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N-acetylcysteine attenuates renal alterations induced by senescence in the rat

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ABSTRACT

The aim of this study was to evaluate the effects of N-acetylcysteine (NAC) on renal function, as well as on sodium and water transporters, in the kidneys of aged rats. Normal, 8-month-old male Wistar rats were treated ($n=6$) or not ($n=6$) with NAC (600 mg/L in drinking water) and followed for 16 months. At the end of the follow-up period, we determined inulin clearance, serum thiobarbituric acid reactive substances (TBARS), serum cholesterol, and urinary phosphate excretion. In addition, we performed immunohistochemical staining for p53 and for ED-1-positive cells (macrophages/monocytes), together with Western blotting of kidney tissue for NKCC2, aquaporin 2 (AQP2), urea transporter A1 (UT-A1) and Klotho protein. At baseline, the two groups were similar in terms of creatinine clearance, proteinuria, cholesterol, and TBARS. At the end of the follow-up period, NAC-treated rats presented greater inulin clearance and reduced proteinuria, as well as lower serum cholesterol, serum TBARS, and urinary phosphate excretion, in comparison with untreated rats. In addition, NAC-treated rats showed upregulated expression of NKCC2, AQP2, and UT-A1; elevated Klotho protein expression, low p53 expression, and few ED-1 positive cells. In conclusion, we attribute these beneficial effects of NAC (the significant improvements in inulin clearance and in the expression of NKCC2, AQP2, and UT-A1) to its ability to decrease oxidative stress, inhibit p53 expression, minimize kidney inflammation, and stimulate Klotho expression.

1. Introduction

Renal aging is associated with alterations in renal morphology and a decline in renal function (Choudhury and Levi, 2011; Zhou et al., 2008). Experimental studies in rats have shown that there is a decline in the glomerular filtration rate, as well as increased proteinuria, with aging. The physiological changes that occur during the normal aging process include a decrease in the maximum ability to concentrate and dilute urine, due to reduced expression of sodium transporters, including NKCC2, in the thick ascending limb of the loop of Henle, as well as reduced expression of the water channel aquaporin 2 (AQP2) and urea transporter A1 (UT-A1) in the inner medullary collecting duct (Sands, 2009).

Numerous mechanisms of injury contribute to age-related organ dysfunction, including increased concentrations of oxygen free radicals and fibrogenic mediators; mitochondrial injury; and imbalances between cell repair/proliferation and cell death. Although various mechanisms are involved in aging, cellular senescence is a key element that is closely linked to aging-related diseases. Oxidative stress can lead to stress-induced premature senescence (SIPS) by increasing apoptosis or DNA mutations (Yang and Fogo, 2010). Physiologic stressors can also cause SIPS, which can be driven by the signaling pathway of the cytoplasmic protein p53, a regulator of apoptosis.

A functional decline in the repair or regeneration of cells is a hallmark of aging and can be aggravated by excessive apoptosis. Despite the fact that cellular senescence is a biological process, senescent endothelial cells are more susceptible to apoptosis (Anderson et al., 2009). The apoptotic p53 signaling pathway has been associated with the perturbation of mitochondrial membrane potential, and the release of apoptogenic factors from the mitochondrial intermembrane space into the cytoplasm triggers a cascade of events leading to caspase activation and cell death (Danial and Korsmeyer, 2004). In addition, p53 expression has been correlated with interstitial fibrosis and tubular atrophy in aged kidneys.

The recently discovered Klotho gene is an aging-suppressor gene, identified in mice, that has been shown to accelerate aging when disrupted and to extend life when overexpressed. The Klotho gene is expressed in specific tissues, such as those of the distal convoluted tubules in the kidney and the choroid plexus in the brain (Kuro-o et al., 1997). The Klotho gene encodes a novel protein that appears to function outside of the cell (Takahashi et al., 2000). It has been shown that Klotho-deficient mice can develop age-related disorders prematurely, such disorders including stroke, arteriosclerosis, osteoporosis, hypogonadism, premature thymic involution, ectopic calcification, decreased bone mineral density, skin atrophy, pulmonary

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emphysema, cognitive impairment, and hearing loss (Kuro-o et al., 1997; Masuda et al., 2005).

There is some evidence that elevated Klotho expression can minimize oxidative stress. Yamamoto et al. (2005) demonstrated that Klotho overexpression in transgenic mice increases the mitochondrial expression of manganese-superoxide dismutase in muscles and reduced oxidative stress.

Factors that could directly retard cellular senescence could be useful in slowing the progression of certain diseases or of the aging process itself. Antioxidants can reduce the production of intracellular oxygen free radicals and could therefore also delay cellular senescence (Yang and Fogo, 2010). N-acetylcysteine (NAC) has been shown to be a potent antioxidant. In a model of indoxyl sulfate-induced oxidative stress (Shimizu et al., 2010), NAC was found to attenuate cellular senescence by inhibiting expression of p53.

The aims of this study were to identify the main alterations in aging kidneys and to evaluate the effect that long-term treatment with NAC has on renal function, as well as on the protein expression of Klotho and p53.

2. Methods

2.1. Animals and experimental protocol

Eight-month-old male Wistar rats were obtained from the Central Animal Facility of the University of São Paulo School of Medicine. As a control for the Klotho protein expression study only, an additional group of 3-month-old male Wistar rats was later obtained from the facility. The experimental protocol was approved by Animal Research Ethics Committee of the School of Medicine.

