-ouabain antibodies.\textsuperscript{13} Cells grown in media previously incubated with normal rabbit IgG or untreated media served as the control. As shown in Figure 1, similarly to the effect seen in cultured cell lines, the viability of the three primary cells tested was significantly lowered after a reduction of the ouabain in the culture medium.

**Reduction in Maternal Circulating Ouabain Reduces Offspring Body Weight**

To test whether ouabain participates in the regulation of cell viability \textit{in vivo}, we studied the involvement of the steroid in mouse fetal development. First, circulating ouabain levels were determined in nonpregnant and pregnant mice. As shown in other studies,\textsuperscript{15} the levels of the steroid are increased by \textgreater3-fold during pregnancy (Figure 2). Notably, because the determination of ouabain is based on the interaction with antibodies, immunoreactive material is actually being measured. However, because the anti-ouabain antibodies used were previously shown to be highly specific, recognizing predominantly ouabain (see Concise Methods), the term \textit{circulating ouabain} is being used. Next we examined the effect of a reduction in the hormone levels in pregnant mice on offspring weight and length. To this end, mice in the second half of pregnancy were treated daily (intraperitoneally) with anti-ouabain antibodies (10 mg/kg) or with non-specific IgG (10 mg/kg) as the control. As shown in Figure 2, the administration of anti-ouabain antibodies reduced circulating ouabain in the pregnant mice by 80\% compared with that in IgG-treated animals. The reduced maternal circulating ouabain did not affect maternal weight gain during the second half of pregnancy, litter size, or average offspring length at birth (Table 1). However, offspring weight on the day of delivery was significantly lowered (Table 1). To test whether this effect is preserved after birth, the offspring were weighed every other day until age 3 weeks. Daily observations of the pups’ behavior (age of first-detected eye openings and rolling over) did not reveal any developmental deficits in the experimental group. However, the lower birth weight of offspring of mothers treated with anti-ouabain antibodies was not compensated postnatally and persisted throughout the examined period, reaching a maximal reduction of 12\% on day 18 (Figure 3). These results suggest that reduced maternal circulating ouabain inhibits fetal and postnatal mice growth.

**Reduction in Maternal Circulating Ouabain Causes Asymmetric Growth Alterations**

To study the involvement of maternal circulating ouabain in organ growth while having a minimal effect on organogenesis, the steroid concentrations were altered during the second phase of mice pregnancy. Weights of several organs, taken from the offspring of the two groups, were examined 17 days after birth. As shown in Figure 4A, a reduction in maternal circulating ouabain, by the administration of the anti-ouabain antibody, caused a significant decrease in the offsprings’ kidney (13\%) and liver (16\%) weights. Interestingly, no changes in the weights of the brain and heart were detected, even though a reduction in total body weight was observed (Figure 3). Similar results were obtained with organs taken 21 days after birth (data not shown). Because a reduction in maternal circulating ouabain affected body weight and organ weights, the effect of the treatment on relative organ weight (organ weight/body weight) was calculated. As shown in Figure 4B, the reduction in kidney and liver weights exceeded the reduction in body weight, resulting in a 12\% and 8.5\% decrease in kidney and liver relative weights, respectively. Notably, the relative weights of the brain and the heart were either not affected or even increased after a reduction in maternal circulating ouabain (Figure 4B). These findings imply that reduced maternal circulating ouabain promotes asymmetric growth alterations in which reduced body weight is manifested by a decline in the weight of peripheral organs and preservation of the weights of organs essential for fetal growth.
Reduction in Offspring Kidney Weight Is Manifested by Reduced Apparent Glomerular Density and Size

As mentioned above, previous studies pointed to the possible involvement of ouabain in kidney development. Hence we tested whether the reduction in kidney weight was accompanied by structural changes in the organ. Indeed, as shown in Figure 5, A and B, histologic examination of the kidney cortex, taken from 17-day-old offspring, revealed a significant (12.5%) reduction in apparent glomerular density in animals exposed to low concentrations of ouabain. Furthermore, the average size of individual glomerulus, as measured by cross-sectional area, was also smaller (8%) in these animals (Figure 5, C and D). These results suggest that maternal ouabain level is involved in kidney development.

