High-salt diet increases glomerular ACE/ACE2 ratio leading to oxidative stress and kidney damage

Stella Bernardi¹,²,* Barbara Toffoli³,* Cristina Zennaro⁴, Christos Tikellis², Silvia Monticone⁵, Pasquale Losurdo⁴, Giuseppe Bellini⁴, Merlin C. Thomas³, Francesco Fallo⁶, Franco Veglio⁵, Colin I. Johnston² and Bruno Fabris⁴

¹Department of Morphology and Embriology, University of Ferrara, Ferrara, Italy, ²Diabetic Complications, Baker IDI, Melbourne, Australia, ³Institute for Maternal and Child Health, IRCCS Burlo Garofalo, Trieste, Italy, ⁴Department of Medical, Surgical and Health Sciences, Cattinara Hospital, University of Trieste, Trieste, Italy, ⁵Department of Internal Medicine and Hypertension, University of Torino, Torino, Italy and ⁶Department of Medical and Surgical Sciences, University of Padova, Padova, Italy

Correspondence and offprint requests to: Stella Bernardi; E-mail: stella.bernardi@bakeridi.edu.au

*Both authors contributed equally to this work.

Abstract
Background. Angiotensin II (AngII) contributes to salt-driven kidney damage. In this study, we aimed at investigating whether and how the renal damage associated with a high-salt diet could result from changes in the ratio between angiotensin-converting enzyme (ACE) and angiotensin-converting enzyme 2 (ACE2). Methods. Forty-eight rats randomly allocated to three different dietary contents of salt were studied for 4 weeks after undergoing a left uninephrectomy. We focussed on kidney functional, structural and molecular changes. At the same time, we studied kidney molecular changes in 20 weeks old Ace2-knockout mice (Ace2KO), with and without ACE inhibition. Results. A high salt content diet significantly increased the glomerular ACE/ACE2 ratio. This was associated with increased oxidative stress. To assess whether these events were related, we measured renal oxidative stress in Ace2KO, and found that the absence of ACE2 promoted oxidative stress, which could be prevented by ACE inhibition. Conclusion. One of the mechanisms by which a high-salt diet leads to renal damage seems to be the modulation of the ACE/ACE2 ratio which in turn is critical for the cause of oxidative stress, through AngII.

Keywords: ACE2; glomerulosclerosis; high-salt diet; oxidative stress; sodium

Introduction

The renin–angiotensin system (RAS) has been described as a group of enzymes and peptides where beside the classical pathway, whose primary effectors are angiotensin-converting enzyme (ACE) and angiotensin II (AngII), an alternative pathway has been recently characterized. This new pathway includes angiotensin-converting enzyme 2 (ACE2), a carboxypeptidase which converts AngII to Angiotensin 1–7 (Ang1–7), and Ang1–7, a peptide exerting opposite actions to those of AngII. Overall, ACE2 seems to counterbalance the effects of AngII by metabolizing active AngII and producing bioactive Ang1-7 so that the ratio ACE/ACE2 determines the final circulating and tissue levels of AngII [1–3] and ACE2 deficiency results in an increase of AngII levels [1].

The kidney was one of the first organs where ACE2 was localized in both tubules and glomeruli [4]. At this level, ACE2 deficiency has been shown to induce the development of an age-dependent glomerulosclerosis with associated proteinuria in male mice [5]. In addition, the systemic delivery of ACE2 activators in spontaneously hypertensive rats significantly attenuated interstitial renal fibrosis [6], and moreover, adenoviral-mediated ACE2 overexpression in diabetic mice significantly ameliorated their glomerular injury [7], therefore pointing out ACE2 protective effect for the kidney.

It is well known that an inappropriate increase of intrarenal AngII is one of the major culprits responsible for salt-related kidney damage [8]. Experimental evidence has shown that exposure to high-salt intake decreases cardiac ACE2 gene expression [9]. Based on these observations, we hypothesized that a high-salt diet could reduce ACE2 in the kidney and therefore increase ACE/ACE2 ratio and AngII, and hence cause kidney damage.

