Towards the Elucidation of the Role of the Chloride Anion in Arterial Hypertension: Its Link with Oxidative Damage in the Kidney

Hacia el esclarecimiento del rol del anión cloruro en la hipertensión arterial: su vínculo con el daño oxidativo en el riñón

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ABSTRACT

Background: The role of the chloride anion on the deleterious effects of excessive salt (NaCl) intake is unknown and whether its effects are independent of the presence of sodium.

Objective: The aim of this study was to demonstrate that both chloride and sodium overload in the diet produce independent deleterious effects on systolic blood pressure (SBP), renal function and kidney markers of oxidative stress.

Methods: Male Wistar rats were divided into four groups (n=8/group) and fed different diets for three weeks: C: control (standard diet), NaCl: high sodium-high chloride diet; Na: high sodium without chloride diet and Cl: high chloride without sodium diet. Systolic blood pressure (SBP) and renal function were measured, and thiobarbituric acid reactive species (TBARS) production, and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymatic activity and expression were evaluated in the renal cortical tissue.

Results: After three weeks, SBP increased (*) in the two groups fed with chloride. Fractional excretion of sodium and chloride increased (*) in the NaCl and Na groups. Diuresis and TBARS increased (*) in the renal cortex with the three diets, with no changes in SOD and CAT activity and expression. GPx activity increased (*) in the two groups that received chloride (* p <0.05 vs. C).

Conclusions: Both sodium and chloride overload are associated with a higher oxidative state characterized by increased lipid peroxidation in the renal cortex. However, only chloride overload is associated with higher GPx activity and hypertension without changes in urinary chloride excretion, suggesting a higher renal pro-oxidant state in this experimental group with respect to the Na group.

Key words: Chloride - Hypertension - Kidney - Lipid Peroxidation - Glutathione

RESUMEN:

Introducción: Se desconoce el papel del anión cloruro en los efectos deletéreos del consumo excesivo de sal (NaCl) y si sus efectos son independientes de la presencia del sodio.

Objetivo: Demostrar que tanto una sobrecarga de cloruro como una sobrecarga de sodio en la dieta producen efectos deletéreos, en forma independiente, sobre la presión arterial sistólica (PAS), la función renal y los marcadores de estrés oxidativo en el riñón.

Materiales y métodos: Ratas Wistar macho fueron divididas en cuatro grupos (n = 8/grupo) y fueron alimentadas con diferentes dietas durante tres semanas: C: control (dieta estándar), NaCl: hipersódica-hiperclórica, Na: hipersódica sin cloruro, Cl: hiperclórica sin sodio. Se determinaron la presión arterial sistólica (PAS) y la función renal y en la corteza renal se evaluó la producción de especies reactivas del ácido tiobarbitúrico (en inglés: TBARS) y la actividad y la expresión de las enzimas superóxido dismutasa (SOD), catalasa (CAT) y glutatión peroxidasa (GPx).

Resultados: Al cabo de tres semanas, la PAS aumentó (*) en los dos grupos alimentados con cloruro. La excreción fraccional de sodio y de cloruro aumentó (*) en los grupos NaCl y Na. La diuresis y los TBARS en la corteza renal aumentaron (*) con las tres dietas, sin cambios en la actividad y en la expresión de SOD y CAT. La actividad de la GPx aumentó (*) en los dos grupos que recibieron cloruro; (*p < 0,05 vs C).

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Conclusión: Tanto la sobrecarga de sodio como la de cloruro se asocian a mayor estado oxidativo caracterizado por un incremento en la peroxidación lipídica en la corteza renal. Sin embargo, solo el exceso de cloruro se asocia a mayor actividad de la GPx y de la hipertensión, sin cambios en la excreción urinaria de cloruro, sugiriendo un mayor estado prooxidante renal en comparación con el grupo Na.

