



HAL
open science

Renal aging in WKY rats: Changes in Na,K-ATPase function and oxidative stress

E. J. Silva, V. Pinto, S. Simão, M.P. Serrão, J. Afonso, J. Amaral, M.J. Pinho, P. Gomes, P. Soares-Da-Silva

► **To cite this version:**

E. J. Silva, V. Pinto, S. Simão, M.P. Serrão, J. Afonso, et al.. Renal aging in WKY rats: Changes in Na,K-ATPase function and oxidative stress. *Experimental Gerontology*, 2010, 45 (12), pp.977. 10.1016/j.exger.2010.09.012 . hal-00641498

HAL Id: hal-00641498

<https://hal.science/hal-00641498>

Submitted on 16 Nov 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Renal aging in WKY rats: Changes in Na⁺,K⁺-ATPase function and oxidative stress

E. Silva, V. Pinto, S. Simão, M.P. Serrão, J. Afonso, J. Amaral, M.J. Pinho, P. Gomes, P. Soares-da-Silva

PII: S0531-5565(10)00304-9
DOI: doi: [10.1016/j.exger.2010.09.012](https://doi.org/10.1016/j.exger.2010.09.012)
Reference: EXG 8811

To appear in: *Experimental Gerontology*

Received date: 22 June 2010
Revised date: 25 August 2010
Accepted date: 21 September 2010



Please cite this article as: Silva, E., Pinto, V., Simão, S., Serrão, M.P., Afonso, J., Amaral, J., Pinho, M.J., Gomes, P., Soares-da-Silva, P., Renal aging in WKY rats: Changes in Na⁺,K⁺-ATPase function and oxidative stress, *Experimental Gerontology* (2010), doi: [10.1016/j.exger.2010.09.012](https://doi.org/10.1016/j.exger.2010.09.012)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Title: Renal aging in WKY rats: changes in Na⁺,K⁺-ATPase function and oxidative stress.

Author names: E. Silva^a, V. Pinto^a, S. Simão^a, M.P. Serrão^a, J. Afonso^a, J. Amaral^a, M.J. Pinho^a, P. Gomes^{a1} & P. Soares-da-Silva^{a*}

Author affiliations: ^a Institute of Pharmacology & Therapeutics, Faculty of Medicine, University of Porto. 4200-319 Porto, Portugal

Corresponding author: * Correspondence to: P. Soares-da-Silva, Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200-319 Porto, Portugal, Telephone: 351-22-55193642, FAX: 351-22-55193643, E-mail: pss@med.up.pt

Present address: ¹ Department of Biochemistry, Faculty of Medicine, University of Porto. 4200-319 Porto, Portugal

Abstract

It has been suggested that alterations in Na^+, K^+ -ATPase mediate the development of several aging-related pathologies, such as hypertension and diabetes. Thus, we evaluated Na^+, K^+ -ATPase function and H_2O_2 production in the renal cortex and medulla of Wistar Kyoto (WKY) rats at 13, 52 and 91 weeks of age. Creatinine clearance, proteinuria, urinary excretion of Na^+ and K^+ and fractional excretion of Na^+ were also determined.

The results show that at 91 weeks old WKY rats had increased creatinine clearance and did not have proteinuria. Despite aging having had no effect on urinary Na^+ excretion, urinary K^+ excretion was increased and fractional Na^+ excretion was decreased with age. In renal proximal tubules and isolated renal cortical cells, 91 weeks old rats had decreased Na^+, K^+ -ATPase activity when compared to 13 and 52 weeks old rats. In renal medulla, 91 weeks old rats had increased Na^+, K^+ -ATPase activity, paralleled by an increase in protein expression of α_1 -subunit of Na^+, K^+ -ATPase. In addition, renal H_2O_2 production increased with age and at 91 weeks of age renal medulla H_2O_2 production was significantly higher than renal cortex production.

The present work demonstrates that although at 91 weeks of age WKY rats were able to maintain Na^+ homeostasis, aging was accompanied by alterations in renal Na^+, K^+ -ATPase function. The observed increase in oxidative stress may account, in part, for the observed changes. Possibly, altered Na^+, K^+ -ATPase renal function may precede the development of age-related pathologies and loss of renal function.

Keywords: Aging; Na⁺,K⁺-ATPase; Renal cortex; Renal Medulla; Hydrogen Peroxide.

Abbreviations: Wistar Kyoto – WKY; Hydrogen peroxide – H₂O₂; Fractional excretion of Na⁺; Glomerular filtration rate – GFR.

ACCEPTED MANUSCRIPT

1. Introduction

The kidney is one of the body organs severely affected by aging even in the absence of other pathologies. The aged kidney is characterized by a decrease in renal function and increased susceptibility to develop renal diseases, mainly due to increased glomerulosclerosis, tubular atrophy, interstitial fibrosis and loss of functional renal mass (Percy et al., 2008). Under normal conditions, the proximal tubules are responsible for the bulk electrolyte regulation, reabsorbing approximately 70% of Na^+ . More distal parts of the nephron such as the thick ascending limb and distal tubules also contribute to Na^+ reabsorption and the fine electrolyte adjustment is made in the cortical collecting ducts (Koeppen and Stanton, 2001).

One key enzyme in the electrolyte regulation is Na^+, K^+ -ATPase. In the kidney, Na^+, K^+ -ATPase catalyzes active transport of Na^+ in exchange for K^+ , maintains intracellular ion balance and membrane potential and is also responsible for maintaining Na^+ gradient (Bengele et al., 1983; Wang et al., 2009). In proximal tubules, glucose and aminoacid reabsorption is achieved due to Na^+, K^+ -ATPase generated Na^+ gradient. Thus, alterations in Na^+, K^+ -ATPase function are related with development of several age-related pathologies (Drozdowski et al., 2003; Epstein, 1996; Epstein and Hollenberg, 1976; Knapowski et al., 2002).

According to the free radical theory of aging a progressive increase in free radical and reactive products of oxygen metabolism are suggested to contribute to cellular aging and the development of aging associated conditions such as diabetes and cardiovascular and renal diseases (Touyz and Schiffrin, 2004; Valko et al., 2007). Aging of the kidney with associated loss of renal function and end-stage renal disease is therefore a problem of clinical and basic interest. Presently, being a major concern to the health system.

Gerontologic investigations both in humans and in experimental animal models can be compromised if the differences observed are actually differences between normal and age-associated disease states. In fact, most works regarding aging are performed in Fisher 344 rats and it is possible that the observed changes are related not only with age but also with the strain of the animals used in the studies. Experiments using other normotensive rodent strains will provide additional data and help elucidate which changes are truly related with age. Thus, in this study we used Wistar Kyoto (WKY) rats, a normotensive strain commonly used as control for spontaneously hypertensive rats, with 13, 52 and 91 weeks of age to evaluate the effects of aging on the activity and expression of Na^+, K^+ -ATPase in renal cortex and medulla and molecular mechanism related to development of age-associated changes.

