Lipoic acid does not improve renal function markers in 5/6 nephrectomy model: possible role of Nrf2 inactivation

Sze M. Lo, Fernando T. Dal Lin, Maria F. Soares, Aline B. Hauser, Roberto Pecoits-Filho and Lia S. Nakao

ABSTRACT
Chronic kidney disease (CKD) progression and complications are associated with increased oxidative stress, as well as with Nrf2 inactivation. Lipoic acid (LA) has been considered an inducer of Nrf2 antioxidant response. We tested whether oral administration of LA provides beneficial effects in experimental CKD in rats. Wistar rats underwent 5/6 nephrectomy (CKD group) or sham laparotomy. Seven days later, CKD group was divided into three subgroups that received: (i) LA continuously in the drinking water (100 mg/kg/day), (ii) LA by gavage every other day (100 mg/kg), or (iii) no LA treatment. LA treatment lasted until day 60. Plasma urea and creatinine, 24 h-proteinuria, glomerulosclerosis, interstitial fibrosis/tubular atrophy, and Nrf2 activation were analyzed. All parameters measured were significantly altered in the untreated CKD group, compared with the sham group, as expected. Oral LA administration, either in the drinking water or by gavage, did not improve significantly any parameter, comparing the treated-groups with the untreated CKD group. These results indicate that oral LA administration for 53 days was ineffective to reactivate Nrf2 in the remnant kidney of uremic rats, likely preventing improvements in biochemical and histopathological markers of renal function.

Introduction
Chronic kidney disease (CKD) is a systemic and progressive disease, leading to a poor quality of life in the later stages of the disease. Because oxidative stress contributes to both progression and complications of CKD, antioxidant therapy has been clinically tested in CKD patients, providing conflicting results.

Supplementation with a-lipoic acid (LA) has been recently assessed in antioxidant therapy in CKD. Although some studies showed that oral administration of LA, particularly to hemodialysis (HD) patients for several weeks, led to an improvement in asymmetric dimethylarginine (ADMA) and C-reactive protein, levels, or erythropoietin resistance index, oxidative stress biomarkers levels were not altered in the LA-treated groups of CKD or HD patients. In experimental CKD, intraperitoneally-injected LA for 8 weeks has been shown to improve blood pressure, renal function, and oxidative markers after 5/6 nephrectomy in rats, while oral LA for 8 weeks decreased blood pressure, as well as vascular injury, compared with the non-treated nephrectomized rats. In acetaminophen-induced uremia, orally-fed LA for 15 days prevented an increase in plasma urea, creatinine, and malondialdehyde levels in rats.

Lipoic acid has received particular attention for several reasons. Its reduced form dihydrolipoic acid (DHLA) is a strong reductant; both LA and DHLA chelate redox-active metal ions and scavenge reactive species; LA has anti-inflammatory properties, which makes it interesting, considering the contribution of inflammation in CKD pathogenesis and progression; LA is an insulin-mimetic and can successfully control glycemic levels in diabetes, a major cause of CKD; its pharmacokinetic, metabolism, and toxicity are relatively well known, even in CKD patients; finally, LA has been shown to activate the Nrf2 pathway, improving the endogenous antioxidant status of cells. This latter ability is especially relevant, since CKD is associated with an increased oxidative stress and with an impaired Nrf2 activation in the remnant kidney and in vascular tissue. Therefore, here we investigated whether oral LA supplementation, initiated at early stages of the disease, could activate Nrf2 antioxidant response in the remnant kidney of the 5/6 nephrectomy model, thus retarding CKD progression. Our results showed that administration
of LA, either in the drinking water or by gavage, did not provide beneficial effects under our experimental conditions.

