

POSSIBLE SENESCENCE ASSOCIATED CHANGE IN THE PREDOMINANT
 α -Na⁺/K⁺ ATP-ase ISOFORM IN THE RENAL CORTEX OF THE RAT

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Abstract With aging the kidney exhibits progressive deterioration, with a decrease in renal function. Most of the filtered Na⁺ is actively reabsorbed in the proximal tubules through different transporters located in apical membrane. This process is possible because basolateral Na⁺/K⁺-ATP-ase generates electrochemical conditions necessary for energetically favorable Na⁺ transport. The α -subunit is the catalytic domain of Na⁺/K⁺-ATP-ase. There are three isoforms of the α /subunit present in rat kidney. The present study was undertaken to examine the expression pattern of rat α -Na⁺/K⁺-ATP-ase during senescence. We tested the impact of aging on mRNA expression of α -Na⁺/K⁺-ATP-ase in cortex and medulla of aged Wistar rats. We observed a significant expression decrease in mRNA levels and a possible change of isoform in the cortex of aged animals. These expression changes observed for α subunit could be contributing to affect the renal function in conditions of water and salt stress.

Key words: aging, kidney, sodium-potassium-exchanging ATPase

Resumen *Posible cambio en la isoforma predominante de la α -Na⁺/K⁺-ATP-asa asociada a la senescencia en corteza renal de rata.* Con el avance de la edad los riñones exhiben un deterioro funcional progresivo con disminución de la función renal. La mayor parte del sodio (Na⁺) filtrado es reabsorbido activamente en los túbulos proximales a través de diferentes transportadores ubicados en la membrana apical. Este proceso es posible por la existencia de la Na⁺/K⁺-ATP-asa basolateral, que genera las condiciones electroquímicas necesarias para que el transporte de Na⁺ sea energéticamente favorable. La subunidad α de la Na⁺/K⁺-ATP-asa es el dominio catalítico de la enzima. Existen tres isoformas de subunidad α , que están presentes en el riñón de la rata. En este trabajo se examinan los patrones de expresión de la α -Na⁺/K⁺-ATP-asa durante la senescencia. Se estudió así si el aumento de la edad incidía en la expresión del ARNm de la α -Na⁺/K⁺-ATP-asa en corteza y médula renal de ratas Wistar senescentes. Se observó una disminución en la expresión del ARNm de la subunidad α y un posible cambio de isoforma predominante en la corteza de los animales senescentes. Los cambios observados para la expresión de la subunidad α podrían contribuir a afectar la función renal en condiciones de estrés hídrico y salino.

Palabras clave: envejecimiento, riñón, ATPasa-intercambiador sodio potasio

As a consequence of the normal aging process there is a decrease in renal function and an increased susceptibility to develop renal diseases¹. As a result, both a decrease in the glomerular filtration rate and renal blood flow as well have been described beside an impairment in the urinary concentrating mechanisms^{2,3}.

Approximately 70% of the filtered Na⁺ is actively reabsorbed by an active process in the proximal tubules, along with chloride and water. Net apical reabsorption of NaCl involves both coupling of Na⁺/H⁺ exchange with

Cl⁻/base exchange and acid recycling or triple coupling of Na⁺-sulfate cotransport, sulfate-anion exchange and Cl⁻/anion exchange⁴. This system operates in series with the basolateral Na⁺/K⁺-ATP-ase, which generates the electrochemical gradient across the cell, providing the energy for Na⁺ transport.

Acidification of the proximal convoluted tubule (PCT) fluid is also altered with age. In previous studies, we have observed in old rats a decrease in the capacity of proximal tubular H⁺ secretion, without changes in the acid-base balance⁵. This reduction is due to a diminished expression of the NHE8 isoform of the Na⁺/H⁺⁶.

The Na⁺/K⁺-ATP-ase is a heteromeric transmembrane protein composed of an α - and a β -subunit. The α -subunit is the catalytic subunit, responsible for the transport activities of the enzyme, and contains the binding site for Na⁺,

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K⁺, ATP and cardiac glycosides like ouabain, an specific inhibitor of the enzyme^{7, 8}. There are four isoforms of the catalytic α -subunit, each of them with a tissue-specific distribution. The relative distribution of α isoform mRNA in the kidney has been reported to be approximately 70% for α 1, 20% for α 2 and 10% for α 3⁹.