2. Material and Methods

2.1. *Animal preparation and experimental design*

Five-week-old male Wistar Kyoto rats were obtained from Harlan-Interfauna Ibérica (Barcelona, Spain). Animals were housed under controlled conditions (12 hour light/dark cycle and room temperature at 22 ± 2 °C) and had free access to tap water and standard rat chow (PANLAB, Barcelona, Spain). The animals were maintained and monitored until 13, 52 and 91 weeks of age.

2.2. *Metabolic study*

Fourty-eight hours before experiments rats were placed in metabolic cages (Tecniplast, Buguggiate, Italy) for a 24 hour urine collection. The urine samples were stored at -80 °C until assayed. After completion of this protocol, rats were anesthetized with sodium

pentobarbital (60 mg/kg, i.p.). The animals were then sacrificed by exsanguination using cardiac puncture and the blood collected into tubes containing K₃ EDTA. Before excising the jejunum and the kidneys, the right ventricle of the heart was perfused with ice-cold saline (0.9% NaCl) to remove all blood from the kidneys. A portion of the kidneys was then rapidly removed through an abdominal midline incision. The kidneys were rinsed with ice-cold saline (0.9% NaCl), weighted and decapsulated. Sagittal sections were made and one section was placed in ice-cold saline for measurement of Na⁺-K⁺-ATPase and another was stored at -80 °C for western blot analysis.

2.3. *Plasma and urine biochemistry*

The quantification of Na⁺ and K⁺ in plasma and urine was performed by ion-selective electrodes. Creatinine was measured by the Jaffé method. All assays were performed by Cobas Mira Plus analyzer (ABX Diagnostics, Switzerland). Creatinine clearance (in ml min⁻¹) was calculated using the formula $C_{cr}=(U_{creat} \times Vu)/(P_{creat} \times 24h \times 60)$ where U_{creat} and Vu are the urinary creatinine concentration and urinary 24 hour volume and P_{creat} is the plasma creatinine concentration.

2.4. *Na⁺,K⁺-ATPase activity in renal cortex*

For the isolation of renal proximal tubules and renal cortex cells the outer cortex was cut out with fine scissors and minced with a scalpel into a fine paste. The cortical paste was filtered sequentially through a series of Nybolt nylon sieves, first 180 µm and then 75 µm. Undissociated cortex remained on the upper (180 µm) sieve, while the lower one (75 µm) retained predominantly proximal nephron segments and cortex cells passed through 75 µm Nybolt nylon sieve and were recovered in a goblet. The retained renal proximal nephron segments and renal cortex cells were then washed with cold

Hanks' solution and collected into a pellet by centrifugation at 200 g, 5 min, 4 °C; renal cells used in incubation experiments were suspended in Hanks' solution.

Na^+, K^+ -ATPase activity in renal proximal tubules and isolated renal cortex cells was measured, as previously described (Quigley and Gotterer, 1969) and adapted in our laboratory with slight modifications (Vieira-Coelho et al., 1999). Briefly, isolated renal proximal tubules and isolated renal cortex cells obtained as described above were pre-incubated for 20 min at 37 °C. After the pre-incubation period the cells were permeabilized by rapid freezing in dry ice-acetone and thawing. The reaction mixture contained (in mM) 37.5 imidazole buffer, 75 NaCl, 5 KCl, 1 sodium-EDTA, 5 MgCl_2 , 6 NaN_3 , 75 tris(hydroxymethyl)aminomethane(tris) hydrochloride and 100 μl cell suspension (100 μg protein). The reaction was initiated by the addition of 4 mM ATP (25 μl). For determination of ouabain-sensitive ATPase, NaCl and KCl were omitted, and 100 μl of ouabain (final concentration 1 mM) or vehicle (water) was added to the assay. After incubation at 37 °C for 20 min, the reaction was terminated by the addition of 50 μl of ice-cold trichloroacetic acid. Samples were centrifuged (1500 g), and liberated Pi (free phosphorus) in the supernatant was measured by spectrophotometry at 740 nm. Na^+, K^+ -ATPase activity is expressed as nanomoles Pi per mg protein per min and determined as the difference between total and ouabain-insensitive ATPase. The protein content was determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with human serum albumin as a standard.

2.5. Na^+, K^+ -ATPase activity in renal medulla

Renal medulla was separated from renal cortex by dissection with fine scissors and homogenized in ice-cold solution containing 0.25 M sucrose and 0.03 M histidine (pH 7.2) (10ml/g tissue) using a Polytron® (Kinematica AG, Littau-Lucerne, Switzerland).

The homogenate was centrifuged at 8000 g for 20 min at 4 °C. The sediment was resuspended in 6 ml of sucrose-histidine solution and centrifuged again. The combined supernatants were used for Na⁺,K⁺-ATPase activity assay (Beltowski and Wojcicka, 2002).

Na⁺,K⁺-ATPase activity in renal medulla was determined as described above with slight modifications. For enzyme assay, 100 µl of the microsomal protein (100 µg of protein) were pre-incubated with 0.02% saponin and reaction mixture for 30 minutes at room temperature, followed by additional 10 minutes incubation at 37 °C in the presence of ouabain or vehicle. The reaction was initiated by the addition of 4 mM ATP (25 µl) and the following procedures were common to what was described for the assay in proximal tubules.

2.6. *Protein expression*

Isolated renal cortex and medulla were sliced very thinly and lysed by the addition of RIPA buffer [150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% NP-40 (IGEPAL), 0.25% sodium deoxycholate] containing protease inhibitors: 1 mM PMSF, 1 µg/ml leupeptine and 1 µg/ml aprotinin; and phosphatase inhibitors: 1 mM Na₃VO₄ and 1mM NaF. Lysed tissue was briefly sonicated, incubated on ice for 1 hour and centrifuged (13,000 g for 45 min). Proteins were mixed with 6x sample Laemmli buffer (0.35 M Tris-HCl, 4% SDS, 30% glycerol, 9.3% DTT, pH 6.8, 0.01% bromophenol blue) and warmed at 37 °C for 15 minutes. Equal amounts of total protein (30 µg), determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with human serum albumin as a standard, were separated on a 7.5% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane in Tris-Glycine transfer buffer containing 20% methanol. Membranes were blocked in 3% non-fat dry

milk in PBS for 1 hour. As in prior experiments no unspecific band were detected with the primary antibodies against Na^+, K^+ -ATPase or GAPDH (data not shown), membranes were cut between the 50 and 70 KDa marker and then incubated overnight at 4 °C with specific primary antibodies (α_1 -subunit Na^+, K^+ -ATPase, sc-21712 and GAPDH, sc-32233 were purchased from Santa Cruz Biotechnology, Inc). The upper half was incubated with the primary antibody against Na^+, K^+ -ATPase and the lower half with the primary antibody against GAPDH. The membranes were subsequently washed and incubated with the fluorescently labeled goat anti-mouse secondary antibody (AlexaFlour 680, Molecular Probes) for 1 hour at room temperature and protected from light. Membranes were washed and imaged by scanning at 700 nm with the Odyssey Infrared System (LICOR Biosciences). Data were normalized to the expression of GAPDH.