Materials and methods

Experimental protocol

To evaluate the effect of different dietary contents of salt on the kidney, we studied 48 wild-type male Wistar rats, weighing 250 g. Before starting the
diets, all the rats underwent left uninephrectomy to accelerate kidney damage [10]. The animals were anesthetized by intraperitoneal injection of 2,2,2-tribromoethanol (Sigma Chemical, St Louis, MO), at a dose of 25 mg/100 g of body weight. Buprenorphine (Temgesic, Reckitt, Benckiser) was used as analgesic and was injected subcutaneously at a dose of 0.05 mg/kg the day of the intervention and at a dose of 0.025 mg/kg the day after. One week after surgery, the rats were randomly allocated to three different dietary contents of salt; 16 rats were fed a 0.2% NaCl diet, considered the control diet, 16 rats were fed a 1.2% NaCl diet, considered the high-salt diet and 16 rats were fed a 8.2% NaCl diet, considered the very high-salt diet. All the rats were then followed for 4 weeks. At the end of the study, systolic blood pressure (SBP) was assessed by tail-cuff plethysmography, and the rats were then placed in individual metabolic cages for 24 h so that water intake could be recorded and total urine output collected. After performing these procedures, the animals were sacrificed by injection of 2,2,2-tribromoethanol at a dose of 25 mg/100 g of body weight. Bloods and kidneys were collected for biochemical, morphological and molecular analyses.

To evaluate the effect of ACE2 reduction on the kidney, we studied 9 male mice C57Bl6 10 weeks old (controls or CNT) and 18 male mice Ace2-knockout (Ace2KO) 10 weeks old on a C57Bl6 background [11]. Ace2KO mice were kindly provided by Prof. Josef Penninger and generated as previously described [1]. To evaluate whether the changes observed in Ace2KO were due to increased levels of AngII [1], Ace2KO mice were further randomly allocated to receive treatment with the ACE inhibitor (ACEi) perindopril (Servier, Neully, France) at a dose of 2 mg/kg/day in drinking water (Ace2KO + ACEi, n = 9 mice) or no treatment (Ace2KO, n = 9 mice) for 10 weeks. All the mice were fed a diet containing 0.2% NaCl and they were sacrificed at the age of 20 weeks by intraperitoneal injection of Euthal (Delvet Ltd, Seven Hills, Australia) at a dose of 10 mg/kg of body weight. Kidneys were removed, weighed and divided to be either fixed in paraformaldehyde or snap frozen for further analyses. The rats were housed at the animal house of Trieste University and the mice were housed at the animal house of the Baker IDI Heart and Cardiovascular Research Institute of Melbourne and were studied according to Institutional guidelines.

**General parameters and biochemical data**

Body weight (at the beginning and end of the study), relative kidney mass, SBP, daily water intake, daily urinary volume, daily sodium and potassium excretion, urinary creatinine, serum creatinine, creatinine clearance, albumin excretion rate (AER) and circulating aldosterone were measured in all the rats studied. Urinary sodium, potassium and creatinine as well as serum creatinine were measured on a analyzer. The creatinine clearance was expressed as millilitre per minute per square metre, by urinary creatinine (milligram per millilitre), volume of urine (millilitre per minute), circulating creatinine (milligram per millilitre) and body weight. AER was measured by enzyme-linked immunosorbent assay (Bethyl Laboratories and IMTEC Diagnostics, Antwerpen, Belgium) in the supernatant of 24-h urine specimens. Circulating aldosterone was determined by enzymatic immunoassay (Bethyl Laboratories, Antwerp, Belgium) in the plasma (DRG diagnostics international, Marburg, Germany).