The expression profile of the alpha isoform mRNA is also subjected to a temporal regulation in the kidney and other organs¹⁰. However, most of the work in the field has been done over the developmental and growth processes, and not in the age-associated changes occurring during senescence. The aim of this work is to explore the expression pattern of α -Na⁺/K⁺-ATP-ase mRNA in young and aged Wistar rats, in order to shed light into the changes that may take place with both aging and decline of renal function.

Twelve Wistar rats were randomly assigned to two groups and sacrificed either at 3 months old (3M, control group) or 18 month old (18M, aged group). The animals were maintained under a cycle of 12 hour light/dark and room temperature at 22 ± 2 °C with food and water *ad libitum*. They were anaesthetized using 5% Inhelthran® enflurane (Abbott, Italy) by inhalation. Adequate anesthesia was assured by the absence of reflexes prior to cervical dislocation and subsequent sample collection. This protocol was approved by the University of San Martin Ethical Review Committee and conformed to the "Revised guide for the care and use of laboratory animals" of NIH.

Total RNA was isolated from both renal cortex and renal medulla using TRIzol reagent (Invitrogen, USA) and according to the manufacturer's instructions. Reverse transcription was carried out using random primers and RevertAid M-MuLV Reverse Transcriptase (Fermentas, Lithuania), using the standard protocol provided by the manufacturer. Primers were designed to amplify all alpha isoforms (α 1: NM_012504.1; α 2: NM_012505.2; α 3: NM_012506.1; α 4: NM_001271030.1) (α For: 5' CTGACTGCCAAGCGCATGGC 3'; α Rev: 5' GGCCACTGTCATGCGGTTCC 3'). The housekeeping gene *gapdh* (NM_008084.2) was amplified with the following primers: *gapdh*For 5' TGCATCCTGCACCACCAACT3'; *gapdh*Rev 5' CTTGGCAGCACCAGTGGATG 3'.

The amplification was performed in a Step One (Applied Biosystems, USA) equipment using a final volume of 12.5 μ l (FastStart Universal SYBR Green Master (Roche, Switzerland) with 0.1 μ g cDNA and 300 μ M primers) with the following cycling steps: 50 °C for 2', 95 °C for 10' and 40 cycles at 95 °C for 20", 61 °C for 1', 72 °C for 20". Transcriptional level was determined by RT-PCR and normalized to the expression of *gapdh* using the 2- $\Delta\Delta$ CT method, efficiency of each reaction (*gapdh* and α isoforms) were determined before the correspondent analysis¹¹. PCR products corresponding to different melting temperatures were purified using QIAquick gel extraction kit (QIAGEN, Netherlands) and sequenced in

a ABI prism 3100 Genetic Analyzer (Applied Biosystems, USA) with the same primer set used to quantify mRNA. Sequences were exported from chromatograms using Chromas Lite 2.1.1 (Technelysium, Australia), manually curated and aligned with ClustalW (www.expasy.org). Phylogram was performed with ClustalW2-phylogeny (EMBL-EBI).

Results, were expressed as mean \pm standard error of the mean (SEM) and analyzed by Student's *t*-test. Differences were deemed significant at $P < 0.05$.

The first step of our study was to determine the total expression level of α subunit of the Na⁺/K⁺-ATP-ase. The abundance of specific mRNA was quantified by RT-PCR using primers that allowed the amplification of all α isoforms (α 1-4). In the renal cortex, we observed a reduction in the 18M group, compared to the level in 3M rats ($p < 0.01$; Fig. 1A). There were no significant changes in the expression of α -Na⁺/K⁺-ATP-ase in the renal medulla (Fig. 1B).

Analyzing melting curves of PCR products a right-shift in the melting temperature was observed in the renal cortex of the 18M group (Fig. 2A) that was absent in the renal medulla (Fig. 2B). Melting curves show if sequences with different AT/GC contents are present in PCR products. This analysis does not allow knowing the amounts of each sequence.