Materials and methods

5/6 Nephrectomy and treatments

Male Wistar rats, weighing between 200 and 300 g, were obtained from the facility at the Universidade Federal do Paraná (UFPR). Five-sixths nephrectomy or sham-operation were performed as previously described, except that 100 μL of Lidovet® (0.01 mg/mL) were added in renal hilum during ligation to prevent bleeding. At the end of the procedure, oral veterinary Buscopan® (dipyrene and scopolamine, 0.25 mL/kg) and intramuscular enrofloxacin (5 mg/kg) were administered. Seven days after the surgery, the CKD group was randomized into three subgroups: (i) control, which received no intervention, (ii) LA-DW, which received LA (Lidifarma, Curitiba, Brazil) in the drinking water ad libitum (600 mg/L solution prepared every 2 days in light-protected flasks, that provided a mean LA ingestion of approximately 100 mg/kg/day), and (iii) LA-G, which received LA by oral gavage (60 mg/mL in 0.1% carboxymethylcellulose suspension) every other day, providing a dose of 100 mg/kg. The treatment lasted until day 60 post-operation. On days 0 and 60, the animals were weighted and blood was collected from the inferior vena cava or from cardiac puncture, respectively. On day 60, the animals were euthanized with a lethal dose of anesthetics (ketamine and xylazine, 100 mg/kg each, i.p.). This protocol has been approved by the Research Ethics Committee (CEUA) of the Biological Sciences Building, protocol number 692/2013.

Samples collection

Plasma was isolated from the blood that had been collected in heparin for urea and creatinine determinations. Twenty-four hour urine was collected at the 59th day after the surgery, in metabolic cages. After determining the total volume, 1 mL of the urine was cleared by centrifugation (1000g, 4 °C). Supernatants were stocked at −20 °C. The remnant kidneys were washed in cold PBS. Half of the tissue was immediately frozen at −80 °C and half was fixed in 4% paraformaldehyde for 24 h.

Determination of plasma urea and creatinine

Urea and creatinine concentrations were determined on the microplate scale using commercial kits (Katal, São Paulo, Brazil, and Labtest, Lagoa Santa, MG, Brazil, respectively). Absorbances were determined on a microplate reader (BioRad, Hercules, CA).

Proteinuria

Protein content in 24-h-urine was determined photometrically, by Bradford. The total amount was calculated considering the total urine output in 24 h.

Histological analysis

Glomerulosclerosis (GS) and interstitial fibrosis and tubular atrophy (IF-TA) were analyzed in paraffin-embedded, routinely processed, and periodic acid of Schiff (PAS)-stained tissues, obtained from the remnant kidneys. GS was determined as the % of sclerosed glomeruli in 10 high power fields (40 objective; 0.44 mm), while the IF-TA index was estimated by the % of damaged renal cortex area relative to total cortex area. This analysis was performed with an optical microscope Olympus BX41 (Olympus, Tokyo, Japan).

SDS-PAGE and Western blotting

The expression of catalytic subunit of glutamate-cysteine ligase (GCLC) in remnant kidneys was analyzed by Western blotting. For this, tissue extracts were prepared in RIPA buffer containing a cocktail of protease inhibitors (Roche, Mannheim, Germany). After electrophoretic separation in a 10% acrylamide/bis-acrylamide gel, proteins were blotted on a nitrocellulose membrane (BioRad, Hercules, CA). The membrane was incubated with 5% skim milk, then with anti-GCLC (Abcam ab41463, Cambridge, MA) 1:500 or with anti-β-actin (Sigma A5441, Saint Louis, MO) 1:4000, followed by anti-rabbit IgG coupled to HRP (Sigma A0545) 1:6000 or anti-mouse IgG coupled to HRP (Sigma A0412) 1:9000. Reactions were developed with Westar Sun ECL chemiluminescence kit (Cyanagen, Bologna, Italy), in autoradiogram films (GE Healthcare, Piscataway, NJ). Bands intensities were measured by densitometry with ImageJ (http://imagej.nih.gov/ij/).

Statistical analysis

The results are expressed as mean ± SD. The exact number of rats (n) is described in each figure. Statistical analysis was performed with ANOVA followed by Dunn’s or Tukey’s (Western blotting analysis) post-hoc test, using SigmaStat v 3.5 (San Jose, CA). p < 0.05 was considered statistically significant.
Results