Glomerular Changes Are Accompanied by Alterations in Protein Expression

Glomerular growth was shown to be associated with overexpression of the cell cycle progression factor, cyclin D1, in the hypertrophic kidney,16,17 whereas a reduction in this protein’s level suppressed the proliferation of glomerular mesangial cells.18 In addition, Lucas et al. recently demonstrated that the ouabain-induced proliferation of Sertoli cells depends on the upregulation of cyclin D1.19 Testing for possible changes in this protein in our system revealed that the expression of cyclin D1 was reduced by 25% in kidneys from offspring subjected to low concentrations of ouabain during their fetal development (Figure 6). This result is in accord with the reduced glomerular density and size observed under these conditions described above.

The levels of Na⁺,K⁺-ATPase, the established receptor for ouabain, were examined in the kidney of the offspring after a reduction in maternal ouabain. As shown in Figure 6, the expression of the α1 isoform of the Na⁺,K⁺-ATPase, the major isofrom present in the kidney, was upregulated (approximately 200%) in response to reduced maternal circulating ouabain. This upregulation of the receptor may represent an attempt to compensate for the reduction in the ligand concentration and may reflect mechanisms coping with the inhibition of kidney growth (see below).

Reduction in Maternal Circulating Ouabain Stimulates Proliferation and Reduces Apoptosis in the Offspring Kidney

The possibility that the inhibition of kidney development elicits postnatal compensatory mechanisms was further investigated.

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**Figure 1.** Effect of anti-ouabain antibodies on the viability of different primary cells. Primary human fibroblasts, bovine aortic endothelial cells, and rat smooth muscle cells are transferred to 96-well flat-bottom tissue culture plates and grown in complete media, as described in the Concise Methods, for 24 hours. The medium is replaced with the complete media previously incubated for 24 hours at 4°C in the presence or absence of anti-ouabain antibodies (100 μg/ml) or IgG (100 μg/ml). Cell viability is assayed after 48 hours using the MTT viability test. Values are expressed as the mean±SEM (error bars) (n=15). *P<0.01; **P<0.001 (significantly lower than the nontreated medium).

**Figure 2.** Effect of anti-ouabain antibody administration on maternal circulating ouabain concentration. Nonspecific IgG (10 mg/kg per day) or anti-ouabain antibodies (10 mg/kg per day) are injected into pregnant mice during days 9–18 of pregnancy. At the day of delivery, the mice are anesthetized, blood samples are collected, and ouabain-like immunoreactivity is measured in these mice and in nonpregnant mice, as described in the Concise Methods. The values are expressed as the mean±SEM (error bars) (n=3). *P<0.001 (significantly lower than the IgG-treated pregnant mice).
Because extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt pathways are involved in the stimulation of cell viability and proliferation, their activation was tested in kidneys taken from 17-day-old offspring. As shown in Figure 7, ERK1/2 and Akt were activated by >2- and 5-fold, respectively, by the reduction in maternal circulating ouabain. Furthermore, we found that the activation of ERK1/2 in these kidneys was manifested by a 20% activation of p90 ribosomal S6 kinase (RSK), a protein kinase activated by this kinase. Cell proliferation in these kidneys was also examined by proliferating cell nuclear antigen (PCNA) and Ki-67 staining. The number of PCNA-positive cells was elevated by 50% and 120% in the cortex and medulla, respectively (Figure 8, A–E). No difference was found in PCNA staining in the glomeruli. Similar results were obtained with Ki-67 staining (Figure 8, F–L). Cell apoptosis in the tissues was evaluated using active caspase 3, Bcl-2-associated X protein (Bax), and terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining. As shown in Figure 9, all tested apoptotic factors were significantly reduced in the kidney cortex and Bax was also significantly decreased in the medulla of offspring subjected to reduced maternal circulating ouabain. These findings strongly suggest that postnatal compensatory mechanisms manifested by increased proliferation and decreased apoptosis follow the growth inhibition effect ensued by the reduction of maternal circulating ouabain.