The targeted region differs in %GC content between α 1 (56%) and the other three isoforms (62-63%). Partial DNA sequences obtained from PCR products from both peaks, found in the cortex products, confirmed that the predominant isoform in the 3M group was α 1, while the right-shift in the 18M group corresponded to α 2 isoform (Fig. 2C).

The present study inspects the changes in the renal expression of the alpha subunit of the Na⁺/K⁺-ATP-ase, associated with the senescence process in Wistar rats. In addition, we report a method based on RT-PCR coupled to melting curve analysis that enables both the simultaneous amplification of α 1-4 isoforms and the rapid screening of isoform switch from α 1 to α 2-4.

The drastic fall in total α -Na⁺/K⁺-ATP-ase transcription level in the renal cortex was puzzling, given that previous reports performed in F344 x BNF1 and WYK rats have shown no age-specific alteration in its expression¹². However, differences in the physiology of aging features among rat strains are well documented and may account for the discrepancy between our results using outbreed Wistar animals and those of former studies¹³. Defining a suitable animal model to test hypotheses concerning senescence is without doubt an important issue. In this sense, we believe our results provide an alternative perspective for renal aging that differs from that observed in the long-lived F344 x BNF1 and the inbreed WYK strains. Nonetheless, to address the question of which model is readily applicable to study the aging process in humans is beyond the scope of this work.

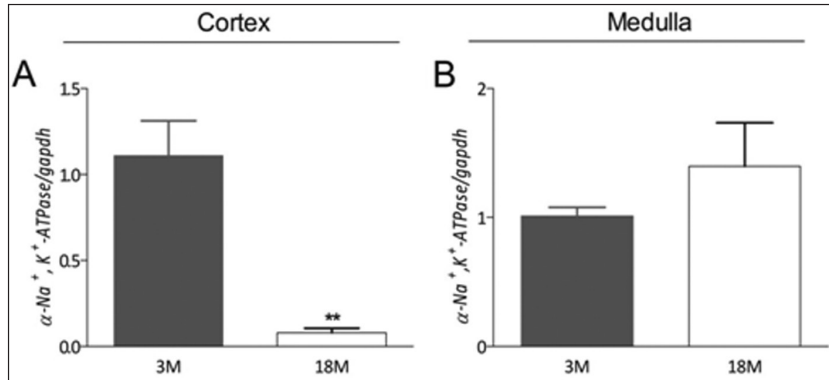


Fig. 1.– Transcriptional level and melting curves of total α -Na⁺/K⁺-ATP-ase in renal cortex and renal medulla in 3 months old and 18 months old rats. Transcriptional level was determined by RT-PCR and relativized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), (A) cortex and (B) medulla. Values were normalized to the 3M control group and reported as means \pm SEM. ** p < 0.01.

