Renal and cardiac oxidative/nitrosative stress in salt-loaded pregnant rat

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Beauséjour A, Houde V, Bibeau K, Gaudet R, St-Louis J, Brochu M. Renal and cardiac oxidative/nitrosative stress in salt-loaded pregnant rat. Am J Physiol Regul Integr Comp Physiol 293: R1657–R1665, 2007. First published July 25, 2007; doi:10.1152/ajpregu.00090.2007.—Sodium supplementation given for 1 wk to nonpregnant rats induces changes that are adequate to maintain renal and circulatory homeostasis as well as arterial blood pressure. However, in pregnant rats, proteinuria, fetal growth restriction, and placental oxidative stress are observed. Moreover, the decrease in blood pressure and expansion of circulatory volume, normally associated with pregnancy, are prevented by high-sodium intake. We hypothesized that, in these pregnant rats, a loss of the balance between prooxidation and antioxidation, particularly in kidneys and heart, disturbs the normal course of pregnancy and leads to manifestations such as gestational hypertension. We thus investigated the presence of oxidative/nitrosative stress in heart and kidneys following high-sodium intake in pregnant rats.

Markers of this stress (8-isoprostaglandin F₂α, 8-iso-PGF₂α, and nitrotyrosine), producer of nitric oxide [nitric oxide synthases (NOSs)], and antioxidants [superoxide dismutase (SOD) and catalase] were measured. Then, molecules (Na⁺·K⁺-ATPase and aconitase) or process [apoptosis (Bax and Bcl-2), inflammation (monocyte chemotactant protein-1, connective tissue growth factor, and TNF-α)] susceptible to free radicals was determined. In kidneys from pregnant rats on 1.8% NaCl-water, NOSs, apoptotic index, and nitrotyrosine expression were increased, whereas Na⁺·K⁺-ATPase mRNA and activity were decreased. In the left cardiac ventricle of these rats, heightened nitrotyrosine, 8-iso-PGF₂α, and catalase activity together with reduced endothelial NOS protein expression and SOD and catalase activities were observed. These findings suggest that oxidative/nitrosative stress in kidney and left cardiac ventricle destabilizes the normal course of pregnancy and could lead to gestational hypertension.

Na⁺·K⁺-ATPase; rat model of preeclampsia; high-sodium supplementation

DURING PREGNANCY, METABOLISM and sodium homeostasis are regulated differently relative to the nonpregnant state. For decades, the relationship between sodium intake and extracellular fluid volume, arterial pressure, and the renin-angiotensin system has been investigated extensively (22, 29). However, only a few studies have focused on the effect of sodium intake during pregnancy. We have demonstrated previously that high-sodium intake brings physiological manifestations that are different between pregnant and nonpregnant Sprague-Dawley rats (10). In the latter, sodium supplementation did not change arterial blood pressure and elicited physiological manifestations such as increased natriuresis and diuresis that were adequate to maintain renal and circulatory homeostasis. However, sodium supplementation during rat gestation prevented the pregnancy-induced decrease in blood pressure and evoked many clinical features of human preeclampsia, such as proteinuria, fetal growth restriction, decreased renin-angiotensin-aldosterone system functioning, reduced circulatory volume, diminished uterine artery diameters, and reversal of gestation-associated resistance to vasopressors (2, 10, 46). Moreover, we demonstrated recently that high-salt intake causes increases in placental oxidative markers (11). Thus we wanted to further characterize this animal model of gestational hypertension.

Although there is no indication in the literature that the incidence of preeclampsia increases with higher salt consumption, or that, alternatively, low-salt intake prevents the development of gestational hypertension, epidemiological studies do demonstrate a small, yet significant correlation between salt intake and hypertension in humans (34). It has been proposed that reactive oxygen species (ROS) generation might explain some of the negative effects of salt-induced hypertension (3). ROS as well as reactive nitrogen species (RNS) are compounds derived, respectively, from oxygen (superoxide, O₂⁻; hydroxyl radical, •OH; and hydrogen peroxide, H₂O₂) and nitrogen (nitric oxide, NO; peroxynitrite anion, ONOO⁻; and nitrogen dioxide, NO₂⁻) (27). Most organs have both nonenzymatic and enzymatic defense mechanisms that protect tissues from the harmful effects of ROS and RNS. Superoxide dismutase (SOD) and catalase are first-line enzymes that counterbalance the deleterious effects of ROS and RNS. SOD catalyzes the reduction of O₂⁻ to H₂O₂, while catalase promotes the conversion of H₂O₂ to water and molecular oxygen. An imbalance between antioxidant defenses and ROS/RNS production in favor of the latter could lead to oxidative/nitrosative stress and induce protein, lipid, and DNA damage in cells, tissues, and organs that might be implicated in diseases such as hypertension (16). To evaluate oxidative and nitrosative stress, F₂-isoprostanes and nitrotyrosine production are considered as markers (32, 43).