**Abundance of Circulating Ouabain in Human Fetal and Maternal Compartments**

Ouabain concentrations were determined in nonpregnant women and were compared with the levels found in pregnant women 1 week before delivery, at delivery, and 1 day after delivery. In agreement with previous studies, we found higher levels of circulating ouabain in pregnant women (Figure 10A). However, no significant differences in the steroid concentration were found at the different times tested. Ouabain concentrations were also tested in different fetal and maternal compartments (Figure 10B). We show that relatively high levels of endogenous ouabain are present in the placenta, implying that this organ is the source of these compounds during pregnancy.

**Circulating Ouabain Level Is Lower in Mothers of Small-for-Gestational Age Neonates**

To elucidate the physiologic role of human maternal circulating ouabain in fetal growth, plasma samples were taken from pregnant women admitted to the maternity ward at 37–42 weeks gestation. The steroid level was evaluated and a comparison was made between plasma taken from mothers of infants presenting with different birth weights within the normal birth weight range (2.5–3, 3–3.5, and 3.5–4 kg) and mothers of small-for-gestational age (SGA) infants (2–2.5 kg). As shown in Figure 10C, mothers with SGA neonates had significantly reduced circulating ouabain levels (0.38±0.15 pmol/ml) compared with the levels in mothers of infants presenting with a normal birth weight range of 3–3.5 and 3.5–4 kg (2.67±0.74 and 6.01±3.1 pmol/ml, respectively).

**DISCUSSION**

Low (nanomolar) concentrations of ouabain are known to induce the growth and proliferation of different cells in culture. In this study, we demonstrate that the same perturbation has a similar effect of reduced viability in primary cells (Figure 1), suggesting that endogenous ouabain acts as a growth factor in vitro. To test whether ouabain participates in the regulation of cell growth in vivo, we examined the involvement of the steroid, which is elevated during pregnancy (Figure 2), on fetal development under physiologic conditions. We show here that a reduction in maternal circulating ouabain concentrations during the second half of mouse pregnancy causes a decrease in the offsprings’ total body weight.

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<thead>
<tr>
<th>Treatment</th>
<th>IgG</th>
<th>Anti-Ouabain Antibodies</th>
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<td>Maternal body weight gain at days 9–18 of gestation (g)</td>
<td>23.05±3.85</td>
<td>22.76±1.4</td>
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<tr>
<td>Litter size</td>
<td>11±1</td>
<td>11.66±0.81</td>
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<tr>
<td>Offspring body length at delivery (cm)</td>
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<td>3.46±0.05</td>
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<tr>
<td>Offspring body weight at delivery (g)</td>
<td>1.73±0.02*</td>
<td>1.67±0.02*</td>
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Values are expressed as the mean±SEM. Pregnant mice were treated daily (intraperitoneally) during the second half of pregnancy (days 9–18) with anti-ouabain antibodies (10 mg/kg) or IgG (10 mg/kg). *P<0.05.
which was manifested by lower weights of kidney and liver and preservation of brain and heart weights. The reduction in offspring kidney weight was accompanied by reduced apparent organ preservation of brain and heart weights. The reduction in offspring organ growth while having a minimal effect on organogenesis, the possible involvement of maternal circulating ouabain in alterations in the steroid level may be involved in the etiology of fetal growth restriction.