2.2. Metabolic cage studies at baseline

From 8 months of age, rats were housed in individual cages and maintained on a 12/12-h light/dark cycle, with ad libitum access to standard rat chow and either pure drinking water (untreated group) or drinking water supplemented with NAC (600 mg/L; NAC-treated group). For the first 24 h, the rats were housed in metabolic cages and urine samples were collected. Each 24-h urine sample was centrifuged in aliquots to remove suspended material, and the supernatants were analyzed. A 0.5-ml blood sample was collected from the tail vein of each animal. Creatinine, proteinuria, and thiobarbituric acid reactive substances (TBARS) were measured in blood samples, urine samples, or both.

2.3. Post-treatment metabolic cage studies

For 16 months (up to the age of 24 months), the rats were treated or not with NAC, as described above, and monitored. At the end of this follow-up period, the animals in both groups were again housed in metabolic cages. We collected 24-h urine and blood samples for the quantification of proteinuria and the determination of TBARS levels, as well as for that of urinary phosphate excretion.

2.4. Post-treatment clearance studies

At the end of the follow-up period, we conducted inulin clearance studies. Each designated animal was first anesthetized with an intraperitoneal injection of sodium thiopental (50 mg/kg body weight). The trachea was cannulated with a polyethylene (PE)-240 catheter, and spontaneous breathing was maintained. To monitor mean arterial pressure and allow blood sampling, the right carotid artery was catheterized with a PE-60 catheter. For infusion of inulin and fluids, the left jugular vein was cannulated, also with a PE-60 catheter. In order to collect urine samples, a suprapubic incision was made, and the urinary bladder was cannulated with a PE-240 catheter. Following the surgical procedure, a loading dose of inulin (100 mg/kg body weight diluted in 0.9% saline) was administered through the jugular vein. A constant infusion of inulin (10 mg/kg body weight in 0.9% saline) was then started and continued at 0.04 ml/min throughout the experiment. A total of three urine samples were collected at 30-min intervals. Blood samples were obtained at the beginning and end of the experiment. At the end of the clearance study, the kidneys were flushed with saline and fixed with methacarn for immunohistochemical analysis. We used the anthrone method in order to quantify inulin in blood and urine samples.

Mean arterial pressure was assessed with a Biopac acquisition system (Biopac Systems Inc., USA). At the end of the inulin clearance study, the renal artery was carefully dissected and blood flow was measured by an electromagnetic flow probe (TS 420; Transonic Systems Inc., USA), placed around the artery.

2.5. Urinary protein excretion and serum levels of TBARS

In 24-h urine samples collected during the post-treatment metabolic cage studies, we determined protein excretion and levels of TBARS. Urinary protein excretion was determined using a Sensiprot Kit (Labtest, Brazil).

Serum levels of thiobarbituric acid reactive substances (TBARS), which are markers of lipid peroxidation, were determined using thiobarbituric acid assay. In brief, a 0.2-ml serum sample was diluted in 0.8 ml of distilled water. Immediately thereafter, 1 ml of 17.5% trichloroacetic acid was added. Following the addition of 1 ml of 0.6% thiobarbituric acid, pH 2, the sample was placed in a boiling water bath for 15 min, after which it was allowed to cool. Subsequently, 1 ml of 70% trichloroacetic acid was added, and the mixture was incubated for 20 min. The sample was then centrifuged for 15 min at 2000 rpm. The optical density of the supernatant was read at 534 nm against a reagent blank using a spectrophotometer. The quantity of TBARS was calculated using a molar extinction coefficient of $1.56\times10^5\ M^{-1}\ cm^{-1}$. Serum levels of TBARS are expressed as nmol/ml (Shimizu et al., 2008).

2.6. Biochemical parameters

The colorimetric method (Labtest, Brazil) was used in order to quantify proteinuria and urinary levels of creatinine. Phosphate was determined only in urine. Serum total cholesterol was measured with the specified reagents (Labtest). Serum aldosterone was measured with a commercial kit (Coat-a-Count Immunoassay kit; Diagnostic Products Corp, USA).

2.7. Immunochemistry for ED-1 and p53

At the end of the follow-up period, tissue samples for immunohistochemical analysis were obtained from rats in both groups (untreated and NAC-treated). The primary antibodies were a monoclonal antibody to a cytoplasmic antigen present in macrophages and monocytes (ED-1; Serotec, UK); and a polyclonal antibody (anti-p53; Santa Cruz Biotechnology, USA). The sections were incubated overnight with the respective antibodies. The reaction product was detected with an avidin–biotin peroxidase complex (Vector Laboratories, USA). The color reaction was developed with 3,3-diaminobenzidine (Sigma Chemical Company, USA). The material was then counterstained (with methyl green), dehydrated, and mounted. Nonspecific protein binding was blocked by incubation with 20% goat serum in phosphate-buffered saline (PBS) for 20 min. Replacing the primary antibody with mouse or rabbit IgG, at an equivalent concentration, created a negative control. The sections were examined under light microscopy at a magnification of 400×. The number of ED-1-positive cells in each section was calculated by counting the number of positive cells in 30 sequential (0.087 mm^2) grid fields per renal cortex. For the evaluation of immunoperoxidase staining for p53,