Renal sodium handling is primarily modulated by the rate of its reabsorption, a process driven by Na⁺·K⁺-ATPase contained in the basolateral membrane of tubular cells (17). It has been shown in guinea pigs that lipopolysaccharide injection, which mediates inflammation and increased NO⁻ and ONOO⁻ production, inhibits renal Na⁺·K⁺-ATPase activity (45). These data suggest that the activity of this transmembrane enzyme is susceptible to free radicals. In addition, inflammation and oxidative/nitrosative stress are closely related in a positive-feedback loop involving cytokine release and ROS/RNS production (26, 47). In fact, ROS can trigger an inflammatory response through activation of the tumor necrosis factor-α (TNF-α) pathway (21). Moreover, inflammation of glomerular...
endothelial cells in culture is associated with increased apoptosis, which can also be induced by oxidative/nitrosative stress (37).

Aconitase, a tricarboxylic acid cycle (Krebs cycle) enzyme, is a member of a growing family of \(\text{O}_2^{-} \) -sensitive dehydratases that contain sites of \(\text{O}_2^{-} \) toxicity where \(\text{O}_2^{-} \) binding leads to inactivation of the enzyme (19, 20). Its suppression may serve as a useful marker for oxidative stress as we have shown in cardiac hypertrophy (7).

With the above considerations, we hypothesized that in pregnant rats receiving high-sodium supplementation, a loss of the balance between prooxidation and antioxidation in the kidney and the heart may disturb the normal course of pregnancy and lead to manifestations such as gestational hypertension. We investigated in the kidneys and left cardiac ventricle of salt-loaded pregnant rats pathways that are suspected to be linked to oxidative/nitrosative stress or to be consequences of this stress (apoptosis, inflammation, reduced Na\(^+\)-K\(^+\)-ATPase or aconitase activity).

**STUDY DESIGN**

**Animals.** Experiments were performed on female Sprague-Dawley rats (Charles River, St-Constant, QC, Canada) weighing between 225 and 250 g. They were bred with a known fertile male. Day 1 of pregnancy was established when spermatozoa were found in morning vaginal smears. All animals were housed under controlled light (6 AM to 6 PM) and temperature (21 ± 3°C). They were fed a normal diet containing 0.23% NaCl (Teklad global 18% protein rodent diet, Harlan Teklad, Montreal, QC, Canada). Control animals received tap water during the entire treatment period, while the experimental groups were exposed to 0.9 or 1.8% NaCl solution ad libitum for 7 days, starting on the last week of gestation. At the end of treatment (day 22 of gestation, term = day 23), the animals were decapitated (9–9:30 AM). Kidneys and the left cardiac ventricle were rapidly removed from the animals and snap-frozen in liquid nitrogen. This study was approved by the local Animal Care Committee, which is accredited by the Canadian Council on Animal Care.

**8-Isoprostaglandin F-2α measurements.** Heart left ventricle samples (100 mg) were homogenized in 1 ml of 100 mM phosphate buffer (pH 7.4) containing 1.5 mM NaF, 0.4 M NaCl, 1 mM EDTA, and 10 μM indomethacin. Samples were loaded on octadecylsilyl silica columns, and 8-isoprostaglandin F-2α (8-iso-PGF\(_2α\)) was eluted by methyl formate and evaporated under vacuum to dryness. An enzyme immunoassay was used to measure 8-iso-PGF\(_2α\) (Cayman Chemical, Ann Arbor, MI). The specificity of the assay was 100% for 8-isoprostanes; cross-reactivity of the antibody with thromboxane B\(_2\), prostaglandin E\(_2\), and PGF\(_2α\) was ≤ 0.1%.