**Isolation of glomeruli and assessment of glomerular albumin permeability**

After isolating the glomeruli from one-half of the kidney, $P_{\text{A}}$ (albumin permeability) was assessed in all the rats studied. Glomeruli were isolated by standard sieving technique in media containing 50 g/L of bovine serum albumin (BSA) as an oncotic agent. The isolated glomeruli were then washed in 1 mL of fresh media, whose 0.9 mL was used for RNA extraction. The remaining 0.1 mL was added to 0.9 mL of media and incubated for 10 min at 37°C and then transferred to a glass coverslip where the glomeruli were videotaped after a passage from a media containing 50 g/L of BSA to one containing 10 g/L of BSA for measuring the $P_{\text{A}}$. The rationale for the determination of $P_{\text{A}}$ has been previously described in detail [12]. Briefly, the media change creates an oncotic gradient across the basement membrane, resulting in a glomerular volume change $\Delta V = (V_{\text{final}} - V_{\text{initial}})/V_{\text{initial}}$, which we measured off-line by a video-based image analysis program (SigmaScan Pro; Jandel Scientific Software, Erkrath, Germany). The computer program determines the average radius of the glomerulus in a two-dimensional space so that the glomerular volume can be calculated by the formula $V = 4/3\pi r^3$. The magnitude of $\Delta V$ is related to the albumin reflection coefficient ($\sigma_A$) by the following equation: $\sigma_A = (\Delta V_{\text{experimental}} - \Delta V_{\text{control}})/\Delta V_{\text{control}}$, where $\Delta V_{\text{control}}$ is a parameter that expresses the degree of membrane permeability or ‘leakiness’. $\sigma_A$ is defined as (1 – $\sigma_A$) and it refers to the movement of albumin subsequent to water flux [13].

**Kidney structural features**

Two coronal paraffin kidney sections of 3-μm obtained from the rats were stained with hematoxylin–eosin, for tubular and glomerular area measurement, and with periodic acid-Schiff, for glomerulosclerosis assessment. Each section was examined by light microscopy (Olympus BX50WI, Hamburg, Germany) and digitized with a high-resolution camera (Q-Imaging Fast 1394, Surrey, Canada). The tubular area was determined in 70 proximal tubules for each section. The tubular area corresponded to the area of proximal tubular cells and the brush border, and it was obtained by subtracting the cross-sectional area of the tubular inner surface from the tubular total cross-sectional area (including inner and outer surface). Glomerular area was determined by measuring the area of 100 consecutive glomeruli in duplicate by two independent observers using an image analysis system (Image ProPlus 6.3 Software, Media-Cybernetics, Silver Spring, MD). Glomerulosclerosis was graded from 0 to 4+ in 40 glomeruli for each section. Sclerosis graded 1+ corresponded to the involvement of <25% of the glomerulus, while sclerosis graded 4+ corresponded to the involvement of >75% of the glomerulus. The values of the glomerular area and sclerosis are reported as the mean of the measurements performed by both examiners.

**Molecular biology**

Molecular biology techniques, microarray analysis, quantitative reverse-transcription polymerase chain reaction (RT–PCR) and immunostainings were performed as previously described [11, 14–16]. Briefly, ACE, ACE2, AT1 receptor, MAS1 receptor, NOX4 (NADPH oxidase 4), CuZnSOD (copper zinc superoxide dismutase), MnSOD (manganese superoxide dismutase) gene expression was also analyzed on the glomeruli isolated from the rats by real-time quantitative RT–PCR using the TaqMan system based on real-time detection of accumulated fluorescence. ACE, CuZnSOD (copper zinc superoxide dismutase), MnSOD (manganese superoxide dismutase), NOX4 (NADPH oxidase 4) gene expression was also analyzed in the kidneys of CNT, Ace2KO and Ace2KO + ACEi mice.

Glomerular ACE, ACE2 and nitrotyrosilated proteins were quantified by immunostainings. Kidney sections were incubated with the following primary antibodies: mouse anti-ACE (Chemicon, Temecula, CA) dilution 1:100 on the rat sections and dilution 1:200 on the mouse sections; goat anti-ACE2 (R&D Systems, Minneapolis, MN, dilution 1:100); rabbit anti-nitrotyrosine (Upstate, Lake Placid, NY, dilution 1:1000 on rat sections; Millipore, Billerica, MA, dilution 1:1000 on mouse sections). Biotinylated anti-ACE2 (R&D Systems, Minneapolis, MN, dilution 1:100); rabbit anti-ACE2 (R&D Systems, Minneapolis, MN, dilution 1:200 on rat sections and dilution 1:200; goat anti-ACE (R&D Systems, Minneapolis, MN, dilution 1:1000 on the rat sections and dilution 1:200 on the mouse sections); goat anti-ANG (Nordic, Abingdon, UK, dilution 1:1000 on the mouse sections); goat anti-ACE (R&D Systems, Minneapolis, MN, dilution 1:1000 on the mouse sections). Kidney sections were stained with hematoxylin–eosin, for tubular and glomerular area measurements, and with periodic acid-Schiff, for glomerulosclerosis assessment.