Normal fetal growth consists of two different phases. The first phase (embryonic life) entails generation, organization, proliferation, and differentiation of the embryo. The second phase (fetal life) involves continuation of growth and functional maturation of different tissues and organs. To study the possible involvement of maternal circulating ouabain in organ growth while having a minimal effect on organogenesis, we altered the steroid’s concentration during the second phase of mouse pregnancy. During embryonic life, the growth in length is known to be most intensive, whereas the increase in weight is most evident during fetal life. Indeed, a reduction in maternal circulating ouabain during the second phase of mouse pregnancy, altered only offspring weight, without significantly affecting length (Table 1).

Kidney formation is extremely sensitive to environmental changes during fetal development. Different perturbations in the feto-maternal environment, such as a low-protein maternal diet and placental insufficiency, have been known to alter kidney weight and glomerular number and size. Because a final wave of nephrogenesis is observed during the first week of life in the mouse, kidney formation and protein activation were examined at postnatal days 17 and 21. We demonstrate that a reduction in maternal circulating ouabain significantly reduced absolute and relative renal weight (Figure 4) and caused a decrease in glomerular size and density (Figure 5). Interestingly, a low glomerular density in humans at birth was found to increase the risk of hypertension and progressive kidney diseases at a later stage of life. Hence, a reduction in maternal circulating ouabain may have significant pathologic implications. Notably, our results are in complete accord with those of Li et al., who studied the influence of ouabain administration to pregnant animals. The increase in maternal ouabain in animals on a low-protein diet should be examined. In addition, Jacobs et al. recently demonstrated that the administration of ouabain to rats under normal conditions impaired fetal and placental growth. We also examined the effect of an elevation in maternal circulating ouabain on offspring development using the experimental system described in this study. The administration of ouabain (15 μg/kg per day) to pregnant mice resulted in similar effects to that of a reduction in the steroid levels—namely, a reduction in offspring body weight (Supplemental Figure 1) and a decrease in kidney and liver weights, without changes in heart and brain weights (Supplemental Figure 2). These results suggest that in normal pregnancy, a defined range of circulating ouabain is required for adequate fetal development and both reduction and elevation in the steroid’s level have deleterious effects.

It is reasonable to suggest that the impairments in kidney development resulting from the reduction in maternal circulating ouabain would be followed by processes aimed at overcoming this deficiency. Our results support this notion: the alterations in kidney development were accompanied by the activation of ERK1/2, Akt, and p90 RSK (Figure 7), an elevation in PCNA and Ki-67 (Figure 8), and a reduction in Caspase 3, Bax, and TUNEL (Figure 9). These molecular events may represent compensatory mechanisms intended to rescue the retarded growth.
In this study, we addressed the possibility that maternal circulating ouabain is involved in the regulation of human fetal development. The steroid level was elevated during pregnancy and relatively high concentrations were found in the human placenta (Figure 10). These results suggest that endogenous ouabain is accumulated or synthesized in the placenta. The latter is in accord with a previous study showing that human placental tissue has the ability to synthesize and release endogenous digitalis-like factors.\textsuperscript{29} In addition, the elevation in maternal circulating ouabain implies that the steroid may play a role in human pregnancy. A possible function of maternal circulating ouabain in mice was recently suggested by Oshiro et al., who showed that pregnant mice possessing a mutation in the ouabain binding site on the α2 subunit of the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase manifest a decreased systolic BP.\textsuperscript{30} This advocates the involvement of maternal circulating ouabain in the elevated systolic BP during pregnancy. The low levels of maternal circulating ouabain found in mothers of SGA infants (Figure 10C) suggest that the compound may be involved in the regulation of human fetal growth. The mechanisms responsible may be either alteration in placental functions, maternal physiology (i.e., systolic BP), or a direct effect on fetal development.

Taken together, our work supports the notion that ouabain acts as a growth factor \textit{in vitro} and participates \textit{in vivo} in the process of fetal development and kidney growth, suggesting a new hormonal function for this steroid.