LA does not improve renal function parameters

We used the 5/6 nephrectomy model in rat to investigate the renoprotective effects of LA. LA was administered to nephrectomized rats from day 7 to 60 after the surgery, either continuously in the drinking water (100 mg/kg/day) or every other day by oral gavage (100 mg/kg). The levels of plasma urea and plasma creatinine were measured at days 0 and 60 of the nephrectomy. Both urea and creatinine levels were in the normal range immediately after the nephrectomy, i.e. day 0 (Figure 1A and B) in both sham and CKD groups. Twenty-three sixty days later, both parameters were significantly increased in the CKD control group compared with the sham group or with the CKD group at day 0 (Figure 1A and B), indicating renal failure. LA treatment, however, did not alter the high urea and creatinine levels observed in the untreated CKD group at day 60 (Figure 1A and B). The CKD control group gained significantly less body weight than the sham group (Figure 1C). LA administration improved this parameter, but not at a statistically significant level (Figure 1C). Proteinuria was determined by measuring the total protein amount in 24 h-urine samples. The results showed that the CKD control group presented a significantly increased protein excretion compared with the sham group 60 days after the nephrectomy (Figure 1D), confirming renal injury. However, LA administration did not significantly inhibit proteinuria. Finally, the remnant renal tissue was analyzed. While no sclerosed glomeruli were detected in the sham group at the end of the treatment, CKD control kidneys presented a significant increase in GS index (Figure 2A and B). LA in the drinking water had no effect. LA administered by gavage inhibited

Figure 1. LA effects on biochemical and clinical markers of renal dysfunction in CKD rats. Notes: Rats were 5/6 nephrectomized or sham operated. Seven days later, CKD rats were divided into three subgroups: CKD, which received no intervention, LA-DW, which received LA in the drinking water or LA-G, which received LA by gavage every other day. LA (100 mg/kg) was administered until day 60, when rats were euthanized. Plasma from day 0 (before the surgery) and day 60 (end of the experiment) were analyzed for urea (A) and creatinine (B) levels. The body weight gain between these two-time points was also determined (C). At day 59, the 24 h-urine was collected and determined (D). The number of rats in each group in shown. The data represent the mean ± SD. *p < 0.05.
GS, but not at a statistically significant level. IF-TA was absent in the sham group, but significantly increased in the CKD group after 60 days. LA treatment, either in the drinking water or by gavage, provided no improvement in such histological parameters (Figure 2C and D).

**LA does not activate Nrf2 in the remnant kidney**

Since LA has been described as an activator of the antioxidant pathway Nrf2, we analyzed the induction of GCLC expression in the remnant kidneys. GCLC is upregulated by Nrf2 activation. Its expression was significantly decreased in CKD control kidneys after 60 days, compared with the sham group, in agreement with reports demonstrating that CKD inhibits Nrf2 activation *in vivo*.

LA treatment, in the drinking water or by gavage, did not restore its expression (Figure 3).

**Discussion**

The Nrf2 pathway has been increasingly recognized as a powerful antioxidant strategy that cells use to deal with oxidative stress. Hyperglycemia, for instance, increases mitochondrial superoxide generation in endothelial cells, which then activates Nrf2. Similarly, streptozotocin-treated Nrf2 knockout mice present more oxidative stress in their kidneys. The results from our study suggest that LA may not be an effective antioxidant in the context of chronic kidney disease, and further investigation into alternative therapeutic strategies is warranted.
stress and renal injury than the treated wild type-mice.\textsuperscript{26} These observations illustrate the cytoprotective effects of Nrf2 activation. However, previous reports showed that the later stages of CKD, such as pre-dialysis and HD, inactivate this antioxidant response by increasing Keap1 expression, in spite of the enhanced oxidative stress present in such situations.\textsuperscript{21,22} The cause of the impairment is not known, but may possibly involve the participation of uremic toxins.\textsuperscript{27} In this context, reactivation of Nrf2 pathway could be a mechanism to re-establish the redox status in CKD, but no agent has been clinically approved so far. Preliminary clinical studies with the potent Nrf2-activator bardoxolone methyl showed promising results, but this trial was halted in phase 3, due to increased mortality.\textsuperscript{28} Besides the different route of LA administration, an additional possibility to explain such contrasting results is based on the fact that LA has different actions depending on the clinical situation.\textsuperscript{16} For instance, LA may have toxic effects in healthy organisms, leading to renal tissue damage.\textsuperscript{13,30} Yu et al.\textsuperscript{13} performed the 5/6 nephrectomy in 2-steps (ablation of 2/3 of the left kidney and removal of the right kidney one week later) and we did it in 1-step (infarction of 2/3 of the left kidney and removal of the right kidney in the same surgery). These two methods of inducing renal insufficiency produce pathological consequences in dissimilar extensions.\textsuperscript{31} Thus, it is possible that CKD progressed differently in these two studies, leading to distinct clinical situations when LA treatment started. In conclusion, oral administration of LA from day 7 to 60 after the 5/6 nephrectomy in rats was not effective in preventing urea and creatinine accumulation in plasma, proteinuria, and kidney tissue damage, possibly as a result of LA inability to reactivate Nrf2 in the remnant kidney.