**RT-PCR analysis.** Total cellular RNA from frozen kidneys was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) as described previously (10). PCR primers specific for the Na\(^+\)-K\(^+\)-ATPase-α1 subunit, the Na\(^+\)-K\(^+\)-ATPase-β1 subunit, monocyte chemoattractant protein-1 (MCP-1), connective tissue growth factor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

**Table 1. PCR primers designed (by Alpha DNA, Montreal, QC, Canada) for genes of interest**

<table>
<thead>
<tr>
<th>Gene (GenBank No.)</th>
<th>Oligonucleotide Sequence</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (X-66539)</td>
<td>TCCTGCTCTCAGGCTTCTTCT</td>
<td>112</td>
</tr>
<tr>
<td>FP</td>
<td>TCCTCCTCAAGGTTTACCC</td>
<td></td>
</tr>
<tr>
<td>RP</td>
<td>AGATTGGACGGAGGCCTAAACCC</td>
<td>418</td>
</tr>
<tr>
<td>Na(^+)-K(^+)-ATPase-α1 (NM_012504)</td>
<td>TCCGGCTTCTCACCTACCAAGAT</td>
<td></td>
</tr>
<tr>
<td>FP</td>
<td>GCTGATCGGAGAAGATTTGCC</td>
<td>357</td>
</tr>
<tr>
<td>RP</td>
<td>TGGAAGCTTACCGCCTGACGATG</td>
<td></td>
</tr>
<tr>
<td>MCP-1 (NM_031530)</td>
<td>CCAGAAACGAGCAACTCTCT</td>
<td>223</td>
</tr>
<tr>
<td>FP</td>
<td>TGCTGCTGTTGATTCTCTTG</td>
<td></td>
</tr>
<tr>
<td>CTGF (NM_022266)</td>
<td>ATGGAGGATGGTCCTAAGAC</td>
<td>303</td>
</tr>
<tr>
<td>FP</td>
<td>CACTCTTCCAGGAGGCTCAC</td>
<td></td>
</tr>
<tr>
<td>GAPDH (XM234175)</td>
<td>GGTGATGTCTGTTGCTGATA</td>
<td>369</td>
</tr>
<tr>
<td>FP</td>
<td>GGATCAGGGGAGATTCTTCT</td>
<td></td>
</tr>
</tbody>
</table>

cDNA was generated by reverse transcription (SuperScript II RNase H Reverse Transcriptase, Invitrogen). PCR (Taq DNA Polymerase, Invitrogen) was performed according to the procedure recommended by the manufacturer. The annealing step was carried out for 1 min at 52°C for TNF-α, 59°C for Na\(^+\)-K\(^+\)-ATPase-α1, 58°C for Na\(^+\)-K\(^+\)-ATPase-β1, 58°C for MCP-1, 61°C for CTGF, and 51°C for GAPDH. The extension step was performed for 1 min at 72°C. The PCR data were collected during the exponential phase. The number of cycles needed for amplification was determined for each gene (32 for TNF-α, Na\(^+\)-K\(^+\)-ATPase-α1, and MCP-1; 28 for CTGF; 22 for Na\(^+\)-K\(^+\)-ATPase-β1; and 20 for GAPDH). Water was used as negative control. PCR products were electrophoresed on 1% agarose gels containing ethidium bromide. Amplification products (Table 1) were quantified with Alpha Imager software (Alpha Innotech, San Leandro, CA).