**Statistical analysis**

The data were evaluated by analysis of variance calculated using Statview 512 software for Apple Macintosh computer (Brainpower, Calabasas, CA). Mean comparisons were performed by Fisher least significant difference method. Linear regression analysis was used for testing two variable relationships. Results are expressed as mean ± SEM, unless otherwise specified. The criterion for statistical significance was $P < 0.05$.

**Results**

**General parameters and biochemical data of rats fed with different dietary contents of salt**

The 8.2% salt or very high-salt diet led to a significant reduction of body weight gain over the length of the study and to a significant increase in SBP (Table 1). Both 1.2 and 8.2% salt diets significantly increased water intake, urinary volume and sodium urinary excretion >24 h compared to the controls; however, potassium urinary excretion did not change between the groups studied (Table 1). Circulating...
aldoSTone was reduced in both the 1.2 and 8.2% salt content diet fed rats, although the reduction was not significant, because of a high variability in the concentrations between the groups studied (Table 1). However, there was a close inverse relationship between aldosterone levels and 24-h urinary sodium excretion \( (r = 0.66; \ P < 0.001) \).

**Renal functional and structural features in rats fed with different dietary contents of salt**

Both 1.2 and 8.2% salt content diets led to kidney hypertrophy and increased tubular area compared to the controls (Table 2). However, only the very high-salt diet significantly increased glomerular area and was associated with glomerulosclerosis (Table 2). Consistent with this, the very high-salt diet led also to the development of glomerular functional abnormalities since it significantly increased glomerular \( P_{\text{alt}} \) and AER, without affecting serum creatinine nor creatinine clearance (Table 2).

**Glomerular oxidative stress in rats fed with different dietary contents of salt**

Since oxidative stress plays a role in salt-driven kidney damage [17], we decided to evaluate the expression of molecules involved in the generation of reactive oxygen species. Following a microarray analysis (Supplementary table), we focussed on CuZnSOD and MnSOD and NOX4, whose gene expression was measured in the glomeruli previously isolated from the kidneys of all the dietary groups of rats. Among these, both CuZnSOD and MnSOD, which are both antioxidants, were significantly down-regulated in the glomeruli of the very high-salt diet fed rats, whereas the gene expression of NOX4 was only slightly increased (n.s., not significant) in the same group of rats (Table 3). The staining for glomerular nitrotyrosine showed a significant increase in nitrosylated proteins after both 1.2 and 8.2% salt content diets, which is a marker of oxidative stress. In the control group, the percentage of the glomerular area stained for nitrotyrosine was 3.76 ± 0.3%, whereas in the high-salt diet group this was 6.58 ± 0.5% \( (P < 0.001 \text{ versus control}) \) and in the very high-salt diet group this was 8.72 ± 0.5% \( (P < 0.001 \text{ versus control}) \) (Figure 1).