**CONCISE METHODS**

**Chemicals**

Rabbit nonspecific purified IgG, Protease Inhibitor Cocktail, N,N\textsubscript{-}dimethyl formamide, and SDS were obtained from Sigma-Aldrich (St. Louis, MO). An enhanced chemiluminescence kit was purchased from Biologic Industries (Bet-Haemek, Israel). Antibodies against Cyclin D1, total Akt, and Pathscan Multiplex Western Cocktail I containing antibodies against phospho-p90RSK, phospho-Akt, phospho-p42/44 mitogen-activated protein kinase (ERK1/2), phospho-S6, and Rab11 were obtained from Cell Signaling Technology (Danvers, MA). Horse-radish peroxidase–conjugated secondary goat anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Bio-Rad protein reagent, Laemmli sample buffer, and Precision plus protein standards were obtained from Bio-Rad (Munich, Germany). Polyvinylidene fluoride membranes were from EMD Millipore (Billerica, MA). Protein A Sepharose CL-4B beads were purchased from Pharmacia Biotech (Uppsala, Sweden) and a Sep-pak C\textsubscript{18} column was purchased from Waters, (Milford, MA).

**Human Samples**

The collection of human plasma and tissues samples was approved by the Hadassah Medical Organization Helsinki Committee (2412/09/97-HMO and 0346-13-HMO). Plasma samples were obtained from women at different stages of pregnancy and from different fetal and maternal compartments at the third stage of delivery. All samples were coded and no personal identifiers were collected. Gestational age was assessed by dating the pregnancy from the first day of the last menstrual period or from the first trimester transvaginal ultrasound. Mothers with gestational diabetes, preeclampsia, coagulation disorders, or multiple gestations were excluded.

**Animals**

The Joint Ethics Committee (Institutional Animal Care and Use Committee) of the Hebrew University and Hadassah Medical Center...
approved the study protocol for animal welfare. The Hebrew University is an internationally accredited institute of the American Association for the Accreditation of Laboratory Animal Care. Timed pregnant HSD:ICR mice were housed in the specific pathogen-free facility according to a 12-hour/12-hour light-dark cycle. After an acclimation period, mice (four animals per group) were injected (intraperitoneally) with either anti-ouabain antibodies (10 mg/kg per day) or rabbit nonspecific IgG-purified IgGs as control (10 mg/kg per day) on days 9–18 of pregnancy. The animals were weighed every other day. At the day of delivery, several mice were anesthetized and a blood sample (1 ml) was collected by cardiac puncture and transferred to cold lithium-heparin tubes (Vacutainer, Oakville, ON, Canada) and centrifuged (3000 × g, 5 minutes) for plasma separation and extraction of ouabain-like substances.

After birth, body size was measured (anal to nasal length). All of the offspring were weighed every other day and some were euthanized at age 17–21 days by cervical dislocation. The offspring were decapitated; the head, abdomen, and thoracic cavities were opened; and the brain, heart, kidney, and liver were removed. The organs were weighed and snap-frozen for protein extraction or fixed in 4% paraformaldehyde overnight for histologic measurements.

**Kidney Histology and Immunostaining**

After fixation, kidneys (10 animals per group) were rinsed with PBS and processed by dehydration in graded ethanol (50%, 70%, 80%, 95%, and 100%). The tissues were cleared through two changes of xylene and embedded in paraffin blocks at 60°C. Each paraffin block was serially sectioned into 4-μm-thick slices and routinely stained with hematoxylin and eosin. To identify cell proliferation and apoptosis, antigen retrieval was performed by incubating the slides in a 80°C water bath for 1 hour. Anti-PCNA antibodies (Abcam, Inc., Cambridge, UK) were used to determine cell proliferation and anti-active caspase 3 and anti-Bax (Abcam, Inc.) antibodies were...

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**Figure 6.** Effect of a reduction in maternal circulating ouabain on the expression of Cyclin D1 and the α1 subunit of the Na⁺, K⁺-ATPase in offspring kidney. Kidneys from 17-day-old offspring of mice that were subjected to different treatments during pregnancy (Figure 2) are lysed and Western blot analysis is performed as described in the Concise Methods. (A and B) Representative immunoblots (A) and their quantification (B). The values are expressed as the mean±SEM (error bars) (n=9). *P<0.05 (significantly different from that of the IgG-treated animals).