**Nitric oxide synthase expression and apoptosis.** These parameters were measured by Western blotting. For nitric oxide synthase (NOS) expression, 100 mg of the kidney cortex or left cardiac ventricle were homogenized in 1 ml of HEPES buffer (20 mmol/l, pH 7.5) containing 5 μM peptatin A, 5 μM leupeptin, and 1 μM aprotinin. The measurement of Bax or Bcl-2 was done solely in the kidney, because the antibodies available were not specific in cardiac ventricle preparations. One hundred milligrams of kidney cortex were homogenized in a lysis buffer containing 10 mM Tris-HCl, 4 mM β-glycerophosphate, 4 mM NaF, 1 mM EDTA, 2 mM EGTA, 0.2 mM Na\(_3\)VO\(_4\), 1% Triton X-100, 0.5 mM PMSE, 5 μM leupeptin, 5 mM dithiotreitol, 1 μM microcystine, and 5 μM benzamidine. The homogenates were centrifuged at either 850 g for 10 min at 4°C (for NOSs) or 16,000 g × 15 min at 4°C (for Bax or Bcl-2). Protein concentrations were determined with the Bio-
Rad assay kit (Bio-Rad, Mississauga, ON, Canada), with bovine serum albumin as standard. The supernatants collected were then resuspended in Laemmli buffer. For comparative purposes, equal amounts of protein (60 μg/lane for NOSs or 65 μg/lane for Bax or Bcl-2) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide (7.5% for NOSs or 15% for Bax or Bcl-2) gel electrophoresis and then transferred electrophoretically to nitrocellulose membranes (Hybond ECL, Amersham, Little Chalfon, Buckinghamshire, UK). The membranes were blocked for 1 h at room temperature in 5% skim milk in Tris-buffered saline-0.1% Tween 20 (TBST) and then incubated with either mouse anti-endothelial NOS (eNOS) or mouse anti-inducible NOS (iNOS) (BD Transduction Laboratories, Mississauga, ON, Canada), or mouse anti-Bax (BD Transduction Laboratories) or mouse anti-Bcl-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) (all diluted 1:1,000 in TBST) overnight at 4°C, washed, and incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (IgG, 1:5,000 in TBST; Amersham) for 45 min at room temperature. Equal protein loading was determined on the same membrane with mouse anti-β-actin primary antibodies (Abcam, Cambridge, MA). Immunoreactive bands were visualized with the enhanced ECL chemiluminescence detection system (Amersham, Piscataway, NJ).

\[ Na^+\cdotK^+\cdotATPase \text{ activity assay.} \] Na⁺-K⁺-ATPase activity in the kidney (cortex and medulla) and left cardiac ventricle was quantified by the production of inorganic phosphate (Pi) liberated from ATP. In brief, 30 mg of tissues were homogenized in 1 ml of buffer containing 250 mM sucrose, 5 mM EDTA, and 20 mM imidazole. Homogenates (200 μl) were preincubated for 15 min at room temperature in an equal volume of 2.6 mM SDS solution. Then, 12.5 μl of the homogenate were added to 112.5 μl of a solution containing 100 mM NaCl, 83.3 mM Tris, 15 mM KCl, 5 mM MgCl₂, and 5 mM Na₃ in the absence or presence of 1 mM ouabain and incubated at 37°C for 10 min. Ouabain served as a selective inhibitor of Na⁺-K⁺-ATPase. Then, 30 mM Na₃ATP was added. After 5 min, the enzymatic reaction was terminated by adding 25 μl of ice-cold 3.9% HClO₄. The samples were centrifuged at 1,500 g × 15 min at 2°C. The supernatant (200 μl) was added to 400 μl of coloration solution containing 144 mM FeSO₄·7H₂O, 8 mM (NH₄)₆MoO₄·24·H₂O, and 3% H₂SO₄. Absorbance was read at 750 nm to assess Pi production. Na⁺-K⁺-ATPase activity (ouabain-sensitive fraction), calculated as the difference between total ATPase (assayed in the absence of ouabain) and the ouabain-resistant fraction, was expressed in micromoles of Pi liberated by 1 mg of protein during 1 h (μmol·h⁻¹·mg protein⁻¹). Each sample was assayed in triplicate, and the difference between averaged total ATPase and averaged ouabain-resistant ATPase was used in further calculations. Protein concentrations were determined with the Bio-Rad assay kit.

\[ SOD \text{ and catalase assay.} \] One hundred milligrams of kidney and left ventricle were homogenized in 1 ml of either 50 mM KH₂PO₄ and 1 mM EDTA, pH 7.4 (for catalase), or 20 mM HEPES, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 7 mM sucrose (for SOD) and centrifuged at either 10,000 g × 15 min at 4°C (for catalase) or 1,500 g × 5 min at 4°C (for SOD). Supernatants were used to measure enzyme activity by commercial kits according to the procedure detailed by the manufacturer (Cayman Chemical, Ann Arbor, MI).