**Table 1. General parameters and biochemical data**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>1.2%</th>
<th>8.2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>328.6 ± 4.8</td>
<td>317.7 ± 8.1</td>
<td>304.8 ± 5.2*</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>117.9 ± 1.4</td>
<td>121.1 ± 3.1</td>
<td>150.1 ± 3.7*†</td>
</tr>
<tr>
<td>Water intake (mL/100 g body weight)</td>
<td>6.77 ± 0.75</td>
<td>16.26 ± 1.1*</td>
<td>21.52 ± 2.6*†</td>
</tr>
<tr>
<td>Urinary volume (mL/100 g body weight)</td>
<td>3.06 ± 0.33</td>
<td>9.78 ± 0.82*</td>
<td>17.02 ± 1.6*†</td>
</tr>
<tr>
<td>Urinary sodium (mEq/100 g body weight/day)</td>
<td>0.39 ± 0.04</td>
<td>3.03 ± 0.26*</td>
<td>4.7 ± 0.6*†</td>
</tr>
<tr>
<td>Urinary potassium (mEq/100 g body weight/day)</td>
<td>0.63 ± 0.12</td>
<td>0.81 ± 0.09</td>
<td>0.61 ± 0.06</td>
</tr>
<tr>
<td>Serum aldosterone (pg/mL)</td>
<td>249 ± 27</td>
<td>213 ± 32</td>
<td>171 ± 25</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.41 ± 0.02</td>
<td>0.40 ± 0.01</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>AER (mg/24 h)</td>
<td>0.62 ± 0.07</td>
<td>0.55 ± 0.1</td>
<td>2.51 ± 0.35*</td>
</tr>
<tr>
<td>Clearance creatinine (mL/min/m²)</td>
<td>35.6 ± 2.4</td>
<td>36.3 ± 3.6</td>
<td>33.5 ± 2.2</td>
</tr>
<tr>
<td>*Data are expressed as mean ± SEM. Control, 0.2% NaCl diet; 1.2%, 1.2% NaCl diet; 8.2%, 8.2% NaCl diet. †P &lt; 0.05 versus control.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Kidney structural and functional features in high-salt-treated rats**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>1.2%</th>
<th>8.2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney structural features</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal mass (mg kidney weight/g body weight)</td>
<td>3.85 ± 0.08</td>
<td>4.16 ± 0.05*</td>
<td>4.41 ± 0.13*</td>
</tr>
<tr>
<td>Tubular area (µm²)</td>
<td>1403 ± 19.9</td>
<td>1473 ± 25.8*</td>
<td>1494 ± 30.9*</td>
</tr>
<tr>
<td>Glomerular area (µm²)</td>
<td>8112 ± 77</td>
<td>8209 ± 84</td>
<td>11130 ± 143*</td>
</tr>
<tr>
<td>Glomerulosclerosis (arbitrary units)</td>
<td>1.07 ± 0.03</td>
<td>1.17 ± 0.04</td>
<td>2.81 ± 0.2*†</td>
</tr>
<tr>
<td>Kidney functional features</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( P_{\text{alt}} )</td>
<td>0.08 ± 0.03</td>
<td>0.11 ± 0.07</td>
<td>0.2 ± 0.35*</td>
</tr>
<tr>
<td>AER</td>
<td>0.62 ± 0.07</td>
<td>0.55 ± 0.1</td>
<td>2.51 ± 0.35*</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.41 ± 0.02</td>
<td>0.40 ± 0.01</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>Clearance creatinine (mL/min/m²)</td>
<td>35.6 ± 2.4</td>
<td>36.3 ± 3.6</td>
<td>33.5 ± 2.2</td>
</tr>
<tr>
<td>*Data are expressed as mean ± SEM. Control, 0.2% NaCl diet; 1.2%, 1.2% NaCl diet; 8.2%, 8.2% NaCl diet. †P &lt; 0.05 versus control.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ACE protein expression significantly increased with salt intake, being 0.7 ± 0.1% in the control diet group, 1.7 ± 0.2% in the high-salt diet group and 2.2 ± 0.3% in the very high-salt diet group of rats, whereas ACE2 protein expression slightly decreased with an increased salt intake. Overall, the ACE/ACE2 ratio was significantly increased in both 1.2% (P < 0.05 versus controls) and 8.2% salt content diet fed rats (P < 0.005 versus controls) (Figure 2B); thus, the immunohistochemistry confirmed the significant increase in the glomerular ratio ACE/ACE2, which was proportional to the dietary content of salt (Figure 3).