2.7. *H₂O₂ production in plasma, renal cortex and renal medulla.*

Renal cortex and medulla were cut into square pieces and incubated at 37 °C in Krebs-HEPES buffer (in mM: NaCl 118, KCl 4.5, CaCl₂ 2.5, MgCl₂ 1.2, K₂HPO₄ 1.2, NaHCO₃ 25.0, Na-HEPES 25.0 and glucose 5; pH 7.4) for 90 minutes. H₂O₂ released from the tissue was detected using the Amplex Red Hydrogen Peroxide Assay kit, as previously described (Furukawa et al., 2004).

2.8. *Drugs*

All chemicals were obtained from Sigma-Aldrich.

2.9. *Statistics analysis*

Arithmetic means are given with standard error mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls

test for multiple comparisons. A *P*-value less than 0.05 was assumed to denote a significant difference.

3. Results

3.1. *General Data*

The effects of age on body weight and tibia length are displayed on table 1. Body weight and tibia length increased with age in WKY rats. However, due to the fact that WKY rats with 52 and 91 weeks of age had increased accumulation of abdominal adipose tissue (data not shown) heart and kidney weight were normalized to tibia length. Both heart/tibia and kidney/tibia ratio increased with age (**Table 1**). No differences in the plasma values of Na^+ were observed at the ages studied (in mmol/L: 142 ± 2 at 13 weeks, 142 ± 2 at 52 weeks and 144 ± 1 at 91 weeks of age).

3.2. *Renal function*

Creatinine clearance and urinary creatinine were used as indicators of renal function and concentration of proteins in the urine as an indicator of renal injury. At the ages studied there was a significant increase in creatinine clearance but no changes were observed in urinary creatinine levels (**Fig. 1A and 1B**). No differences were observed in urinary protein concentration (**Fig. 1B**).

Urinary Na^+ and K^+ excretion were determined as indicators of ability of the aged kidney to maintain electrolyte homeostasis. The results show that aging had no effect on urinary Na^+ excretion (**Fig. 2A**). However, K^+ excretion was significantly affected with

age. An observed increase in urinary K^+ excretion was observed in WKY rats at 52 and 91 weeks of age (**Fig. 2B**). Moreover, evaluation of fractional excretion of Na^+ ($FeNa^+$) showed that aging was accompanied by a significant decrease in this parameter at the ages of 52 and 91 weeks (**Fig. 2C**). As electrolyte excretion may be conditioned by food ingestion, food intake values were evaluated. No changes were observed in the amount of ingested food during aging (**Fig. 2D**).

3.3. *Effect of aging on Na^+ , K^+ -ATPase activity and expression*

To evaluate the effects of age on Na^+ , K^+ -ATPase at the functional level we measured Na^+ , K^+ -ATPase activity (under V_{max} conditions) and expression in renal cortex and renal medulla. Na^+ , K^+ -ATPase activity was determined in the segments from renal proximal tubules, in renal cortical cells and in lysates from renal medulla isolated as mentioned in methods. In renal tubules and renal cortical cells there were no differences in Na^+ , K^+ -ATPase activity between 13 and 52 weeks of age. At 91 weeks of age a significant decrease was observed in Na^+ , K^+ -ATPase activity when compared to 13 weeks of age rats, both in proximal tubules and isolated cortical cells (**Fig 3A and 3B**). In the renal medulla aging was accompanied by an increase in Na^+ , K^+ -ATPase activity. However, this increase did not attain statistical significance (**Fig 3C**). Subsequently, it was evaluated the expression of the catalytic subunit of Na^+ , K^+ -ATPase (α_1 -subunit). In the renal cortex the expression of α_1 -subunit of Na^+ , K^+ -ATPase was not affected by aging (**Fig. 4A**). In renal medulla expression of α_1 -subunit of Na^+ , K^+ -ATPase was significantly increased in with aging (**Fig. 4B**). This increase was significant already at 52 weeks of age.

To have a better insight on Na^+ , K^+ -ATPase function the ratio between Na^+ , K^+ -ATPase activity and protein expression was calculated. In the renal cortex, aging is accompanied

by a decrease in the ratio between Na^+, K^+ -ATPase activity and protein expression, which is statistically significant at 91 weeks of age (**Fig. 5A**). In the renal medulla, the ratio between Na^+, K^+ -ATPase activity and protein expression increases significantly at 52 weeks of age and then decreases between 52 and 91 weeks of age (**Fig. 5B**).

3.4. *Oxidative stress in plasma and kidney*

Hydrogen peroxide (H_2O_2) quantification was used as a marker of oxidative stress in aged rats since this reactive oxygen species is more stable than the superoxide anion. Aging rats had an increase in plasma H_2O_2 concentration, being this increase significant when comparing 13 and 91 weeks of age WKY rats (**Fig 6A**). The aged kidney also had significantly increased levels of H_2O_2 both in the renal cortex and medulla (**Fig. 6B**), as demonstrated by measurement of 90 minutes H_2O_2 tissue release to the reaction medium. Moreover, at 91 weeks of age the levels of H_2O_2 in the renal medulla were significantly higher than in the renal cortex (**Fig. 6B**).

4. Discussion

The present study was designed to examine the effects of aging on Na^+, K^+ -ATPase activity and expression in the kidney of WKY rats. Our results show that aged WKY rats have preserved renal function and were able to maintain body Na^+ homeostasis until 91 weeks of age. Despite Na^+ reabsorption being compromised in the renal proximal tubules, the more distal part of the nephron is still able to compensate by augmenting Na^+ reabsorption in this segment.

In the present work, creatinine clearance was used to estimate glomerular filtration rate (GFR), which is commonly used as an index of renal function. WKY rats showed a

slight but significant increase in creatinine clearance with age which could be taken as an indication of no loss of GFR. Moreover, the fact that WKY rats did not have proteinuria demonstrates that no severe renal injury is present in the kidney of these animals until 91 weeks of age. However, our results show that with age, even in the absence of severe renal injury, WKY kidney is becoming dysfunctional. This is supported by the fact that quantification of Na^+ and K^+ electrolytes in urine revealed that K^+ excretion and FeNa^+ were severely affected. At 52 weeks of age WKY rats had increased kaliuresis and decreased FeNa^+ . These changes were even more pronounced at the age of 91 weeks. Changes in urinary electrolyte excretion could be due to differences in food intake. However, food intake was similar between all studied ages further supporting the possibility of altered electrolyte regulation in aged animals.