Palabras clave: Cloruro – Hipertensión – Riñón - Peroxidación de lípido - Glutatión peroxidasa

INTRODUCTION

Excessive salt intake in the diet is a risk factor for the development of high blood pressure. Saline overload in the kidney induces oxidative stress and inflammation, regardless of the blood pressure value. Clinical studies suggest that blood pressure is not increased by a high sodium diet (Na^+) in the absence of chloride (Cl^-), (1-3) since sodium bicarbonate does not have the same pressor effect as sodium chloride. (NaCl) in hypertensive people. (2, 4) Recent evidence suggests that chloride may have a more specific role in "salt sensitive" hypertension, independent of the hypertensogenic effect of sodium. (5-9)

Our working group has demonstrated the presence of acute and chronic pro-inflammatory and profibrotic effects that NaCl overload causes in the kidney. (10-13) A high NaCl diet induces activation of the angiotensinogen gene, increased synthesis of renal angiotensin II, and increased oxidative stress leading to the development of hypertension. (14-16) However, to date, the possible harmful effects of a chloride overload in the kidney, and whether its effects are independent of the presence of sodium have not been described or clarified.

Hypothesis

The chloride anion (Cl^-), independently of the sodium cation (Na^+), would also be involved in the oxidative stress of the kidney and blood pressure elevation. These alterations would be attenuated if Cl^- were replaced by another anion (for example, citrate) or if Na^+ were replaced by other cations.

Objectives

The aim of this study was to determine the independent effects of dietary chloride and sodium overload on the following parameters:
- systolic blood pressure (SBP)
- renal function
- kidney markers of oxidative stress.

METHODS
Animals used

Thirty–two 7-week-old male Wistar rats, weighing 155-165 g at the beginning of the diet, were used.

Diets

The animals were divided into a control group and three experimental groups (n=8/group). They received drinking tap water ad libitum and were fed the following diets (17) for 3 weeks:
1. Control: standard diet (0.4% W/W NaCl in food)
2. NaCl: high sodium-high chloride diet (8%)
3. Na: high sodium diet without chloride ($Na_3C_6H_5O^7$ 11.8%) (equimolar in $Na+$ with group 2)
4. Cl: high chloride diet without sodium ($CaCl_2$ 3.80%; KCl 3.06% and $MgCl_2$ 1.30%) (equimolar in $Cl-$ with group 2).

Assessment of systolic blood pressure

Baseline SBP was measured at 1, 2 and 3 weeks in the rat tail using a sphygmomanometer (Hatteras Instruments, Cary, NC, US), between 9 and 11 a.m., after training the animals for 3 consecutive days.

Assessment of food, calorie and water intake

During the third week, food (g) and water (mL) intake was assessed in three consecutive days. Calorie intake (kcal) was estimated as: 3.3 kcal/g*food intake (g).

Assessment of urinary and plasma parameters and evaluation of excretory renal function

After 3 weeks of diet, the animals were housed in metabolic cages for two days: one for acclimatization and the other for 24-hour urine collection to measure diuresis, and urinary concentrations of Na^+, Cl^- (mEq/L) and creatinine (mg/dL).

Before euthanizing the animals, the final body weight (BW) was obtained, and blood was drawn from the retroocular sinus under anesthesia with ketamine (60 mg/kg) and xylazine (2 mg/kg). Plasma concentrations of Na^+, Cl^-, creatinine, glucose and urea were assessed by means of an autoanalyzer. Plasma osmolarity (mOsm/kg) was estimated as:

2 * plasma sodium (mEq/L)+1/18*blood glucose (mg/dL)+1/6*plasma urea (mg/dL).

Creatinine clearance was calculated as:

CrCl=(urinary creatinine/plasma creatinine)*diuresis/time/body weight (BW).

Tubular function was assessed by means of filtered load (FL), urinary excretion (UE), fractional excretion (FE), tubular reabsorption (TR) and fractional reabsorption (FR) of the different ions, using the following standard formulas:

FLNa= CrCl * plasma sodium	UENa= diuresis*urinary sodium
TRNa=FLNa–UENa	FENa= (UENa/FLNa)*100
FLCl= CrCl *plasma chloride	UECl= diuresis*urinary chloride
TRCl=FLCl–UECl	FECl= (UECl/FLCl)*100

Diuresis, CrCl and FL, TR and UE were normalized by the BW of each rat and were expressed in mL/day/kg, mL/min/kg or mEq/day/kg, while FE and FR are expressed as percentage (%).

Euthanasia, kidney removal and sample processing.

Under anesthesia, both kidneys were removed by abdominal laparotomy. The renal cortex was dissected, homogenized in saline phosphate buffer (7.6 mM KH_2PO4, 42.4 mM K_2H-PO4, 150 mM NaCl, pH: 7.4) and centrifuged at 600 g for 20 minutes at 4° C. TBARS, and the antioxidant enzyme activity: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were determined in the supernatants. The protein expression of these enzymes was assessed by Western Blot in renal cortex homogenates, and protein content was measured by the Lowry method. (18) The animals were euthanized by decapitation.