**Figure 7.** Effect of reduction in maternal circulating ouabain on the activation of signaling molecules in offspring kidney. Kidneys from 17-day-old offspring of mice that were subjected to different treatments during pregnancy (Figure 2) are lysed and Western blot analysis is conducted as described in the Concise Methods. (A and B) Representative immunoblots (A) and their quantifications (B). The activation of ERK, Akt, and p90 RSK is calculated as the ratio of phosphorylated-ERK (p-ERK) to total ERK, phosphorylated-Akt (p-Akt) to total Akt, and phosphorylated-p90 RSK (P-p90 RSK) to Rab11 as control protein. The values are expressed as the mean±SEM (error bars) (n=9). *P<0.05 (significantly higher than that of the IgG-treated animals).
applied to detect apoptosis. The slides were then incubated with specific peroxidase-conjugated secondary antibodies (Impress-Vector, Burlingame, CA). Diaminobenzidine/hydrogen peroxide was used as the chromogen substrate, producing a brown staining and counterstaining was performed with hematoxylin. Slices treated similarly in the absence of primary antibodies served as control. For immunofluorescence staining, the slices were rehydrated and antigen retrieval was performed using a PickCell pressure cooker in 10 mM citrate buffer (pH 6.0). Rabbit anti-Ki67 (Neo Markers, CA) was used as a marker for cell proliferation. The slides were then incubated with Cy3 conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). To visualize the cells nuclei, specimens were mounted in medium containing 46-diamidino-2-phenyl indole (DAPI) at 1 µg/ml (Thermo Fisher Scientific, Waltham, MA).

Quantification of Renal Phenotype
To quantify PCNA-positive cells, 10 randomly nonoverlapping fields (×200) were recorded for each slice (three slices per kidney) in the medulla and in the cortex, and the number of stained cells in each field was counted. For Ki-67 staining analysis, 10 high-magnification (×400) images were taken for each slice using a confocal Olympus microscope (Tokyo, Japan) equipped with a camera. The number of Ki-67-positive cells was determined among 1000 DAPI-positive cells. To evaluate the level of apoptosis in the tissue, the intensity of the immunostaining was given an arbitrary score from 0 to 3 by a blind observer, with 3 being the most intensive diaminobenzidine staining. Apparent glomerular density and glomerular size were determined using high-resolution recording. Images were recorded using a motorized stage to cover the full area of each section and were automatically stitched together. The number of total glomeruli in each slice was double-blind counted and divided by the area of the renal cortex of the same slice, measured by National Institutes of Health ImageJ software. Glomerular cross-sectional areas were measured in different kidney sections (three slices/kidney) using ImageJ software.

TUNEL Detection
Apoptosis was also evaluated by the TUNEL assay using an apoptotic cell detection kit following the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). To visualize the cell nuclei, specimens were mounted in medium containing DAPI at 1 µg/ml. For image analysis, 20 high-magnification (×400) images were taken for each slice (two slices per kidney) using a confocal or epifluorescent Olympus microscope equipped with a camera. To measure cell apoptosis, the number of TUNEL-positive cells was counted among 1000 DAPI-stained cells.

Cell Culture
Primary cultures of bovine aortic endothelial cells, rat smooth muscle cells, and human fibroblasts were prepared and characterized as...
previously described.31,32 Bovine aortic endothelial cells and human fibroblasts were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ at 37°C. The plates for bovine aortic endothelial cells were precoated with bovine fibronectin (5 μg/ml) and the cultures were studied between the third and seventh passages. Rat smooth muscle cells were cultured in DMEM supplemented with 15% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ at 37°C and were studied between the second and fifth passages.