\[ Protein \text{ nitration assay.} \] Tyrosine nitration, a marker of nitrosative stress, was quantified by slot-blot as described by Beauchamp et al. (9). Briefly, frozen tissues (75 mg) were homogenized in 1 ml of Tris buffer: 50 mM Tris, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1× complete cocktail inhibitor (Roche, Indianapolis, IN). The homogenates were centrifuged at 12,000 g at 4°C for 30 min, and the supernatant was collected. Protein concentrations were determined with the Bio-Rad assay kit. For comparative purposes, equal amounts of protein (15 μg/lane for the kidney and 5 μg/lane for the left ventricle) were loaded in a final volume of 100 μl in phosphate-buffered saline (PBS: 15 mM NaCl, 2 mM KCl, 10 mM KH₂PO₄, 1 mM KH₂PO₄, pH 7.4). Nitrocellulose membrane was placed in a Hybri-Slot manifold apparatus (Bethesda Research Laboratories, Bethesda, MD) and washed with PBS: 100 μl of PBS-diluted protein solution were allowed to passively diffuse for 30 min and then washed three times with PBS. The membrane was removed from the apparatus, washed briefly with PBS, and blocked with PBS-Tween containing 5% dried milk for 45 min at room temperature. The membrane was incubated overnight at 4°C with anti-nitrotyrosine polyclonal antibodies (1:5,000, Upstate Biotechnology, Lake Placid, NY). The washing, secondary incubation, revelation, and densitometry techniques and analysis were the same as described above for Western blotting. The membrane was finally incubated with Ponceau S to normalize for loading.

\[ Tricarboxylic \text{ acid cycle enzyme activities.} \] Frozen powdered tissue (left cardiac ventricle: 30 mg) was homogenized in 1 ml of 0.1 mM Tris·HCl-15 mM tricarboxylic acid solution (pH 7.8) and centrifuged at 9,500 g for 10 min at 4°C, and the supernatant collected was immediately processed for enzyme determinations. Citrate synthase activity was measured at 412 nm using 5,5'-dithiobis-2-nitrobenzoic acid as substrate (40). Aconitase activity was quantified at 340 nm by the formation of NADPH after α-ketoglutarate production from isocitrate (14). Protein concentrations were determined with the Bio-Rad assay kit. Enzyme activities are expressed in units (U) per milligram protein, where U is defined as the amount of enzyme catalyzing the conversion of 1 μmol of substrate per minute at 37°C.

\[ Statistical \text{ analysis.} \] Statistical analysis was performed by one-way ANOVA to assess the effects of 0.9 and 1.8% NaCl supplementation compared with the unsupplemented groups. The results are expressed as means ± SE, with P < 0.05 considered to represent significant differences.

\[ RESULTS \] Oxidative/nitrosative stress in the kidney. NOS protein expression is an index of NO production. Expression of eNOS (Fig. 1A) and iNOS (Fig. 1B) protein was increased in the kidney cortex from pregnant rats receiving sodium supplementation (0.9 or 1.8% NaCl).

The activity of SOD (Fig. 2A) and catalase (Fig. 2B), the first-line antioxidant enzymes acting in the presence of oxidative stress, was not different in the kidney cortex of pregnant rats on sodium supplement compared with their controls. However, nitrotyrosine levels were increased by NaCl supplementation but only reached statistical significance in rats re-
receiving 1.8% NaCl (Fig. 2C). This suggests the presence of nitrosative stress.

The ratio of apoptosis-promoting Bax to apoptosis-inhibiting Bcl-2 is often considered a useful index of cell death or survival (41). As illustrated in Fig. 3, Bax expression was increased in the kidney cortex of pregnant rats receiving salt supplement (Fig. 3A). No change in Bcl-2 protein expression was observed (Fig. 3B). This led to a higher Bax/Bcl-2 in kidney cortex samples from pregnant rats on 1.8% NaCl supplementation (Fig. 3C).

There was no indication of any inflammation process going in the kidney cortex on sodium supplementation, since none of the proinflammatory markers (MCP-1, CTGF, and TNF-α) measured was altered (Table 2).

