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N-acetylcysteine as a potential strategy to attenuate the oxidative stress induced by uremic serum in the vascular system

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ABSTRACT

Aims: Chronic kidney disease (CKD) progression is accompanied by systemic oxidative stress, which contributes to an increase in the risk of cardiovascular diseases (CVDs). N-acetylcysteine (NAC) is among the most studied antioxidants, but its therapeutic benefits in CKD-associated CVDs remain controversial. Here, we investigated whether NAC could inhibit the oxidative stress induced by uremia *in vitro* and *in vivo*.
Main methods: Endothelial and smooth muscle cells were challenged with human uremic or non-uremic sera, and the effects of a pre-treatment with 2 mM NAC were evaluated. Reactive oxygen species (ROS) production, protein oxidation and total glutathione/glutathione disulfide (tGSH/GSSG) ratios were measured. Five-sixths nephrectomized or sham-operated rats were orally treated (in the drinking water) with 60 mg/kg/day NAC or not treated for 53 days. Plasma cysteine/cystine reduction potential $E_{h(Cyss/2Cys)}$ was determined as a novel marker of the systemic oxidative stress.
Key findings: NAC inhibited all the determined oxidative stress parameters, likely by increasing the tGSH/GSSG ratio, in both cell lines exposed to uremic serum. Orally administered NAC attenuated the systemic oxidative stress in uremic rats.
Significance: The present results indicate that NAC, by preventing GSH depletion in vascular cells exposed to uremic serum and by attenuating the systemic oxidative stress during CKD progression, emerges as a potential strategy to prevent the oxidative stress induced by uremic toxicity in the vascular system.

Introduction

Chronic kidney disease (CKD) represents a relevant risk factor for the development of cardiovascular diseases (CVDs) [1]. CKD progression is associated with systemic chronic inflammation [2] and oxidative stress [3–5], which, in combination, are major contributors to the acceleration of CVDs [2,6]. Indeed, the severity of CKD is associated with increased levels of several oxidative stress biomarkers in the blood [6–11].

The participation of uremic toxins, such as advanced glycation end products and indoxyl sulfate, in mechanisms associated with vascular injury has been demonstrated [12]. Smooth muscle cells are sensitive to the toxic effects of uremic serum or toxins, which increase the proliferation rate [13] and induce cellular senescence [14] and the expression of calcification proteins [15,16], leading to arterial degeneration [17,18]. Endothelial cells cultivated in the presence of uremic serum or uremic toxins exhibit increased proliferation [19] and apoptosis [20] rates, increased expression of pro-inflammatory genes [21] and inflammatory proteins [22] and increased reactive oxygen species (ROS) production [23,24]. The inhibition by antioxidants of several of these processes [16,18,23,25] demonstrates the participation of ROS in such events and, most importantly, supports the idea that uremia triggers excessive ROS production.

We have shown that systemic oxidative stress, as determined either by plasma total thiol levels and total protein carbonylation levels [26] or by the cysteine/cystine reduction potential, $E_{h(Cyss/2Cys)}$ [27], increases with CKD progression and decreases after kidney transplant [26], indicating that oxidative stress results from uremia. Indeed, the reduction of serum concentrations of indole-derived toxins with the oral adsorbent AST-120 has been shown to retard CKD progression [28], partially through the inhibition of oxidative stress [29]. In this context, antioxidant therapy should be considered as a putative strategy to attenuate the oxidative stress associated with CKD progression.

Several studies and clinical trials have been performed to assess the effects of antioxidants in CKD patients. A recent systematic review [30] of these trials indicated that orally ingested N-acetylcysteine (NAC) has

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promising effects, decreasing the levels of oxidative stress markers and cardiovascular events [31]. In the 5/6 nephrectomy model, NAC also demonstrated renoprotective effects by attenuating proteinuria and renal tissue damage and improving the glomerular filtration rate [32]. Orally ingested NAC is rapidly absorbed by the gastrointestinal tract because of its neutral charge at the acidic pH of the stomach [33,34]. Most of the NAC undergoes first pass metabolism, intracellular deacetylation and promotes an increased hepatic content of cysteine [33], which acts as an intracellular GSH precursor, at least when GSH demand is increased [35]. However, it was also described that after a single dose, a minor fraction of free NAC reaches the systemic circulation and reduces plasma cystine in healthy volunteers, thereby increasing the concentrations of plasma cysteine in the first hour [36].

In this study, we show that NAC can be considered as a potential strategy to attenuate the oxidative stress induced by uremia in the vascular system. We demonstrate that NAC pre-treatment increases the resistance of smooth muscle and endothelial cells to uremic toxicity and that NAC administration inhibits the systemic oxidative stress, as determined by the plasma $E_{h(Cyss/2Cys)}$, which is a novel biomarker of oxidative stress, in the 5/6 nephrectomy model of CKD in rats.