An increased kaliuresis points to altered distal tubule and collecting duct function because these parts of the nephron have the capacity to either reabsorb or secrete K^+ , unlike the proximal tubules which are responsible for the bulk of K^+ reabsorption (Koeppen and Stanton, 2001). Knowing that the uptake of K^+ to the tubular fluid is controlled by basolateral membrane Na^+, K^+ -ATPase activity (Koeppen and Stanton, 2001), renal Na^+, K^+ -ATPase function was evaluated in aged animals. Na^+, K^+ -ATPase, a key enzyme for normal renal function, mediates not only electrolyte reabsorption but also the maintenance of Na^+ gradient which regulates nutrient reabsorption. To evaluate the effects of age on Na^+, K^+ -ATPase at a functional level we measured Na^+, K^+ -ATPase activity in segments from renal proximal tubules, in renal cortical cells and in lysates from renal medulla which contains collecting ducts. Activity was measured as the difference between activities assayed in the presence of Na^+ and K^+ and the activity in the sample which lacked these ions and contained ouabain. Although other ATPases such as K^+ -stimulated ATPases (Na^+ -independent) and ouabain-insensitive Na^+ -ATPase

contribute to the overall activity, these represent a small fraction of the activity assayed, with Na^+, K^+ -ATPase being the main contributor. Therefore we assume that the differences observed are representative of Na^+, K^+ -ATPase activity.

Renal cortex lysates were used to evaluate Na^+, K^+ -ATPase protein expression due to the fact that renal cortex is heavily enriched in proximal tubules and distribution of Na^+, K^+ -ATPase in renal cortex is representative of that in renal proximal tubules (Magyar et al., 2000; McDonough, 2010).

A slight but not significant decrease was observed in Na^+, K^+ -ATPase activity in isolated renal cortical cells at 52 weeks of age. Decreased Na^+, K^+ -ATPase activity was more pronounced and significant at 91 weeks of age, both in segments of proximal tubules and in isolated cells from the renal cortex. In renal cortex the ratio between Na^+, K^+ -ATPase activity and expression decrease with age due to decreased enzyme activity, only. Decreased Na^+, K^+ -ATPase activity without decreased protein expression has been observed in aged Fischer 344 rats (Asghar et al., 2001). In these rats higher basal PKC activity was responsible for increased basal Na^+, K^+ -ATPase phosphorylation and decreased enzyme activity in the renal cortex. Increased oxidative stress and increased basal PKC activity have also been described in experimental models of age-related pathologies such as hypertension and diabetes (Banday et al., 2007; Marwaha and Lokhandwala, 2006). WKY rats used in this study, is a well studied normotensive strain with no age-related alterations of blood pressure values (Bing et al., 2002; Loch et al., 2009; Moura et al., 2005; Yamamoto et al., 1987). The proposed mechanism was that increased ROS production stimulated PKC- δ via activation of NF- κ B (Banday et al., 2007). This mechanism could explain what is reported in the renal cortex of aged WKY rats, since we observed an increase in plasma concentrations of H_2O_2 which and increased levels of H_2O_2 in the renal cortex.

Reduced Na^+, K^+ -ATPase activity in renal cortex increases Na^+ delivery to Henle's loop and the distal tubules (Koeppen and Stanton, 2001). Stimulation of Na^+ reabsorption mediated by Na^+, K^+ -ATPase to maintain Na^+ homeostasis in the renal medulla could be achieved by an increase in aldosterone levels. Aldosterone, the main regulator of membrane transporters in the renal medulla, was found to be increased in WKY rats aged 52 and 91 weeks (Pinto et al., 2010). As Na, K -ATPase activity was measured under V_{max} conditions it is possible that a decrease in protein activity due to phosphorylation of the enzyme was not detected at 52 weeks of age. The fact that a slight decrease is observed in isolated cells from renal cortex and that aldosterone levels are increased in this animals supports this possibility. However, the observed increase in protein expression between 13 and 52 weeks of age could not have accounted alone for the observed increase in protein activity, since the ratio between Na^+, K^+ -ATPase activity and expression increased. Given that regulation of Na^+, K^+ -ATPase differs in proximal and distal nephron it is possible that in renal medulla increased ROS may activate cell specific signaling pathways that up-regulate other kinases such as ERK1/2, PKA or other PKC isoforms which lead to the phosphorylation of other sites of the enzyme that stimulate Na^+, K^+ -ATPase activity. The lack of change in Na^+, K^+ -ATPase activity between 52 and 91 weeks of age, even when expression of α_1 -subunit of Na^+, K^+ -ATPase is still increasing, may be due to ROS mediated protein carbonylation. In fact, the levels of H_2O_2 in the renal medulla of 91 weeks old rats were 10 fold increased when compared to 13 weeks old WKY rats and higher than the levels in the renal cortex. Thus, increased levels of ROS could be playing a dual role in the regulation of Na^+, K^+ -ATPase activity in the renal medulla. ROS may be acting directly on Na^+, K^+ -ATPase increasing protein carbonylation and indirectly as a signalling molecule. Final Na^+, K^+ -ATPase activity will be dependent on the balance between these

two factors. Undoubtedly, the mechanisms responsible for Na^+, K^+ -ATPase regulation in the kidney are affected by aging and ROS may play a key role in the observed age-related changes.

In conclusion, our results indicate that aging induced changes in the major nephron segments responsible for Na^+ and K^+ electrolyte regulation. Despite aging having affected Na^+, K^+ -ATPase function throughout the nephron WKY rats were still able to maintain Na^+ homeostasis and preserve renal function up to 91 weeks of age. However, if loss of Na^+, K^+ -ATPase activity in proximal tubules progresses with age this could compromise reabsorption of glucose and aminoacids, since these are dependent on the Na^+ gradient generated by Na^+, K^+ -ATPase. If Na^+, K^+ -ATPase activity in the renal medulla increases with aging this could lead to Na^+ retention and lead to the development of hypertension. The present work contributes to a better understanding of the renal molecular changes that take place with aging and may precede the development of age-related pathologies and loss of renal function.

Acknowledgements: E. Silva was supported by PhD grant SFRH/BD/22876/2005.