For the experiments, rat smooth muscle cells and human fibroblasts were cultured in 96-well flat-bottom tissue culture plates, coated with 200 μg/ml type I rat tail collagen. Bovine aortic endothelial cells were cultured in 96-well flat-bottom tissue culture plates coated with fibronectin (5 μg/ml). After 24-hour preincubation in complete medium, the medium was replaced with complete medium previously incubated for 24 hours at 4°C with or without anti-ouabain antibodies (100 μg/ml) or rabbit IgG (100 μg/ml). Cell viability was assayed after 48 hours.

**Determination of Cell Viability**

Cell viability was determined using the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT was dissolved in PBS at a concentration of 5 mg/ml. A 25-μl volume of this solution was added to each well, and the plates were incubated at 37°C for 2 hours. The assay was terminated by the addition of 100 μl/well of an aqueous solution of 20% wt/vol SDS, 50% vol/vol N,N-dimethyl formamide, and incubation at 37°C for 1 hour. Absorbance was measured with an ELISA plate reader (BioTek Instruments, Winooski, VT) at 570 nm to quantify the amount of formazan product, which reflects the number of viable cells in culture.33 In view of the finding that MTT staining in ouabain-treated cells is not a sufficient methodology for quantification of Madin-Darby canine kidney cell viability,34 we performed preliminary experiments matching cell viability determined by MTT, cell count, and protein determinations. The results of these experiments validated the use of the MTT assay in our experimental systems (data not shown).

**Western Blot Analyses**

Kidneys were rinsed with PBS and homogenized with modified RIA buffer supplemented with 1 mM NaVO₄ and Protease Inhibitor Cocktail.35 After centrifugation, protein samples were diluted in Laemmli sample buffer and incubated at 95°C or 60°C for 5 minutes. SDS-PAGE was used to separate proteins on 12% or 10% gel. Proteins were loaded onto the gel (20 μg/40 μl) and subjected to electrophoresis for 1.5–2 hours at 100 V with protein standards and transferred to polyvinylidene fluoride membranes. After blocking with Tris-buffered saline (TBS) containing 0.1% (v/v) Tween and 5% (w/v) skim milk for 1 hour at room temperature, the membranes were incubated overnight at 4°C with one of the following primary antibodies: phospho-ERK1/2, total ERK1/2, phospho-Akt, total Akt, phospho-p90RSK, Rab11, Cyclin-D1, α1 subunit of Na⁺, K⁺-ATPase, and tubulin. The membranes were then washed with TBS containing
Figure 10. Circulating ouabain-like immunoreactivity in sera of pregnant and nonpregnant women and different maternal and fetal compartments. Ouabain-like substance extraction, partial purification, and determination are conducted as described in the Concise Methods. Values are expressed as the mean±SEM (error bars). (A) Ouabain-like immunoreactivity in sera collected from nonpregnant and pregnant women a week before delivery, at delivery, and 1 day postpartum (n=11–19). *P<0.01; **P<0.001 (significantly higher than in nonpregnant women). (B) Ouabain-like immunoreactivity in samples from the indicated maternal and fetal compartments at day of delivery (n=20–37). **P<0.001 (significantly higher than in maternal plasma). (C) Ouabain-like immunoreactivity in sera of pregnant women having neonates of varying birth weight (n=10–30). Sera are collected from pregnant women before delivery and infants are weighed immediately after delivery. *P<0.05 (significantly higher than 2.0–2.5 birth weight range).

0.1% Tween and subsequently incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies. An additional three washings in TBS were followed by antibody detection using an enhanced chemiluminescence kit, according to the manufacturer’s instructions. Signals were visualized on film (Kodak; BioMax, Wellsville, NY) and quantified by densitometry (Fluro-s Multilmager; Bio-Rad, Hercules, CA).