TBARS assessment
The test is based on a method previously described by Fraga et al. (19) Results are expressed in nmol TBARS of malondialdehyde (MDA) equivalents/mg protein.

Assessment of enzyme activity
Superoxide dismutase activity was measured spectrophotometrically following adenochrome formation. Total SOD activity was determined in the absence of potassium cyanide (KCN) (4μM); in its presence, Mn-SOD (mitochondrial isoform) activity was assessed, and by difference, Cu/Zn-SOD (cytosolic isoform) activity was obtained. The results are expressed in arbitrary units (AU)/mg protein. (20)

Catalase activity was determined by spectrophotometry at 240 nm following H_2O_2 consumption. The results are expressed as μmol H_2O_2/mg protein/min. (21, 22)

Glutathione peroxidase activity was measured by spectrophotometry at 340 nm following the enzymatic oxidation of NADPH in the presence of 1 mM glutathione (GSH), 1 mM NaN_3, 0.15 mM NADPH and 0.25 units (U)/mL of glutathione reductase. The results are expressed in μmol of oxidized NADPH/min/mg protein, which is equivalent to μmol oxidized glutathione (GSSG)/min/mg protein. (23)

Western Blot analysis
The methodology was previously described. (24) Primary antibodies were diluted 1: 1000, and the secondary ones, conjugated with horseradish peroxidase 1: 5000 in phosphate-buffered saline (PBS). As a loading control, β-actin was used to normalize the protein content.

Statistical analysis
The results are expressed as mean±standard error of the mean (SEM). A two-way analysis of variance (ANOVA) with Tukey post-hoc test was performed using the InfoStat program, version 2018. Results with $p < 0.05$ were considered significant.

Ethical considerations
The experimental protocol used was approved by the Institutional Committee for the Care and Use of Laboratory Animals of the School of Pharmacy and Biochemistry, University of Buenos Aires (CICUAL), by Res (D) N° 1881/2019, and the procedures were made following the indications of the "Guide for the Care and Use of Laboratory Animals" of the National Academy of Sciences of the United States of North America.

RESULTS
Body weight, food, calorie and water intake
The three groups fed with experimental diets showed a lower final-initial BW difference than the control group, accompanied by a higher intake of water after three weeks of dietary treatment (Table 1).

Evolution of systolic blood pressure
Control rats remained normotensive during the 3 weeks of diet. In the three groups fed with experimental diets, SBP increased since the second week, and the differences were significant with respect to baseline values and the control group for NaCl and Cl diets.

The highest SBP values were reached at 2 and 3 weeks in the NaCl group, while the SBP rises in the Cl and Na groups were lower than those reached in the NaCl group. As can be seen in Figure 1, SBP in the Na group showed a lower increase than in the other two experimental groups, but without reaching significant differences with respect to the control group.

Plasma and urinary parameters
Plasma creatinine, sodium, chloride and osmolarity (estimated from plasma sodium, glucose and urea) did not change in any of the groups. Urinary creatinine decreased in the three groups with respect to the control group, and urinary sodium increased in the groups with a high sodium diet (NaCl and Na) and decreased in the Cl group. The urinary Na$^+$/Cl$^-$ index, which assesses urinary equimolarity between the two ions, increased significantly in the Na group, and reached values very close to equimolarity in the Cl group (Table 2).

Excretory renal function parameters
Diuresis increased in the three groups with respect to control, while CrCl decreased in the NaCl and Na groups.

In the NaCl and Na groups, FLNa, TRNa, FRNa, FLCl, TRCl and FRCl decreased and UENa, FENa, UECl and FECl increased with respect to control.

Compared with the NaCl group, in the Na group rats we observed an increase in UENa and a decrease in UECl. Moreover, in the Na group, FECl was lower than FENa, while FRCl was higher than FRNa.

The Cl group did not show significant changes with respect to the control group, but evidenced differences when compared with the other two groups: it had higher FLNa, TRNa, FRNa, FLCl, TRCl and FRCl with respect to the NaCl group, changes that were accompanied by less urinary and FE of both ions.