In the kidney cortex (Table 3), 1.8% sodium intake decreased the mRNA expression of Na\(^+\)-K\(^+\)-ATPase-α1, the catalytic subunit, as well as the mRNA of the regulatory subunit, β1. This was accompanied by decreased activity of the pump. However, in the kidney medulla, no difference was observed between the high-sodium intake groups and the control group.

**Oxidative/nitrosative stress in the left cardiac ventricle.** iNOS bands were undetectable using Western blotting in left cardiac ventricle. As shown in Fig. 4, eNOS protein expression was not different between sodium-supplemented pregnant rats and their controls.

When compared with control animals, decreases in SOD activity (Fig. 5A) were seen in the left cardiac ventricle from pregnant rats on 0.9 or 1.8% NaCl. Catalase activity (Fig. 5B) increased in rats receiving 1.8% NaCl supplementation. Left cardiac ventricle 8-iso-PGF2α levels were augmented in animals receiving 1.8% NaCl (Fig. 5C). Although nitrotyrosine expression (Fig. 5D) was increased in the left cardiac ventricle from pregnant rats on NaCl-supplemented water, it only reached statistical significance in rats receiving 1.8% NaCl.
We reported previously that high-NaCl supplementation (0.9 or 1.8% in drinking water) given to pregnant rats during the last week of gestation prevented the pregnancy-induced decrease in blood pressure. Moreover, the higher NaCl supplementation (1.8%) induced physiological changes in these pregnant rats reminiscent of those noted in preeclampsia in humans, including placental oxidative stress (10, 11). In the present report, we demonstrate the presence of oxidative/nitrosative stress in the kidneys and heart, which could explain the inappropriate responses to pregnancy in this animal model of gestational hypertension.

**Oxidative/nitrosative stress in kidney.** Figure 7A summarizes our findings in the kidney cortex from pregnant rats on 1.8% NaCl. Increases in eNOS and iNOS protein expression lead to a greater production of NO. Since unchanged SOD or catalase activity was observed, this could suggest that, if oxidative stress is present, NO can be inactivated by O$_2^{-}$, thus limiting its vasodilatory effect. Moreover, in association with O$_2^{-}$, it can form ONOO$^-$ (23), which can nitrosylate tyrosine residues and cause changes in protein functions as well as tissue damage. The augmented nitrotyrosine expression strengthens this hypothesis of augmented O$_2^{-}$. In addition, the increased ratio of apoptosis-promoting Bax to apoptosis-inhibiting Bcl-2 indicated higher level of apoptosis. These cellular alterations together could explain the proteinuria observed in this animal model (10) and indicate glomerular injury. In male Sprague-Dawley rats, a high-sodium diet (6 g/kg NaCl for 10 days) induces an increase in renal O$_2^{-}$ production compared with a normal-salt diet (0.3 g/kg NaCl) (35). Moreover, excessive intrarenal O$_2^{-}$ production elicits sodium retention and blood pressure elevation by compromising local NO availability (36, 53). Contrary to these reports, in our hands, no effect was found in female nonpregnant rats (unpublished data). Sex-related differences could explain the origin of this divergence. In fact, it has been shown that the aorta and mesenteric arteries from male hypertensive rats produce higher levels of O$_2^{-}$ than those of females (12, 15). Moreover, a sexual dimorphism in antioxidant systems could also be implicated, since male Wistar rats show decreased SOD expression in the heart as well as lower SOD and catalase activities in macrophages compared with females (4, 5). We suggest that, during pregnancy, kidney redox state could be modified. This is in accordance with investigations in humans suggesting that pregnancy per se is an oxidative condition (51). Inducing oxidative stress by high-sodium diet could then alter the normal course of pregnancy.