Renal effect of ACE2 deficiency

To determine the renal consequences of a reduction of ACE2, we studied oxidative stress in the kidneys of Ace2KO mice. In addition, to establish whether the changes observed in Ace2KO mice were due to an excess of AngII, we treated Ace2KO mice with the ACEi perindopril. First of all, renal ACE gene expression increased significantly in Ace2KO mice (P < 0.05 versus control), which was completely prevented by treatment with perindopril (P < 0.005 versus Ace2KO) (Figure 4A). Similarly, glomerular ACE protein expression increased significantly in Ace2KO (P < 0.005 versus control), which was completely prevented by treatment with perindopril (P < 0.001 versus Ace2KO) (Figure 4B). Secondly, focusing on the oxidative stress, CuZnSOD and MnSOD gene expression were down-regulated in Ace2KO mice, the first with a P-value < 0.05 versus controls and the second only moderately decreased (n.s.) (Table 5); on the contrary, NOX4 gene expression was significantly up-regulated in Ace2KO mice (P < 0.05 versus controls). However, semiquantitative analyses of proteins have all been performed on the glomeruli. Treatment with perindopril led to a significant increase in the gene expression of CuZnSOD (P < 0.05 versus Ace2KO) and to a significant decrease in the gene expression of NOX4 (P < 0.05 versus Ace2KO) (Table 5). Consistent with this, Ace2KO mice displayed a significant increase of glomerular nitrosylated proteins, marker of oxidative stress. In the control group, the percentage of the glomerular area stained for nitrotyrosine was indeed 0.65 ± 0.1%, whereas in the Ace2KO this was 1.29 ± 0.2% (P < 0.05 versus controls) (Figure 5A and B). Treatment of Ace2KO mice with the ACEi perindopril prevented such a change, being the glomerular area stained for nitrotyrosine 0.30 ± 0.1% in the Ace2KO + ACEi group (P < 0.005 versus Ace2KO) (Figure 5B and C). The fact that the RNA extraction was performed in the glomeruli of the rats, whereas it was performed in the entire kidneys of the mice could explain why the changes in the gene expression of oxidative stress mediators were not entirely congruent between the models.

Discussion

In this work, we have investigated the consequences of a high-salt intake on the kidney, finding that a high dietary

Table 3. Glomerular oxidative stress mediators in the salt-treated rats

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Control</th>
<th>1.2%</th>
<th>8.2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuZnSOD</td>
<td>1 ± 0.16</td>
<td>0.83 ± 0.10</td>
<td>0.46 ± 0.06**</td>
</tr>
<tr>
<td>MnSOD</td>
<td>1 ± 0.11</td>
<td>0.79 ± 0.08</td>
<td>0.64 ± 0.06*</td>
</tr>
<tr>
<td>NOX4</td>
<td>1 ± 0.21</td>
<td>0.80 ± 0.10</td>
<td>1.37 ± 0.43</td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± SEM. Control, 0.2% NaCl diet; 1.2%, 1.2% NaCl diet; 8.2%, 8.2% NaCl diet. NOX4, NADPH oxidase 4; CuZnSOD, copper zinc superoxide dismutase; MnSOD, manganese superoxide dismutase.

**P < 0.05 versus control.

Table 4. Glomerular RAS in the salt-treated rats

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Control</th>
<th>1.2%</th>
<th>8.2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>1 ± 0.2</td>
<td>1.26 ± 0.18</td>
<td>1.52 ± 0.18</td>
</tr>
<tr>
<td>ACE2</td>
<td>1 ± 0.35</td>
<td>0.40 ± 0.06*</td>
<td>0.51 ± 0.05*</td>
</tr>
<tr>
<td>AT1 receptor</td>
<td>1 ± 0.19</td>
<td>1.03 ± 0.11</td>
<td>1.07 ± 0.16</td>
</tr>
<tr>
<td>MAS1 receptor</td>
<td>1 ± 0.24</td>
<td>0.71 ± 0.08</td>
<td>1.25 ± 0.01</td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± SEM. Control, 0.2% NaCl diet; 1.2%, 1.2% NaCl diet; 8.2%, 8.2% NaCl diet.