Table 1. Body weight, food, calorie and water intake

	Control	NaCl	Na	Cl
Number of cases (n)	8	8	8	8
Initial body weight (g)	152 ± 4	151 ± 6	156 ± 10	175 ± 6
Final body weight (g)	293 ± 22	265 ± 21	247 ± 15	290 ± 13
Body weight difference (g)	141 ± 7	114 ± 9*	91 ± 15*	115 ± 3*
Estimated food intake (g)	27 ± 2	29 ± 3	35 ± 8	33 ± 3
Estimated calorie intake (kcal)	91 ± 7	95 ± 9	116 ± 26	110 ± 10
Estimated water intake (mL)	21 ± 1	50 ±4 *	61 ± 9*	31 ± 1*

NaCl: high sodium-high chloride diet; Na: high sodium diet without chloride; Cl: high chloride diet without sodium. * $p < 0.05$ vs. Control.
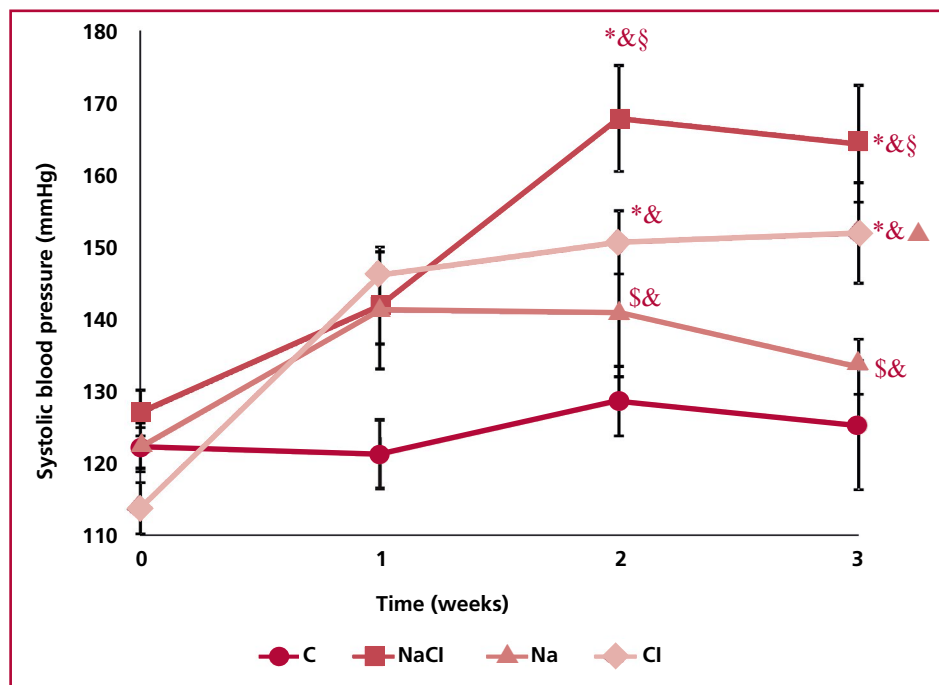
Fig. 1. Time course of systolic blood pressure

NaCl: high sodium-high chloride diet; Na: high sodium diet without chloride; Cl: high chloride diet without sodium. p <0.05: * vs. Control; $ vs. NaCl; Δ vs. Na; & vs. t=0; § vs. 1st week.

Table 2. Plasma and urine parameters

	Control	NaCl	Na	Cl
Plasma creatinine(mg/dL)	0.56 ± 0.04	0.64 ± 0.04	0.62 ± 0.03	0.63 ± 0.04
Plasma sodium (mEq/L)	151 ± 5	144 ± 2	147 ± 3	144 ± 2
Plasma chloride (mEq/L)	102 ± 2	100 ± 1	101 ± 3	99 ± 1
Glycemia (mg/dL)	138 ± 11	152 ± 15	153 ± 13	151 ± 14
Plasma urea (mg/dL)	27 ± 1	38 ± 4 *	49 ± 4 * $	22 ± 2 * $ Δ
Estimated plasma osmolarity (mOsm/kg)	319 ± 9	311 ± 4	321 ± 7	306 ± 5
Urinary creatinine (mg/dL)	316 ± 42	52 ± 17 *	22 ± 4 *	71 ± 24 *Δ
Urinary sodium (mEq/L)	117 ± 31	293 ± 41 *	360 ± 43 *	26 ± 11 * $ Δ
Urinary chloride (mEq/L)	145 ± 37	345 ± 48 *	83 ± 8 * $	29 ± 12 * $ Δ
Urinary Na^+/Cl^- index	0.77 ± 0.09	0.84 ± 0.05	4.30 ± 0.23 * $	0.99 ± 0.26 Δ

NaCl: high sodium-high chloride diet; Na: high sodium diet without chloride; Cl: high chloride diet without sodium. * p <0.05 vs. Control; $ p <0.05 vs. NaCl; Δ p <0.05 vs. Na.