**Table 2. Inflammatory gene mRNA expression (MCP-1, CTGF, and TNF-α) in the kidney cortex of control or salt-loaded pregnant rats**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Not Supplemented</th>
<th>0.9% NaCl</th>
<th>1.8% NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1 mRNA, arbitrary units</td>
<td>1.02±0.14 (5)</td>
<td>1.02±0.17 (5)</td>
<td>0.85±0.17 (5)</td>
</tr>
<tr>
<td>CTGF mRNA, arbitrary units</td>
<td>1.60±0.09 (5)</td>
<td>1.48±0.18 (5)</td>
<td>1.53±0.15 (5)</td>
</tr>
<tr>
<td>TNF-α mRNA, arbitrary units</td>
<td>1.04±0.11 (5)</td>
<td>0.87±0.12 (5)</td>
<td>0.86±0.07 (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Nos. in parentheses represent the no. of rats used.
During normal rat and human pregnancies, sodium and volume retentions are elevated (1, 8). Sodium reabsorption is driven, in part, by the Na⁺-K⁺-ATPase pump present in the basolateral membrane of tubular cells (17). Our results demonstrated that, in the kidney cortex from pregnant rats receiving 1.8% NaCl, decreased mRNA expression of both Na⁺-K⁺-ATPase-α1 and -β1 subunits is associated with diminished Na⁺/K⁺-ATPase activity in an autocrine manner, and this inhibition is accompanied by a reduction in Na⁺-dependent solute transport (44), erythrocytes (48), and kidney proximal tubules (52). In addition, it has been suggested that endogenous NO per se plays a direct inhibitory role in Na⁺-K⁺-ATPase activity in the kidneys (33). In fact, it has been reported that these rats have greater sodium excretion but higher plasma sodium concentration, suggesting that they are unable to excrete the sodium excess, and this may underlie the renal defects and cause the increased blood pressure (10). Increasing NaCl concentration in the lumen of the macula densa activates eNOS (25). In the present work, NOS activities were not measured. Interestingly, the overall remodeling that occurs in these rats seems to be the consequence of an inappropriate response compared with normal pregnant controls. In the present study, no significant differences were observed in aconitase and citrate synthase activities and oxidative and nitrosative stress markers as well as in TNF-α gene expression and Na⁺-K⁺-ATPase activity in the left cardiac ventricle from pregnant rats.

### Table 3. Na⁺-K⁺-ATPase-α1 and -β1 subunit mRNA expression and Na⁺-K⁺-ATPase activity in the kidneys of control or salt-loaded pregnant rats

<table>
<thead>
<tr>
<th></th>
<th>Not Supplemented</th>
<th>0.9% NaCl</th>
<th>1.8% NaCl</th>
</tr>
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<tr>
<td><strong>Na⁺-K⁺-ATPase-α1 mRNA</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Kidney cortex, arbitrary units</td>
<td>1.40±0.09 (5)</td>
<td>1.18±0.21 (5)</td>
<td>0.74±0.11* (5)</td>
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<tr>
<td><strong>Na⁺-K⁺-ATPase-β1 mRNA</strong></td>
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<tr>
<td>Kidney cortex, arbitrary units</td>
<td>2.24±0.13 (5)</td>
<td>1.93±0.22 (5)</td>
<td>1.77±0.12* (5)</td>
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<tr>
<td><strong>Na⁺-K⁺-ATPase activity</strong></td>
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<tr>
<td>Kidney cortex, μmol PO₄⁻·mg⁻¹·h⁻¹</td>
<td>4.90±0.27 (10)</td>
<td>4.56±0.43 (10)</td>
<td>3.53±0.28* (10)</td>
</tr>
<tr>
<td>Kidney medulla, μmol PO₄⁻·mg⁻¹·h⁻¹</td>
<td>3.90±0.57 (10)</td>
<td>4.16±0.34 (10)</td>
<td>4.36±0.26 (10)</td>
</tr>
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</table>

Values are means ± SE. Nos. shown in parentheses represent the no. of rats used. *P < 0.05 compared with not-supplemented rats.

**Fig. 4.** eNOS protein expression in left cardiac ventricle of control or salt-loaded pregnant rats. Representative immunoblot is shown. Human endothelial cell lysate (BD Biosciences) serves as positive control (control⁺) and Laemmli buffer as negative control (control⁻). Data from densitometric analysis are expressed as means ± SE from 6 animals/group.

**Fig. 5.** SOD activity (A), catalase activity (B), 8-isoprostaglandin F₂α (8-isopGF₂α) levels (C), and nitrotyrosine expression (D) in left cardiac ventricle of control or salt-loaded pregnant rats. Results are expressed as means ± SE from 10 rats/group. Values significantly different from unsupplemented control animals are indicated: *P < 0.05, **P < 0.01, ***P < 0.001.
receiving 0.9% NaCl, suggesting that oxidative stress may not be implicated in the cardiac remodeling present in these animals.