References

- Asghar, M., Kansra, V., Hussain, T., Lokhandwala, M.F., 2001. Hyperphosphorylation of Na-pump contributes to defective renal dopamine response in old rats. *J Am Soc Nephrol* 12, 226-32.
- Banday, A.A., Fazili, F.R., Lokhandwala, M.F., 2007. Oxidative stress causes renal dopamine D1 receptor dysfunction and hypertension via mechanisms that involve nuclear factor-kappaB and protein kinase C. *J Am Soc Nephrol* 18, 1446-57.
- Beltowski, J., Wojcicka, G., 2002. Spectrophotometric method for the determination of renal ouabain-sensitive H⁺,K⁺-ATPase activity. *Acta Biochim Pol* 49, 515-27.
- Bengele, H.H., Mathias, R., Perkins, J.H., McNamara, E.R., Alexander, E.A., 1983. Impaired renal and extrarenal potassium adaptation in old rats. *Kidney Int* 23, 684-90.
- Bing, O.H., Conrad, C.H., Boluyt, M.O., Robinson, K.G., Brooks, W.W., 2002. Studies of prevention, treatment and mechanisms of heart failure in the aging spontaneously hypertensive rat. *Heart Fail Rev* 7, 71-88.
- Drozdowski, L., Woudstra, T., Wild, G., Clandinin, M.T., Thomson, A.B., 2003. The age-associated decline in the intestinal uptake of glucose is not accompanied by changes in the mRNA or protein abundance of SGLT1. *Mech Ageing Dev* 124, 1035-45.
- Epstein, M., 1996. Aging and the kidney. *J Am Soc Nephrol* 7, 1106-22.
- Epstein, M., Hollenberg, N.K., 1976. Age as a determinant of renal sodium conservation in normal man. *J Lab Clin Med* 87, 411-7.
- Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M., Shimomura, I., 2004. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 114, 1752-61.
- Knapowski, J., Wieczorowska-Tobis, K., Witowski, J., 2002. Pathophysiology of ageing. *J Physiol Pharmacol* 53, 135-46.
- Koeppen, B.M., Stanton, B.A., 2001. *Renal Physiology*, Third ed. Mosby, St Louis.
- Loch, D., Chan, V., Hoey, A., Brown, L., 2009. Rosuvastatin Attenuates Heart Failure and Cardiac Remodelling in the Ageing Spontaneously Hypertensive Rat. *Basic Clin Pharmacol Toxicol*.
- Magyar, C.E., Zhang, Y., Holstein-Rathlou, N.H., McDonough, A.A., 2000. Proximal tubule Na transporter responses are the same during acute and chronic hypertension. *Am J Physiol Renal Physiol* 279, F358-69.

- Marwaha, A., Lokhandwala, M.F., 2006. Tempol reduces oxidative stress and restores renal dopamine D1-like receptor- G protein coupling and function in hyperglycemic rats. *Am J Physiol Renal Physiol* 291, F58-66.
- McDonough, A.A., 2010. Mechanisms of proximal tubule sodium transport regulation that link extracellular fluid volume and blood pressure. *Am J Physiol Regul Integr Comp Physiol* 298, R851-61.
- Moura, E., Pinho Costa, P.M., Moura, D., Guimaraes, S., Vieira-Coelho, M.A., 2005. Decreased tyrosine hydroxylase activity in the adrenals of spontaneously hypertensive rats. *Life Sci* 76, 2953-64.
- Percy, C.J., Power, D., Gobe, G.C., 2008. Renal ageing: changes in the cellular mechanism of energy metabolism and oxidant handling. *Nephrology* 13, 147-52.
- Pinto, V., Simão, S., Silva, E., Pinho, M., Amaral, J., Afonso, J., Serrão, M., Gomes, P., Soares-da-Silva, P., 2010. Increased Oxidative Stress and Aldosterone Levels in Aged Wistar Kyoto and Spontaneously Hypertensive Rats: Effect of Food Restriction. *Journal of Hypertension* 28, e509.
- Quigley, J.P., Gotterer, G.S., 1969. Distribution of (Na⁺-K⁺)-stimulated ATPase activity in rat intestinal mucosa. *Biochim Biophys Acta* 173, 456-68.
- Touyz, R.M., Schiffrin, E.L., 2004. Reactive oxygen species in vascular biology: implications in hypertension. *Histochem Cell Biol* 122, 339-52.
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39, 44-84.
- Vieira-Coelho, M.A., Hussain, T., Kansra, V., Serrao, M.P., Guimaraes, J.T., Pestana, M., Soares-Da-Silva, P., Lokhandwala, M.F., 1999. Aging, high salt intake, and renal dopaminergic activity in Fischer 344 rats. *Hypertension* 34, 666-72.
- Wang, X., Armando, I., Upadhyay, K., Pascua, A., Jose, P.A., 2009. The regulation of proximal tubular salt transport in hypertension: an update. *Curr Opin Nephrol Hypertens* 18, 412-20.
- Yamamoto, J., Nakai, M., Natsume, T., 1987. Cardiovascular responses to acute stress in young-to-old spontaneously hypertensive rats. *Hypertension* 9, 362-70.

Figure legends

Figure 1 – Creatinine clearance, urinary creatinine excretion and urinary proteins. Creatinine clearance (**A**), urinary creatinine excretion (**B**) and concentration of proteins in urine (**C**) of WKY rats at 13, 52 and 91 weeks of age. Columns represent means and vertical lines show SEM (n= 6 to 18).

Significantly different from values for rats with 13 weeks of age using the one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons (* P<0.05).

Figure 2 – Electrolyte excretion and food intake. Urinary excretion of Na⁺ (**A**), urinary excretion of potassium (**B**), fractional excretion of Na⁺ (Fe Na⁺) (**C**), and 24 hour food intake in WKY rats at 13, 52 and 91 weeks of age. Columns represent means and vertical lines show SEM (n= 6 to 18).

Significantly different from values for rats with 13 weeks of age using the one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons (* P<0.05).

Figure 3 – Renal Na⁺,K⁺-ATPase activity. Renal proximal tubules (**A**), isolated cells from renal cortex (**B**) and isolated renal medulla (**C**) Na⁺,K⁺-ATPase activity in WKY rats at 13, 52 and 91 weeks of age. Columns represent means and vertical lines show SEM (n= 5 to 17).

Significantly different from values for rats with 13 weeks of age using one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons (* P<0.05).

Significantly different from values for rats with 52 weeks of age using one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons (# P<0.05).

Figure 4 – Renal expression of α_1 -subunit of Na^+, K^+ -ATPase. Renal cortex representative blot (**A**) and relative expression of Na^+, K^+ -ATPase (**B**); renal medulla representative blot (**C**) and relative expression of Na^+, K^+ -ATPase (**D**) in WKY rats at 13, 52 and 91 weeks of age. Protein expression was normalized for GAPDH. Columns represent means and vertical lines show SEM (n= 4 to 8).