With respect to the Na group, the Cl group had lower urinary and FE, and greater FR of both ions (Table 3).

Oxidative stress parameters in the renal cortex
The production of TBARS increased in the renal cortex in the NaCl, Na and Cl groups compared with the control group. The activity and protein expression of SOD and CAT mitochondrial and cytosolic isoforms were not modified. While the protein expression of GPx was not modified in any group, the activity of this enzyme increased in the NaCl and Cl groups compared with the control and Na groups (Figure 2).

Summary of Results
Both excess of a high-sodium and high-chloride diet are associated with a higher oxidative state evidenced

by an increase in lipid peroxidation in the renal cortex, demonstrated by an increase in the production of TBARS. However, compared with the Na group, only excess of chlorides is associated with greater GPx activity and the development of hypertension with greater urinary retention of both ions, suggesting a higher pro-oxidant and oxidative stress state in the kidney in the presence of chloride overload.

DISCUSSION
Body weight, food, calorie and water intake
The intake of Na^+, Cl^- or both ions was associated with lower BW gain, with respect to the control group, during the three weeks of diet. These results are consistent with that reported in the literature, where a hypersaline diet was associated with a decrease in total

Table 3. Kidney function parameters

	Control	NaCl	Na	Cl
Urine output (mL/day/kg)	10 ± 2	78 ± 14 *	92 ± 15 *	51 ± 21 * Δ
CrCl (mL/min/kg)	3.55 ± 0.55	2.21 ± 0.29 *	2.41 ± 0.19 *	3.01 ± 0.53
NaFL (mEq/day/kg)	790 ± 141	461 ± 61 *	511 ± 39 *	634 ± 105 $
NaUE (mEq/day/kg)	1.2 ± 0.3	22.9 ± 4.3 *	34.4 ± 6.2 * $	1.1 ±0.3 $ Δ
NaFE (%)	0.15 ± 0.04	5.24 ± 1.74 *	6.82 ± 0.97 *	0.15 ± 0.03 $ Δ
NaTR (mEq/day/kg)	789 ± 141	440 ± 64 *	477 ± 38 *	633 ± 105 $
NaFR (%)	99.85 ± 0.04	94.76 ± 1.74 *	93.18 ± 0.97 *	99.85 ± 0.03 $ Δ
ClFL (mEq/day/kg)	532 ± 92	319 ± 40 *	349 ± 26 *	435 ± 74 $
ClUE (mEq/day/kg)	1.4 ± 0.3	26.5 ± 5.1 *	7.8 ± 1.5 * $	1.1 ± 0.3 $ Δ
ClFE (%)	0.27 ± 0.07	8.39 ± 2.70 *	2.23 ± 0.33 * $ @	0.24 ± 0.04 $ Δ
ClTR (mEq/day/kg)	531 ± 92	295 ± 44 *	341 ± 26 *	434 ± 74 $
ClFR (%)	99.73 ± 0.07	91.61 ± 2.70 *	97.77 ± 0.33 * $ @	99.76 ± 0.04 $ Δ

NaCl: high sodium- high chloride diet; Na: high sodium diet without chloride; Cl: high chloride diet without sodium. CrCl: creatinine clearance, FL: filtered load, UE: Urinary excretion, FE: fractional excretion, TR: tubular reabsorption, FR: fractional reabsorption. * $p < 0.05$ vs. Control; $ $p < 0.05$ vs. NaCl; @ $p < 0.05$ vs. FENa or FRNa; Δ $p < 0.05$ vs. Na.