Preparation and Concentration of Anti-Ouabain Antibody
Anti-ouabain antibodies were prepared as previously described. In brief, ouabain-BSA conjugate, subsequently used to immunize rabbits was prepared as described by Masugi et al. A total 500 mg of ouabain-BSA in CFA were injected intramuscularly and subcutaneously into 3- to 4-month-old rabbits. The animals were rechallenged as above 7 times at 6-week intervals, with the same amount of antigen emulsified in incomplete Freund’s adjuvant. The collected rabbit sera were used at 1:1000 in the ouabain ELISA and without dilution to determine the concentrations of the antibodies (see below). The resulted antibodies are highly specific for ouabain and cross-react only with ouabagenin (53%), strophanthidin (16.5%), digoxin (0.76%), and bufalin (0.6%). Other steroids, including cholesterol, testosterone, progesterone, corticosterone, 17-hydroxy pregnenolone, and 21-deoxycortisol do not cross-react with the antibodies, even at 10 μM. Furthermore, incubation of FBS with the anti-ouabain antibodies resulted in a 70% reduction in endogenous ouabain levels, without a significant effect on the levels of other components such as testosterone, estradiol and insulin.

For concentrating anti-ouabain antibodies, Protein A Sepharose CL-4B beads and columns were prepared according to the manufacturer’s instructions. Rabbit plasma containing anti-ouabain antibodies was loaded onto the columns. Unbound IgGs were then eluted with PBS. Bound IgGs were then eluted with glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 3) into tubes containing 1 M Na2HPO4. The presence and quantification of anti-ouabain antibodies in the eluant was determined using ELISA (see below). The purified antibody was concentrated by centrifugation using Centricon (Amicon; EMD Millipore) centrifugal filter units (3000 NMWL membrane). The concentrated anti-ouabain antibodies were dialyzed overnight against saline in a cellulose tubular dialysis membrane (6000–50,000 cutoff, nominal pore size of approximately 0.002 μm; Spectrotemp Medical, Los Angeles, CA), which was first immersed in boiling double-distilled water containing 1 mM EDTA and 2% Na2CO3 for 20 minutes. The dialyzed antibodies were used in the above-described animal studies.

Extraction of Ouabain-Like Substances
Ouabain-like substances were extracted from mouse plasma, human plasma, and human placenta as previously described. Mouse plasma samples previously treated with anti-ouabain antibodies or rabbit nonspecific IgG were centrifuged (5 minutes, 15,000×g). The supernatant was first separated from high molecular weight compounds, using a 3000 NMWL membrane centrifugal filter (Amicon; EMD Millipore). The lower molecular weight fraction (<3000) containing free ouabain-like substances lacking contaminating anti-ouabain antibodies was diluted (1:1, vol/vol) with 0.1% trifluoroacetic acid (TFA). Human plasma samples were diluted (1:1) with 0.1% TFA. Human placentas were homogenized in methanol (1:10). After centrifugation (15 minutes, 28,500×g), the clear supernatant was evaporated and the dry residue was dissolved in 0.1% TFA. The TFA
samples obtained from the extractions were centrifuged (15 minutes, 28,500g) and the clear supernatant was loaded onto a Sep-Pak C18 column, which was then washed with 10 ml of double-distilled water containing 0.1% TFA. The bound steroid was eluted with 80% acetonitrile and the residue was dissolved in PBS after solvent evaporation. Aliquots from these solutions were used to determine ouabain-like substances by a quantitative competitive ELISA based on anti-ouabain antibodies.

Determination of Ouabain-Like Immunoreactivity by ELISA
A sensitive, competitive inhibition ELISA was designed for the quantification of ouabain-like substances. In this assay, samples were tested for their ability to inhibit the specific binding of the rabbit antibodies to solid phase–bound ouabain. The entire procedure was as previously described, with no modifications.5

Statistical Analyses
The data were expressed as the mean ± SEM. The two-tailed t test was applied when appropriate. Statistical comparisons between ouabain levels in different samples were made using the Kruskal–Wallis test. P < 0.05 was considered statistically significant.

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None.

REFERENCES


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