Significantly different from values for rats with 13 weeks of age using one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons (* P<0.05).

Significantly different from values for rats with 52 weeks of age using one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons (# P<0.05).

Figure 5 – Renal Na^+, K^+ -ATPase activity, expression ratio. Renal cortex Na^+, K^+ -ATPase activity, expression ratio (**A**) and renal medulla Na^+, K^+ -ATPase activity, expression ratio (**B**) in WKY rats at 13, 52 and 91 weeks of age. Columns represent means and vertical lines show SEM (n= 4 to 8).

Significantly different from values for rats with 13 weeks of age using one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons (* P<0.05).

Significantly different from values for rats with 52 weeks of age using one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons (# P<0.05).

Figure 6 – Plasma and renal H_2O_2 . H_2O_2 concentration in plasma (**A**) and 90 minutes H_2O_2 release from renal cortex and medulla (**B**) of WKY rats at 13, 52 and 91 weeks of age. Columns represent means and vertical lines show SEM (n = 4 to 7).

Significantly different from values for rats with 13 weeks of age using one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons (* P<0.05).

Significantly different from values for rats with 52 weeks of age using one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons (# $P < 0.05$).

Significantly different from values for H_2O_2 production in renal medulla at the corresponding age using the one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons (§ $P < 0.05$).