**Disclosure statement**

The authors report no conflicts of interest.

**Funding information**

This study was supported by the INCT Redoxoma (#573530/2008-4), Fundação Araucária (#14620 and #22122), and CNPq (#473450/2007-0 and #475455/2012-6). Fellowships from CNPq/PIBIC (SML), CAPES (FTD), and from CNPq (RP and LSN) are also acknowledged.
References

1. Sohrabi Z, Eftekhari MH, Eskandari MH, Rezaieanzadeh A, Sagheb MM. Malnutrition-inflammation score and quality of life in hemodialysis patients: Is there any correlation? Nephrourol Mon. 2015;7(3):e27445.

2. Aveles PR, CriminiàCR, Gonçalves S, et al. Association between biomarkers of carotid stiffness with increased systemic inflammatory response in different stages of chronic kidney disease and after renal transplantation. Nephron Clin Pract. 2010;116(4):c294–c299.

3. Modaresi A, Nafar M, Sahraei Z. Oxidative stress in chronic kidney disease. Iran J Kidney Dis. 2015;9(3):165–179.

4. Fuji H, Nakai K, Fukagawa M. Role of oxidative stress and indoxyl sulfate in progression of cardiovascular disease in chronic kidney disease. Ther Apher Dial. 2011;15(2):125–128.

5. Rodrigues SD, França KC, Dallin FT, et al. N-acetylcysteine as a potential strategy to attenuate the oxidative stress induced by uremic serum in the vascular system. Life Sci. 2015;121:110–116.

6. Coombes JS, Fassett RG. Antioxidant therapy in hemodialysis patients: A systematic review. Kidney Int. 2012;81(3):233–246.

7. Chang JW, Lee EK, Kim TH, et al. Effects of alpha-lipoic acid on the plasma levels of asymmetric dimethylarginine in diabetic end-stage renal disease patients on hemodialysis: A pilot study. Am J Nephrol. 2007;27(1):70–74.

8. Khabbazi T, Mahdavi R, Safa J, Pour-Abdollahi P. Effects of alpha-lipoic acid supplementation on inflammation, oxidative stress, and serum lipid profile levels in patients with end-stage renal disease on hemodialysis. J Ren Nutr. 2012;22(2):244–250.

9. El-Nakib GA, Mostafa TM, Abbas TM, El-Shishtawy MM, Mabrouk MM, Sobh MA. Role of alpha-lipoic acid in the management of anemia in patients with chronic renal failure undergoing hemodialysis. Int J Nephrol Renovasc Dis. 2013;27(6):161–168.

10. Ramos LF, Kane J, McMonagle E, et al. Effects of combination tocopherols and alpha-lipoic acid therapy on oxidative stress and inflammatory biomarkers in chronic kidney disease. J Ren Nutr. 2011;21(3):211–218.

11. Ahmadi A, Mazooji N, Roozbeh J, Mazloom Z, Hasanzade J. Effect of alpha-lipoic acid and vitamin E supplementation on oxidative stress, inflammation, and malnutrition in hemodialysis patients. Iran J Kidney Dis. 2013;7(6):461–467.

12. Himmelfarb J, Ikizler TA, Ellis C, et al. Provision of antioxidant therapy in hemodialysis (PATH): A randomized clinical trial. J Am Soc Nephrol. 2014;25(3):623–633.

13. Yu X, Liu H, Zou J, Zhu J, Xu X, Ding X. Oxidative stress in 5/6 nephrectomized rat model: Effect of alpha-lipoic acid. Ren Fail. 2012;34(7):907–914.

14. Ergür BU, Çiakler Mıcı: S, Yılmaz O, Akokay P. The effects of α-lipoic acid on aortic injury and hypertension in the rat remnant kidney (5/6 nephrectomy) model. Anatol J Cardiol. 2015;15(6):443–449.