**P < 0.05 versus control.

Fig. 1. Effect of dietary salt intake on glomerular oxidative stress. Representative nitrotyrosine-immunostained sections of kidneys (original magnification ×20). (A) 0.2% salt diet; (B) 1.2% salt diet and (C) 8.2% salt diet.
content of salt significantly increases glomerular ACE/ACE2 ratio which may regulate the degree of glomerular oxidative stress and therefore may be considered one of the mediators of salt-driven kidney damage. Experimental evidence has frequently shown the detrimental effect of salt on renal structure, leading to widespread renal fibrosis [18, 19]. This process has been shown not only to lead to renal impairment but also to increased blood pressure, together creating a vicious cycle, leading to end-stage renal disease. This study investigates some of
the molecular mechanisms that may be involved in the pathogenesis of salt-driven kidney damage. The animal models chosen for this purpose included rats fed with three different contents of dietary salt after a unilateral nephrectomy that was performed to accelerate the natural history of kidney damage [10] and mice with a genetic deficiency of ACE2. Among the molecular mechanisms linking salt to renal damage, we particularly focussed on the glomerular RAS, as it has been recently described [2, 20]. We hypothesized that changes in ACE/ACE2 glomerular ratio could play a causative role in salt-induced kidney damage, particularly as recent evidence support a role for this enzyme in the development of glomerulosclerosis [5] and diabetic nephropathy [4, 21].

The highest dietary salt intake (8.2% salt content diet) significantly raised blood pressure, led to kidney hypertrophy, glomerulosclerosis and caused functional abnormalities. It increased the \( P_{\text{ab}} \), with consequent development of albuminuria. This is in agreement with the concept that a high-salt diet has a detrimental effect on kidney function [18]. We also found that increasing the dietary content of salt promoted glomerular oxidative stress. Oxidative stress was proportionally increased in both the 1.2 and 8.2% salt content diets compared to the controls (Figure 1); whereas 1.2% salt diet significantly increased the nitrosylation of proteins, the 8.2% salt content diet led to a significant change of both the transcriptional and post-transcriptional processes leading to oxidative stress. \( \text{CuZnSOD} \) was the main gene whose expression was changed after a 8.2% salt diet, and this is in line with previous observations reporting that \( \text{CuZnSOD} \)-knockout mice displayed increased superoxide production and vascular dysfunction [22]. These gradual changes in glomerular oxidative stress were consistent with the gradual development of renal abnormalities since the 1.2% salt content diet fed rats only showed signs of renal hypertrophy without the significant signs of structural or functional glomerular abnormalities that the 8.2% salt content diet fed rats showed. This would suggest a causative role for oxidative stress on salt-driven kidney damage, consistent with the current concept that nitric oxide is a critical determinant of salt sensitive (if lacking) and salt-resistant hypertension (if available) [17].

The novel finding of our study was that salt increases glomerular ACE/ACE2 gene and protein ratio (Figures 2 and 3) [23]. On the contrary, none of the receptors seemed to be modulated by the different dietary salt intakes. Even though the relationship between salt and ACE2 has not be fully elucidated yet in the kidney, experimental evidence offers a background suggesting that salt would modulate local AngII production by decreasing ACE2 [9]. In the kidney, this could be interpreted as a compensatory mechanism that takes place when Na+ increases since it is well known that AngII acts by modulating tubuloglomerular feedback (TGF). TGF response is indeed blunted by AngII antagonists or ACEis and TGF has been demonstrated to be absent in mice lacking either the AT1 receptor or ACE, whereas a systemic infusion of AngII in \( \text{ACE} \) knockout mice restores it [24–29]. In this study, the effect of salt on renal oxidative stress and ACE/ACE2 modulation was independent from blood pressure increase and was not due to changes in circulating aldosterone. The blood pressure independent of salt effect—with respect to renal oxidative stress and ACE/ACE2 modulation—is consistent with the report that a high-salt diet produced fibrosis in the kidney of both normotensive and hypertensive rats [18] and that a

### Table 5. Effect of ACE2 deficiency on renal oxidative stress

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>CNT</th>
<th>Ace2KO</th>
<th>Ace2KO + ACEi</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{CuZnSOD} )</td>
<td>1 ± 0.14</td>
<td>0.59 ± 0.10*</td>
<td>1.01 ± 0.10\†</td>
</tr>
<tr>
<td>( \text{MnSOD} )</td>
<td>1 ± 0.08</td>
<td>0.97 ± 0.04</td>
<td>1.10 ± 0.04</td>
</tr>
<tr>
<td>( \text{NOX4} )</td>
<td>1 ± 0.06</td>
<td>1.52 ± 0.07*</td>
<td>1.20 ± 0.06\†</td>
</tr>
</tbody>
</table>

\*Data are expressed as mean ± SEM. CNT, C57Bl6; CuZnSOD, copper zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; NOX4, NADPH oxidase 4.