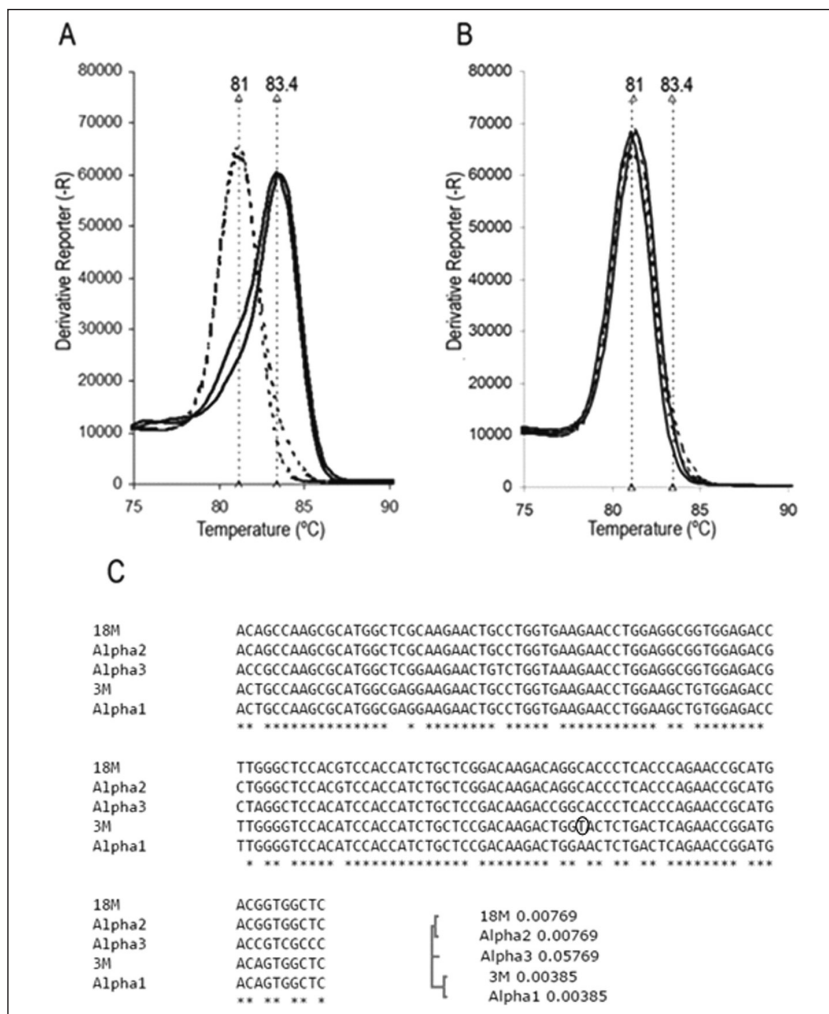


Fig. 2.– Representative melting curves and alignment obtained from amplification and sequencing of total α -Na⁺/K⁺-ATP-ase mRNA. (A) Melting curves from renal cortex; (B) Melting curves from renal medulla. (C) Clustal alignment and phylogram of the sequence obtained from 3M PCR product, 18M PCR product, α 1 isoform, α 2 isoform and α 3 isoform. Notice that 3M corresponds to α 1 while 18M predominant isoform is α 2. 3M sequence show a punctual mutation (T instead C or A).

It was unexpected to find a punctual mutation in 3M sequence; T nucleotide present instead A (in $\alpha 1$ sequence) or C ($\alpha 2$ and $\alpha 3$ sequence). Although transversion mutations are possible (A to T in this case), transitions (C to T) appear more frequent in rat genome¹⁴. Thus, it is more likely that the original sequence was a C than A, which corresponds with $\alpha 2$ sequence, and accordingly with the rest of the sequence. When considering the reading frame, this is a conservative mutation located in the third base encoding for a glycine amino acid in all the sequences ($\alpha 1$, $\alpha 2$ and $\alpha 3$), and the mutation is conservative.

It is generally assumed that $\alpha 1$ isoform comprises up to 70% of the total Na⁺/K⁺-ATP-ase in the kidney⁹. A decrease in the mRNA of total α -Na⁺/K⁺-ATP-ase may be a consequence of a decrease in the $\alpha 1$ mRNA. This means that without changes or in the increase in $\alpha 2$ transcription level will result in a significant increase in the ratio $\alpha 2$ $\alpha 1$. To the best of our knowledge, this is the first study to report a possible age-associated change of the predominant isoform in the renal cortex. Since α -Na⁺/K⁺-ATP-ase isoforms differ in many aspects, such as: substrate affinity (KmNa⁺), sensitivity to cardiotonic steroids and association with both channels and regulatory proteins, the alterations in the isoform expression profile could impact the functionality of the pump.

Considering that our results involve a decrease in the alpha subunit of the Na⁺/K⁺-ATPase, that could be associated with a decrease in proximal sodium reabsorption, we expected a compensatory increase in distal function in order to keep total sodium balance in steady state.

Although there were not statistical differences in Na⁺/K⁺-ATPase mRNA expression in renal medulla, there was a tendency to increase in aged animals. Besides, in unpublished observations, we found an increase in Na⁺/K⁺-ATPase activity in renal medulla. In conclusion, renal aging could be associated to alterations in sodium transporters along the nephron¹⁵.

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Conflict of interests: None to declare

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