15. Pradhan S, Mandal S, Roy S, Mandal A, Das K, Nandi DK. Attenuation of uremia by orally feeding alpha-lipoic acid on acetaminophen induced uremic rats. Saudi Pharm J. 2013;21(2):187–192.

16. Shay KP, Moreau RF, Smith EJ, Smith AR, Hagen TM. Alpha-lipoic acid as a dietary supplement: Molecular mechanisms and therapeutic potential. Biochim Biophys Acta. 2009;1790(10):1149–1160.

17. Teichert J, Tuemmers T, Achenbach H, et al. Pharmacokinetics of alpha-lipoic acid in subjects with severe kidney damage and end-stage renal disease. J Clin Pharmacol. 2005;45(3):313–328.

18. Cao Z, Tsang M, Zhao H, Li Y. Induction of endogenous antioxidants and phase 2 enzymes by alpha-lipoic acid in rat cardiac H9C2 cells: Protection against oxidative injury. Biochem Biophys Res Commun. 2003;310(3):979–985.

19. Suh JH, Shenvi SV, Dixon BM, et al. Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipic acid. Proc Natl Acad Sci USA. 2004;101(10):3381–3386.

20. Rodrigues SD, Batista GB, Ingberman M, Pecoiots-Filho R, Nakao LS. Plasma cysteine/cystine reduction potential correlates with plasma creatinine levels in chronic kidney disease. Blood Purif. 2012;34(3–4):231–237.

21. Kim HJ, Vaziri ND. Contribution of impaired Nrf2-Keap1 pathway to oxidative stress and inflammation in chronic renal failure. Am J Physiol Renal Physiol. 2010;298(3):F662–F671.

22. Aminzadeh MA, Reisman SA, Vaziri ND, et al. The synthetic triterpenoid RTA dh404 (CDDO-dhTFEA) restores endothelial function impaired by reduced Nrf2 activity in chronic kidney disease. Redox Biol. 2013;1:527–531.

23. Sviglerová J, Kuncová J, Nalos L, Tonar Z, Rajdl D, Stengl M. Cardiovascular parameters in rat model of chronic renal failure induced by subtotal nephrectomy. Physiol Res. 2010;59 Suppl 1:S51–S58.

24. Quijano C, Castro L, Peluffo G, Valez V, Radi R. Enhanced mitochondrial superoxide in hyperglycemic endothelial cells: Direct measurements and formation of hydrogen peroxide and peroxy nitrite. Am J Physiol Heart Circ Physiol. 2007;293(6):H3404–H3414.

25. Ungvari Z, Bailey-Downs L, Gautam T, et al. Adaptive induction of NF-E2-related factor-2-driven antioxidant genes in endothelial cells in response to hyperglycemia. Am J Physiol Heart Circ Physiol. 2011;300(4):H1133–H1140.

26. Yoh K, Hirayama A, Ishizaki K, et al. Hyperglycemia induces oxidative and nitrosative stress and increases renal functional impairment in Nrf2-deficient mice. Genes Cells. 2008;13(11):1159–1170.

27. Stockler-Pinto MB, Fouque D, Soulaige CO, Croze M, Mafra D. Indoxyl sulfate and p-cresyl sulfate in chronic kidney disease. Could these toxins modulate the antioxidant Nrf2-Keap1 pathway? J Ren Nutr. 2014;24(5):286–291.

28. Ruiz S, Pergola PE, Zager RA, Vaziri ND. Targeting the transcription factor Nrf2 to ameliorate oxidative stress and inflammation in chronic kidney disease. Kidney Int. 2013;83(6):1029–1041.

29. Biewenga GP, Haenen GR, Bast A. The pharmacology of the antioxidant lipoic acid. Gen Pharmacol. 1997;29(3):315–331.

30. Bhatty F, Mankhey RW, Asico L, Quinn MT, Welch WJ, Maric C. Mechanisms of antioxidant and pro-oxidant effects of α-lipoic acid in the diabetic and nondiabetic kidney. Kidney Int. 2005;67:1371–1380.

31. Yang H-C, Zuo Y, Fogo AB. Models of chronic kidney disease. Drug Discov Today Dis Models. 2010;7(1–2):13–19.