\*P < 0.05 versus C57Bl6.

\†P < 0.05 versus Ace2KO.
0.45% salt diet resulted in increased glomerulosclerosis compared to a 0.09%, SBP not differing between the two groups [18, 30]. Moreover, since there is an inverse relationship between sodium intake and serum aldosterone [31] and aldosterone down-regulates ACE2 in the kidney [32], we measured circulating aldosterone to exclude any influence of this hormone on renal RAS. Both 1.2 and 8.2% salt diets fed rats demonstrated a non-significant reduction of circulating aldosterone, suggesting that the modulation of ACE/ACE2 ratio by the salt content of a diet is independent of this hormone.

To understand if the reduction of ACE2 and the subsequent increase in the ACE/ACE2 ratio could cause salt-driven oxidative stress and thus kidney damage, we studied the renal modifications of Ace2KO mice. In this study, we found that ACE2 deficiency was also associated with an increase in ACE gene and protein expression, which is consistent with a previous work by Soler et al. [33] demonstrating that after pharmacological inhibition of ACE2 there is an increase in ACE at the glomerular level. In addition, in our study, ACE increase seemed to be AngII-dependent since the treatment with ACEi prevented it completely. This data indicates that any reduction of ACE2 would increase ACE, through AngII, and thus further promote an increase of the ratio ACE/ACE2. On the contrary, ACE enzymatic activity, which we measured previously [34], did not change in ACE2 deficiency. Based on the renal changes of the rats fed with different dietary contents of salt, we hypothesized that salt-driven oxidative stress would be caused by a decrease of ACE2 and a subsequent increase of the ACE/ACE2 ratio, which would increase AngII levels. Consistent with this, Ace2KO mice demonstrated higher levels of oxidative stress in their kidneys. The anti-oxidant CuZnSOD was indeed significantly down-regulated, whereas the pro-oxidant NOX4 was significantly up-regulated in the kidney of these mice and was associated with an increase in the glomerular nitrolyation of proteins. These changes were completely prevented by treatment with ACEi, indicating that AngII is the main cause of the increased oxidative stress found in Ace2KO mice. It has indeed been previously shown by Crackower et al. [1] as well as by our group [34] that genetic inactivation of ACE2 results in increased AngII and decreased Ang1-7 peptide levels in the kidneys. Our findings are consistent with the report that AngII infusion increases cortical NOS gene and protein expression [35, 36] and also with the experimental finding that AngII increases NO production in isolated perfused efferent arterioles via activation of the AT1 receptor. However, ACE2 deficiency could also increase the amount of renal oxidative stress by reducing the local production of Ang 1–7, which has been shown to exert antioxidant effects [37].

In conclusion, this work shows that increasing the content of salt in the diet promotes glomerular oxidative stress. This is associated with a reduction of ACE2 and a subsequent increase in the ACE/ACE2 ratio, which causes salt-driven oxidative stress, via AngII. Therefore, we suggest that one of the mechanisms whereby high-salt diet could lead to renal damage, via oxidative stress, is the modulation of ACE/ACE2.

**Supplementary data**

Supplementary table is available online at http://ndt.oxfordjournals.org.

**Acknowledgements.** Stella Bernardi is receiving a scholarship from SIIA (Società’ Italiana dell’Ipertensione Arteriosa).

**Funding.** This work was supported by a grant from Ministero dell’Istruzione, dell’Università e della Ricerca (PRIN 2006) of Italy.

**Conflict of interest statement.** None declared.

**References**

37. Gwathmey TM, Pendergrass KD, Reid SD et al. Angiotensin-(1-7)-angiotensin-converting enzyme 2 attenuates reactive oxygen species formation to angiotensin II within the cell nucleus. *Hypertension* 2010; 55: 166–171

Received for publication: 24.5.11; Accepted in revised form: 9.9.11