ACCEPTED MANUSCRIPT

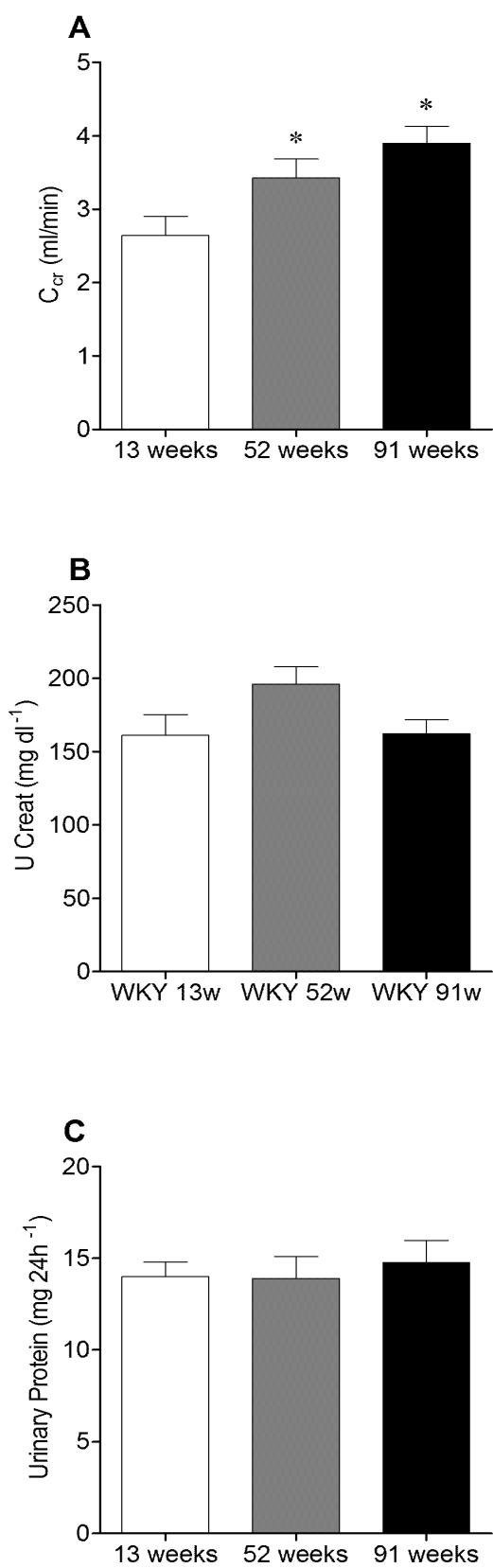


Figure 1

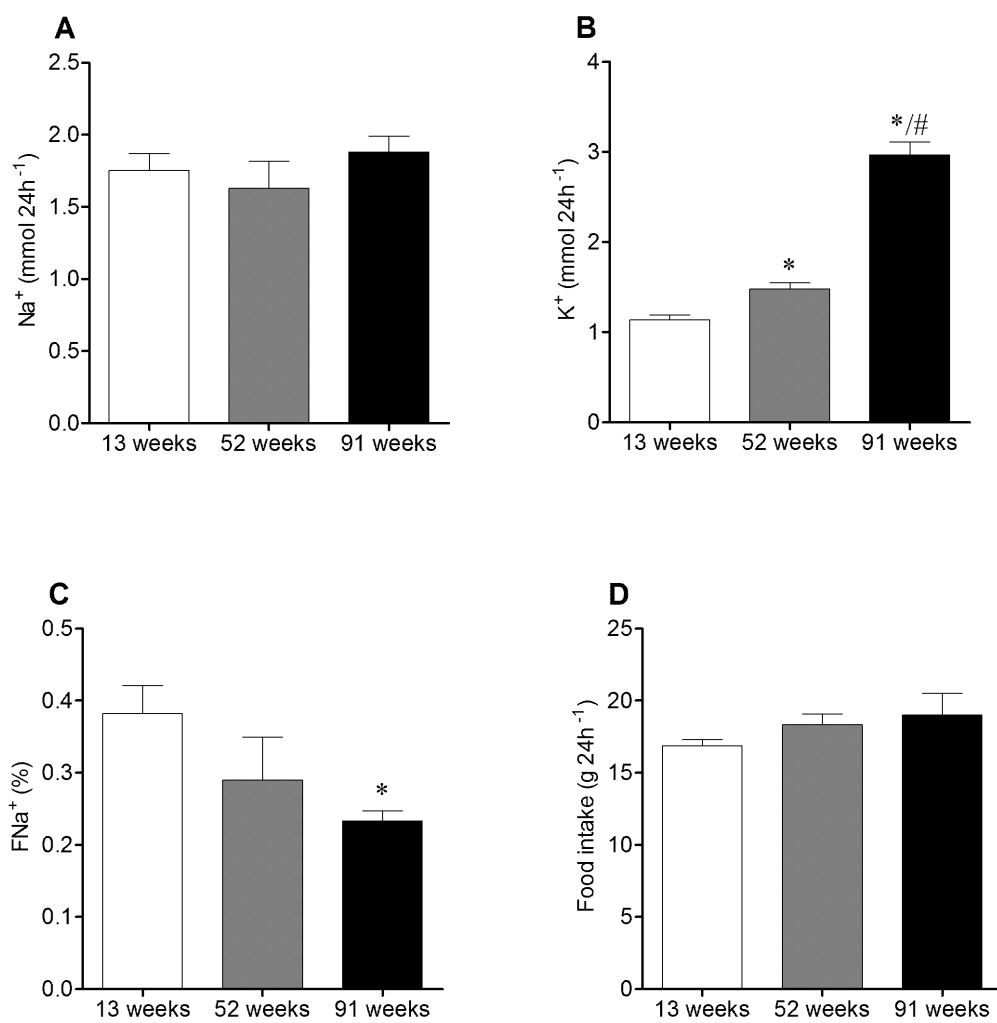


Figure 2

Y

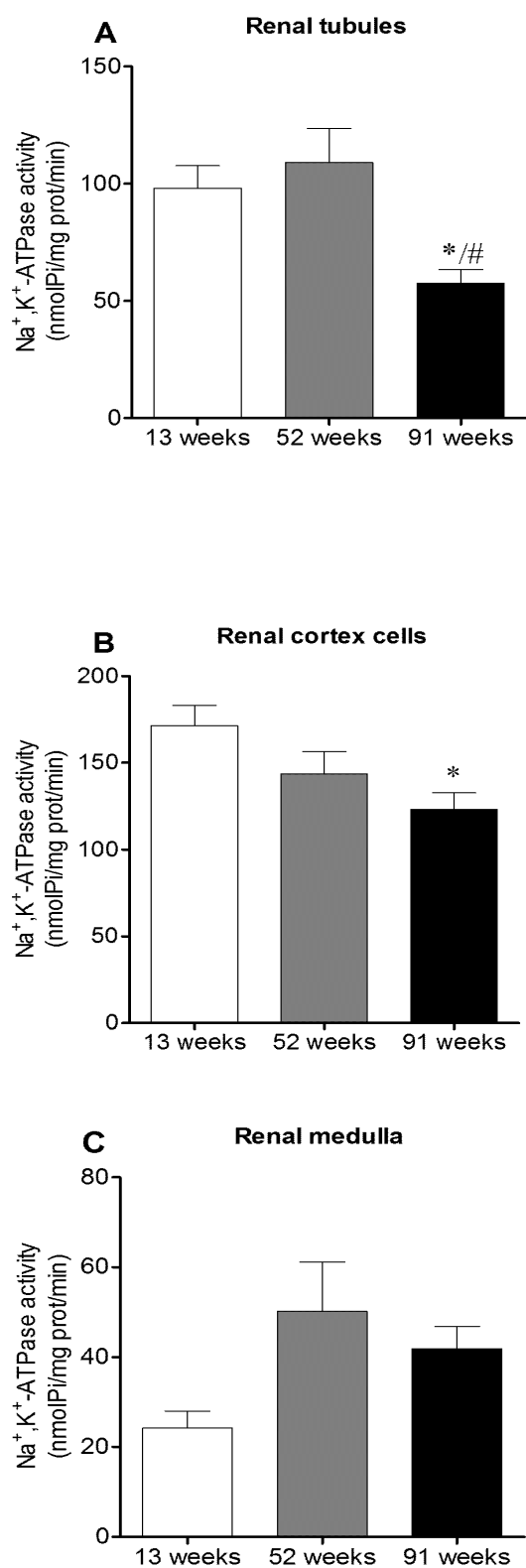


Figure 3

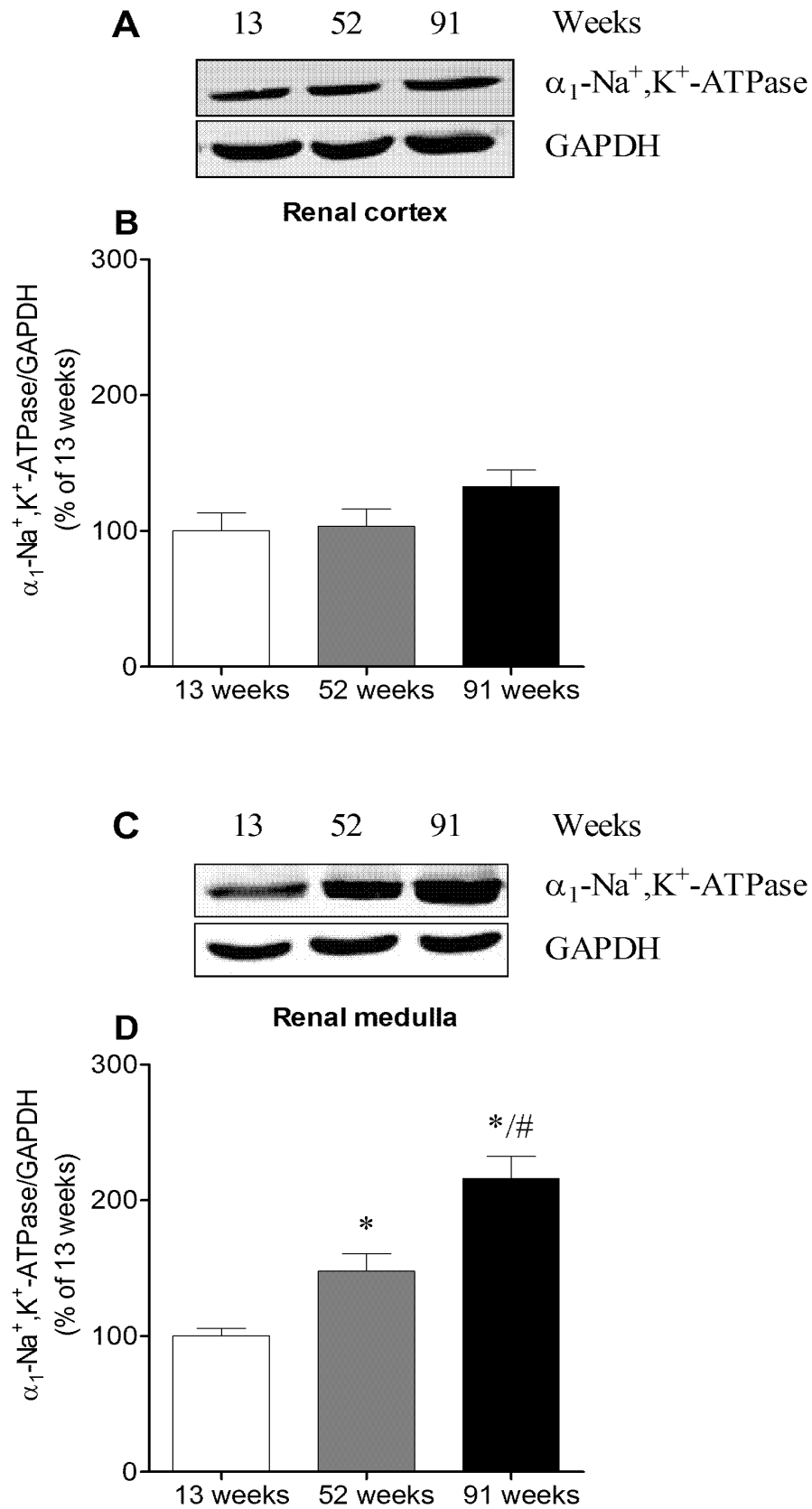


Figure 4

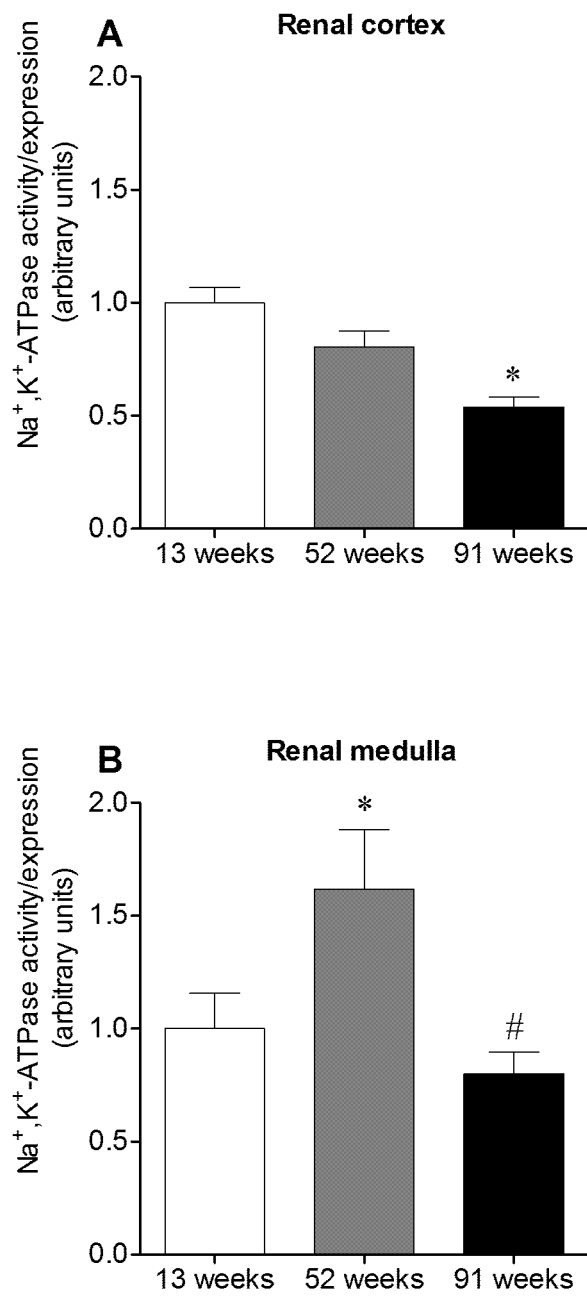


Figure 5

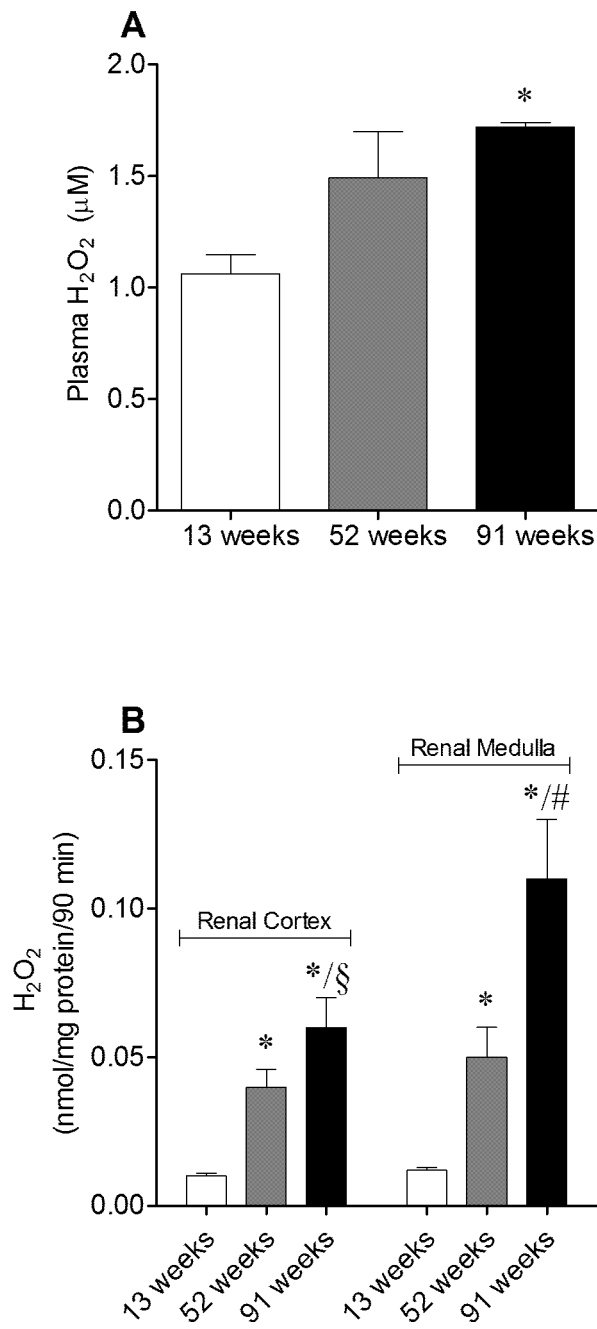


Figure 6

Table 1. Body weight, tibia length, heart tibia ratio and kidney tibia ratio in 13, 52 and 91 weeks old WKY rats.

	13 weeks	52 weeks	91 weeks
Body weight, g	330±10	565±10*	666±11 ^{*/#}
Tibia length, cm	3.96±0.06	4.35±0.04*	4.55±0.02 ^{*/#}
Heart/tibia, g cm ⁻¹	0.29±0.03	0.37±0.01*	0.38±0.01*
Kidney/tibia, g cm ⁻¹	0.30±0.02	0.35±0.03*	0.35±0.01*

Significantly different from values for rats with 13 weeks of age using the Student's *t*-test (* P<0.05).

Significantly different from values for rats with 52 weeks of age using the Student's *t*-test (# P<0.05).

- Aging is accompanied by diminished capacity of proximal tubule Na^+ reabsorption.
- Na^+, K^+ -ATPase activity decreases in renal proximal tubules and increases in renal medulla.
- Aged rats have changes in redox homeostasis with a pronounced increase in renal medulla production of H_2O_2 .