fat mass in mice that presented upregulation of genes involved in lipolysis and downregulation of genes related to lipogenesis. (25) In our work, at the time of euthanasia, we observed a decrease in epididymal and perirenal fat in rats that consumed NaCl and Na with respect to the other two experimental groups (data not included). These findings occurred despite the fact that all the diets were isocaloric. Animals fed with salt-overloaded diets consumed more water than controls. This may be caused by an initial acute increase in plasma osmolarity, which stimulates the thirst center, in order to compensate for that increase. (26)

Evolution of systolic blood pressure
We have shown that male Sprague Dawley rats, subjected to a diet with NaCl overload (8% W/W) increase their SBP after three weeks of diet, with values that exceed those defined as systolic hypertension (140 mmHg). (27)

The results presented suggest that the increase in SBP is also related to chloride overload, since the Cl group reached pressure values greater than 140 mmHg, higher than those of the Na group. The Cl⁻ anion is a component of NaCl that could have a more specific role in salt-sensitivity and that could be even more decisive than Na⁺ (28) Other studies in "salt-sensitive" Dahl rats showed that over several weeks, hypertension developed in NaCl-consuming animals, but not in those fed NaHCO3 or other Na⁺ salts. (29-31)

On the other hand, excess intake of "non-sodium" chloride salts, which is accompanied by a lower urinary excretion of chlorides than that produced in the presence of Na⁺, could be related to a selective accumulation of Cl⁻ in the body, which would lead to the development of "salt-sensitive" hypertension. (32-34)

Plasma and urinary parameters
The absence of changes in plasma sodium and chloride concentrations and osmolarity are evidence of the biological efficiency of physiological mechanisms to compensate for possible hypernatremia and/or hyperchloremia and to preserve plasma osmolarity.

As expected, urinary sodium and chloride increased in the NaCl group compared with the control group and the urinary Na⁺/Cl⁻ index was similar in both groups. In the Na group, it is possible that bicarbonate secretion and excretion increases, a result consistent with the increase in the urinary Na⁺/Cl⁻ index with respect to the other groups, suggesting that Cl⁻ is not the main counterion to excreted Na⁺. The objective of HCO₃⁻ secretion is to compensate for metabolic alkalosis in the animals that receive Na⁺ citrate and, as a consequence, the reabsorption of Cl⁻ would be increased and its excretion, decreased, since the Cl⁻/HCO₃⁻ exchanger, independent of the Na⁺ cation, would present a higher expression in the apical cell membranes of the distal convoluted, cortical collector and connector tubules. (28) Regarding the Cl group, the low urinary Cl⁻ is striking with respect to control rats, suggesting that for its excretion, it is also necessary to eliminate Na+ as a counterion.

These results indicate that the chloride anion is accumulating in some compartment, such as the skin, since its levels are still normal in plasma. (32-34)

Excretory renal function parameters
The increase in diuresis in the three experimental groups, with respect to controls, agrees with the increase in water intake. Di Ciano et al. have reported increased diuresis in Wistar rats fed a saline diet. (35) These changes were accompanied by a lower filtration fraction, a finding that is consistent with the decrease in CrCl and FLNa and FLCl that we observed in the NaCl group.