Material and methods

Human uremic and non-uremic serum

Uremic sera were collected at the hemodialysis (HD) unit of the Cajuru University Hospital. Exclusion criteria included an age of <18 years, history of diabetes mellitus, active infection, autoimmune diseases and malignancy and were verified by detailed analysis of medical records and patient interview. The pool was prepared from the plasma of 29 fasted patients (21 males and 8 females) with end-stage renal disease on maintenance HD for a mean period of 30 months. The mean age was 49 ± 15 years. Causes of renal failure included the following: chronic glomerulonephritis (44.8%), nephrosclerosis (24.1%), polycystic kidney disease (6.9%), obstructive pyelonephritis (6.9%), obstructive uropathy (6.9%), congenital stenosis (3.4%), transplant rejection (3.4%) and hypertension (3.4%). A total of 24 mL of peripheral blood was collected from each patient at three times, before the first HD session of the week. Non-uremic (control) sera were collected in a private laboratory. Exclusion criteria were identical to those used for the patients, in addition to the history of kidney disease. The pool was prepared from the plasma of 30 fasted subjects (22 males and 8 females) with a mean age of 44 ± 11 years. Informed consent to blood collection and serum utilization was obtained from all participants. Uremic or non-uremic sera were pooled and heat inactivated (56 °C, 30 min), as routinely made with fetal calf serum. This protocol was approved by the Ethics Committee of the UFPR, protocol number 02/08.

Sera pool characterization

The uremic and non-uremic sera pools were analyzed at Scribner Laboratory (Curitiba, Brazil) to characterize their uremic, inflammatory and redox state (Table 1). Increased levels of urea, creatinine, uric acid and phosphorus were observed in the uremic pool, reflecting renal failure in the HD patients. The slightly increased level of glucose in the uremic pool is due to a nonfasting condition of some patients attending the HD sessions. Levels of calcium, albumin and cholesterol are compatible to what would be expected from a clinically stable CKD-5 patient treated with HD. The increased C-reactive protein (CRP) levels are in agreement with an inflammatory condition in the uremic pool. Decreased total thiols and increased carbonylated proteins, which were determined as previously described [27], in the uremic pool indicate an oxidative status associated with uremia. Collectively, these results confirm the uremic status and indicate an oxidative milieu in the blood of the HD patients.

Table 1
Biochemical characterization of the non-uremic and uremic sera pools.

Parameters	Non-uremic pool (n = 30)	Uremic pool (n = 29)
Urea (mg/dL)	30.1	151.4
Creatinine (mg/dL)	0.77	8.83
Uric acid (mg/dL)	5.80	7.40
Glucose (mg/dL)	83	115
Calcium (mg/dL)	11	10
Phosphorus (mg/dL)	4.2	6.5
Albumin (mg/dL)	4.7	5.1
Total cholesterol (mg/dL)	215	164
CRP (mg/L)	0.356	1.105
Total thiols (μM)	662	268
Carbonylated proteins (nmol/mg albumin)	0.77	0.97

Cell culture

Rabbit aortic smooth muscle (RASM) and endothelial (RAEC) cells were kindly provided by Dr. Helena B. Nader (UNIFESP, São Paulo). The cells were routinely cultured in an F12 medium (Cultilab, Campinas, Brazil) containing penicillin and streptomycin (Life Technologies) and 10% fetal calf serum (Cultilab).

Cell treatment with human sera pools

Both RASM and RAEC were plated (seeding confluence of approximately 3×10^4 cells/cm^2) and incubated over the day until complete adhesion. Subsequently, the cells were pre-treated or not treated with 2 mM NAC (Sigma-Aldrich, São Paulo, Brazil) in a fresh complete medium for 24 h. This concentration is the approximate NAC concentration reported in human serum following an oral administration of 400–600 mg NAC [37]. To perform a smooth transition between the fetal calf serum and the human serum, the concentration of the fetal calf serum was decreased to 0.5% for 16 h, after which cells were treated with an F12 medium containing 10% human uremic or non-uremic sera pools for 3 or 24 h. Under this condition, RASM and RAEC were viable for at least 24 h in the presence of human sera, without any visible morphological alteration.

Intracellular ROS detection with fluorescent probes

Cells were exposed to human sera pools in 96-well microplates as previously described. The 2′, 7′-dichlorofluorescin diacetate (DCFH2-DA) assay was verified by treating both cell lines with 0.5 mM H_2O_2 (Merck, Darmstadt, Germany) for 4 h in the complete culture medium. In the final 30 min of the incubation time, 20 μM $DCFH_2$-DA (Sigma-Aldrich) was added to the incubation medium. Intracellular DCF fluorescence was determined using a microplate reader (Tecan Infinite M200) set at λ_{exc} = 485 nm and λ_{em} = 530 nm [38]. The dihydroethidium (DHE) assay was verified by treating cells with 30 mM glucose [39] for 4 h in a routine medium. In the final 30 min of the incubation time, 10 μM DHE (Sigma-Aldrich) was added to the incubation medium. After washing with PBS, intracellular 2-OH-Et^+ (which is the product of oxidation by superoxide) fluorescence was specifically detected at λ_{exc} = 396 nm and λ_{em} = 580 nm [40]. In some assays, 20 μM apocynin was added to the cell culture for 15 min at the end of the treatment. After this period, cells were washed with PBS and incubated with DHE. Mitochondrial superoxide was measured using MitoSOX Red® (Invitrogen, Carlsbad, CA). This assay was verified by treating cells with 1 μM rotenone (Sigma-Aldrich) [41] for 4 h in a routine medium. In the final 15 min of the incubation time, 1 μM MitoSOX Red® [39] was added to the incubation medium. After washing with PBS, intracellular MitoOH-Et^+ fluorescence was measured at λ_{exc} = 396 nm and λ_{em} = 580 nm [40].