However, in the left cardiac ventricle from pregnant rats receiving 1.8% NaCl (Fig. 7B), nitrotyrosine and 8-iso-PGF$_{2\alpha}$, respective markers of ONOO$^{-}$ production and lipid peroxidation, were significantly increased. This was associated with decreased aconitase, a tricarboxylic acid cycle enzyme extremely sensitive to ROS (28), thus confirming the presence of an oxidative stress that could alter cardiac function and remodeling and could be associated with the high blood pressure observed in this model (10). SOD activity was reduced. It has been shown that ONOO$^{-}$ inhibited recombinant human Mn-SOD activity and induced the production of nitrotyrosine (38). In our rat model of gestational hypertension, unchanged eNOS expression combined with decreased SOD activity indicated that the half-life of tissue O$_{2}$$^{\cdot-}$ could be potentially lengthened, and this could promote its conversion to other deleterious oxidants. Indeed, a role for both NADPH and mitochondrial oxidases in cardiac ROS production and toxicity has been suggested (24, 39). The function of NADPH oxidases in the left ventricle of our model requires further investigation. In the present work, we noted higher TNF-$\alpha$ expression in the left cardiac ventricle from pregnant rats receiving 1.8% NaCl supplementation, which suggests that the cytokine pathway could be involved. In fact, ROS production was found to correlate positively with the severity of cardiac dysfunction and expression of cytokines, such as TNF-$\alpha$, interleukin-1, and interferon-$\gamma$, possibly via the activation of nuclear factor-$\kappa$B (18).

Normal pregnancy is characterized by decreased maternal plasma glucose. This is explained by its transfer to supply fetal requirements. As demonstrated by Bassien-Capsa et al. (6), pregnancy in rats is also accompanied by higher maternal plasma lactate-to-pyruvate ratios. The latter is a well-recognized marker of the redox state (13). In fact, when glucose metabolism is enhanced, increased NADPH production by glucose-6-phosphate dehydrogenase can cause inhibition of pyruvate dehydrogenase, the enzyme catalyzing pyruvate con-

Fig. 6. Tricarboxylic acid cycle enzyme activities: citrate synthase (A) and aconitase (B) in the left cardiac ventricle of control or salt-loaded pregnant rats. Results are expressed as means ± SE from 10 rats/group. Values significantly different from unsupplemented control animals are indicated: *$P < 0.05$, ***$P < 0.001$.
activation of aconitase A, the first Krebs cycle substrate. This phenomenon leads to higher lactate production and anaerobic metabolism. Thus it indicates that oxidative stress and differential regulation of cofactors could play a role in the adaptive changes that occur during pregnancy. However, increasing O$_2^-$ by salt supplementation could alter the regulation of cofactors, such as NADPH, and evoke considerable increases in oxidative stress status.

Pregnancy in humans and rats is a condition in which several changes occur to ensure normal evolution of mother and fetus. Our results, obtained in the kidney, indicate that pregnancy itself is an oxidative state. However, adaptation of the maternal body to this new environment resets cellular homeostasis. Decreased blood glucose concentration and elevated triglyceride and lactate levels, as reported (6), suggest an effect on redox status. With high-sodium intake, the boundaries of cellular homeostasis are exceeded, and oxidative/nitrosative stress ensues. Our animal pregnancy model, a tool to study gestational hypertension, provides information on kidney and heart damage while maternal perturbations are taking place. In fact, on day 22 of gestation, we demonstrated that high-NaCl supplementation leads to kidney apoptosis and kidney and heart oxidative/nitrosative stress, which destabilize the normal course of gestation. Oxidative stress has been clearly associated with preeclampsia, and our results correlate with human data, providing more information at the cellular level (31, 49, 50). Thus we propose that longitudinal studies must be undertaken to determine whether oxidative stress and NO reduction are the causes or consequences of renal and cardiac alterations.

Recently, a randomized, placebo-controlled trial was conducted on women with risk of preeclampsia (42). They were randomly allocated to vitamins C and E as antioxidant supplementation or placebo. The trial was stopped because not only did it fail to decrease the incidence of preeclampsia, but adverse events were observed. This emphasizes that basic research in an animal model is essential to better understand cellular mechanisms occurring in maternal adaptation to pregnancy.

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