Chloride overload leads to an increase in the supply of this anion and can cause renal vasoconstriction and decrease the glomerular filtration rate as a consequence of the tubule-glomerular feedback due to greater transport of Cl⁻ to the macula densa. This ef-
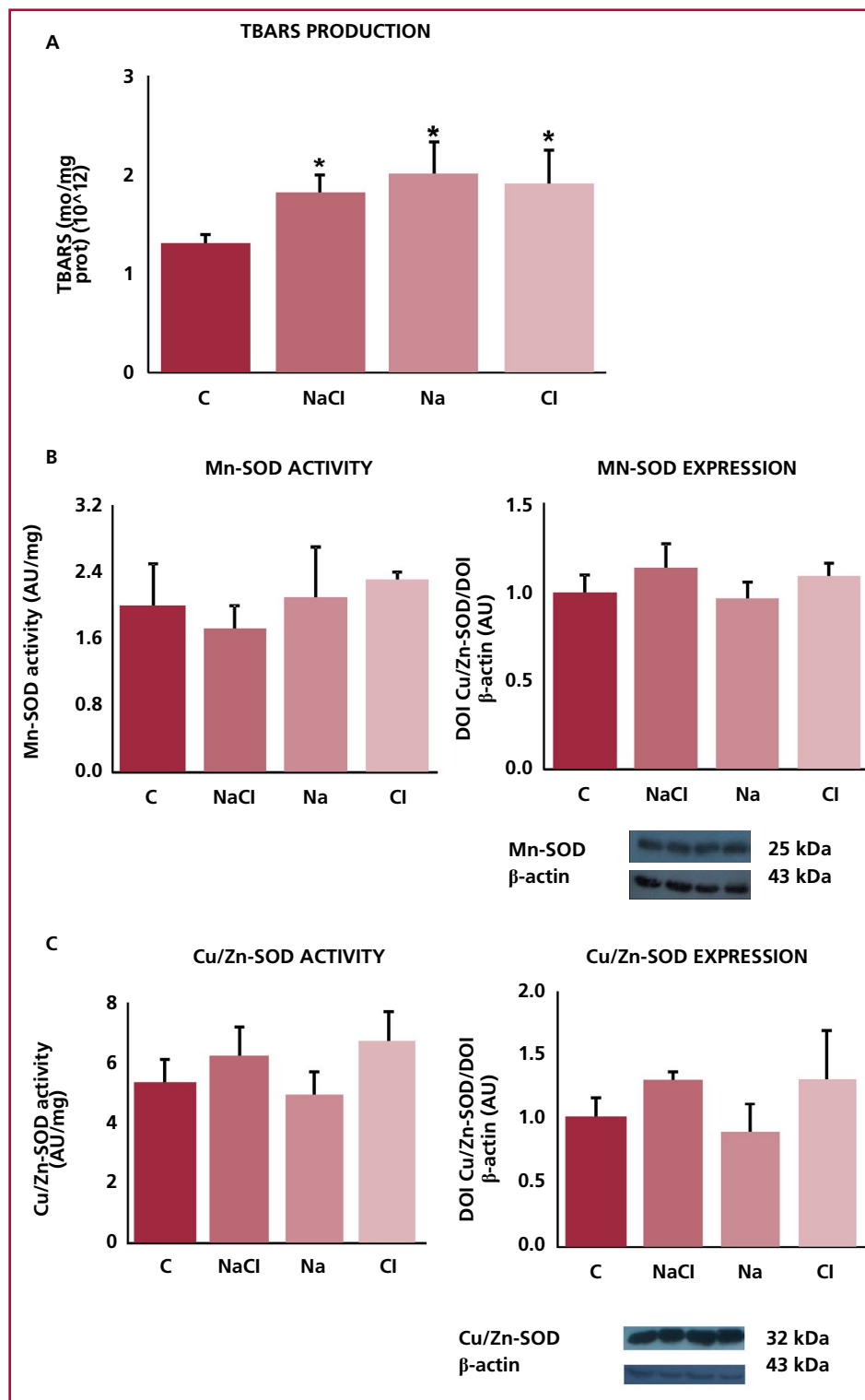
fect would result in increased renal afferent arteriolar resistance, with a reduction in renal plasma flow and glomerular filtration rate and an increase in systemic arterial pressure. (36, 37)

The decrease in CrCl in the NaCl group may explain lower FLNa and FLCl, compared with the con-

trol group. It was expected that both groups that received iso-osmolar Na^+ overloads would present the same profile in terms of ionic excretion and retention parameters. But this profile was not observed in the Cl group, showing that the replacement of the Na^+ ion by other cations causes dissimilar responses. In the Cl

Cont.
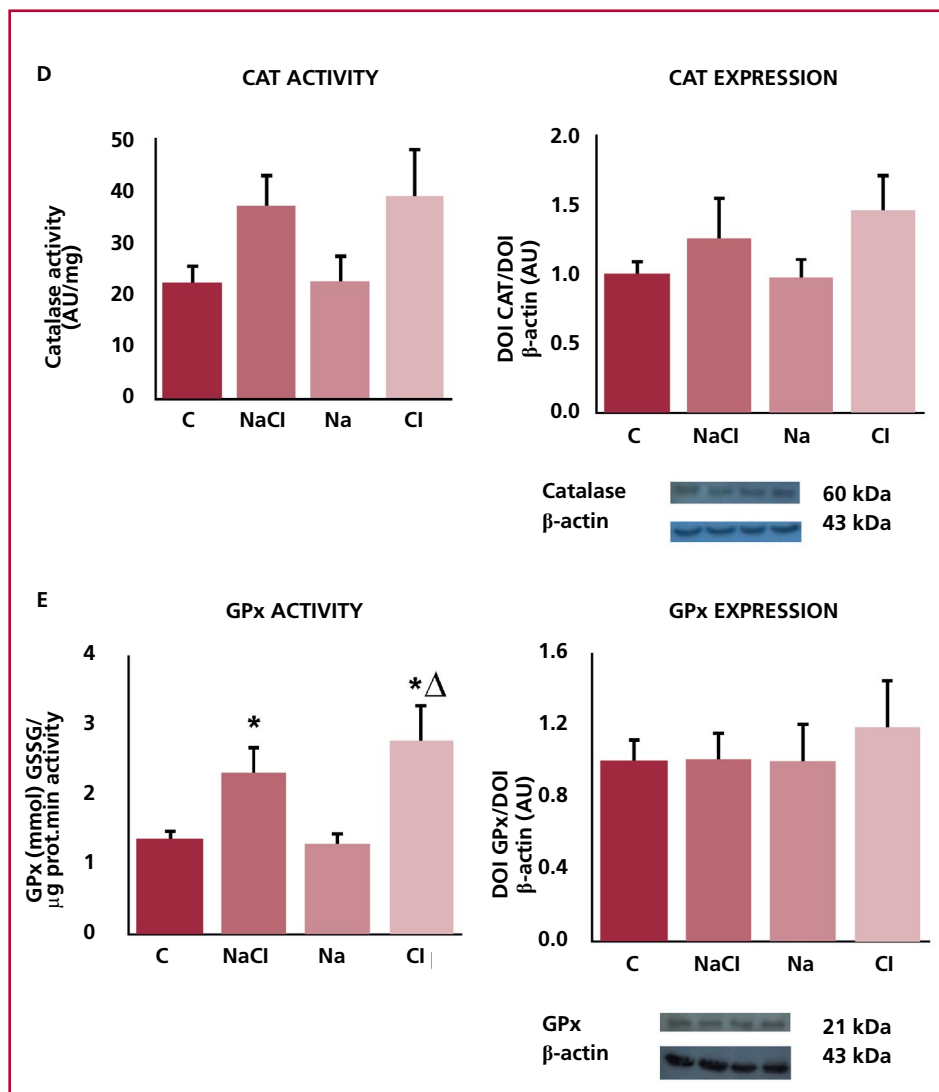
A) TBARS: Thiobarbituric acid reactive species. B) Mn-SOD: Manganese-superoxide dismutase (mitochondrial isoform of the enzyme). C) Cu/Zn-SOD: Copper/Zinc-superoxide dismutase (cytosolic isoform of the enzyme). D) CAT: Catalase. E) GPx: Glutathione peroxidase. NaCl: high sodium-high chloride diet; Na: high sodium diet without chloride; Cl: high chloride diet without sodium. * p < 0,05 vs Control; Δ p < 0,05 vs Na.

group, Na^+ and Cl^- ions presented very similar excretion and reabsorption profiles, evidencing a clear urinary equimolarity, which reflects that the counterion that is eliminated with Cl^- is Na^+.

Oxidative stress parameters in the renal cortex
Na^+, Cl^- or both ions overload in the diet was associated with an increase in lipid peroxidation in the renal cortex, represented by the increase in the production of TBARS. The pro-oxidant state in these cells is characterized by an increase in the production of reactive oxygen species, a situation in which SOD produces the dismutation of the superoxide anion to H_2O_2 and molecular oxygen with a high reaction rate constant ($2.3.10^9$ $M^{-1}s^{-1}$), while CAT converts H_2O_2 to molecular oxygen and water.

It was expected that increased TBARS production was accompanied by increased SOD and CAT activity and/or expression. But in our models these parameters were not affected. However, an increase in GPx activity was registered, which suggests a compensatory effect in the absence of SOD and CAT modifications. The regulation of its activity is related to post-translational modifications that take place in the active site of the enzyme and that occur regardless of whether or not its expression varies. (38)

CONCLUSION
These results suggest that the chloride anion is co-responsible, together with sodium, of triggering oxidative kidney damage and increased blood pressure. It is therefore important to take into account the reduction in the intake of both ions as a measure of non-pharmacological treatment of hypertension, considering that most commercial dietary products, substitutes for table salt, are based on potassium chloride.

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