Guanidino compounds inhibit nitric oxide release in J774A.1 macrophages

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Abstract

Chronic kidney disease (CKD) has been usually associated with accumulation of some nitrogen compounds deriving from protein catabolism like creatine (CRT), creatinine (CRTN), guanidine (GN) and methylguanidine (MG) proposed as responsible of some manifestations of uremic syndrome. Changes in monocyte functions have been recognised as one of the most important key factor responsible for the immunological disorders associated with uremia and it has been demonstrated that high blood concentrations of nitrogen compounds, as MG, could be responsible of immunodisfunctions associated to uremic syndrome. Nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) is a crucial step both in the activation of immunoreponsive cells and in the mechanism of citotoxicity NO mediated. The aim of this study is to evaluate if some uremic toxins like CRT, CRTN, GN, MG could interfere in macrophagic immunoresponse modulating iNOS activity. Our results demonstrated that GN and MG exerted the stronger effect in inhibiting NO release; this effect was reverted by a L-ARG supplementation.

Moreover macrophage co-exposure to GN and MG further enhanced the inhibitory effect on iNOS activity and expression. Our results demonstrated that, among tested compounds, GN and MG significantly affected iNOS activity and expression.

KEY WORDS: GUANIDINO COMPOUNDS, NITRIC OXIDE, MACROPHAGES. LPS
Introduction

Chronic kidney disease (CKD) progression leads to the dysfunction of multiple organs with clinical features constituting the uremic syndrome that, despite pharmacological and dialysis treatment remains associated with multiple complications [1-3]. This syndrome is attributed to the progressive retention of a large number of compounds which, under normal conditions, are excreted by healthy kidneys. These compounds are called uremic toxins because of their harmful effects in various physiological functions in CKD patients [1,2]. The development of CKD is associated with a significant increase in all-cause mortality [4,5]. The main factors responsible for the increased risk of morbidity and mortality in patients with CKD are cardiovascular dysfunctions and infections, both linked to changes in immune responses [6,7]. In uraemia, a diminished immune defence contributes to the high prevalence of infections, whereas pre-activation and priming of immune cells leads to inflammation and consequently also to cardiovascular complications.

There are multiple causes for the immune reaction that characterizes dysfunction related to uraemia and dialysis. They may include accumulation of uremic toxins, strongly present in CKD and only partially corrected by dialysis, dialysis-related factors such as interactions between blood and dialyzer membranes, endotoxin presence in water, access-related infections, and peritoneal dialysis solutions with high glucose concentration, low pH, and the presence of glucose degradation products represent chronic stimuli of the inflammatory response [8]. Oxidative stress and inflammation are crucial for defence against infections, but they initiate a number of deleterious effects if not properly regulated [9]. Oxidative stress increases in parallel with the progression of CKD and correlates with the level of renal functions [10]. Thus the pharmacological manipulation of inflammatory stimulus and the control of oxidative stressors are of particular importance in the uremic syndrome. Uremic toxins are responsible for many uraemia-associated dysfunctions and among them there is an altered immune response [11-15]. CKD has been usually associated with a significant reduction in serum L-arginine (L-ARG) levels, reduced nitric oxide (NO) production and with accumulation of some nitrogen compounds deriving from protein catabolism like creatine (CRT), creatinine (CRTN), guanidine (GND) and methylguanidine (MG) [16,17]. It has been proposed that nitrogen compounds accumulation could be responsible of some manifestations of uremic syndrome [18-20] modifying key biologic functions [21]. We have previously reported the pro-apoptotic effect of MG on hydrogen peroxide-treated astrocytes [22] and its ability to inhibit, both in vitro and in vivo, the inflammatory response [12-15]. In particular we have previously demonstrated the capability of MG to inhibit NO release and iNOS expression. The aim of this study was to evaluate if uremic toxins like CRT, CRTN, GND could interfere in macrophage immunoresponse modulating iNOS activity in vitro on J774A.1 murine/macrophage cell line stimulated with Lipopolysaccharide from E. coli (LPS) in absence or presence of L-ARG supplementation and if their effects were stranger respect to MG.

Methods

Reagents

Unless stated otherwise, all reagents and compounds were purchased from Sigma Chemicals Company (Sigma, Milan, Italy).

Cell culture

J774A.1 murine monocyte macrophage cell line (American Type Culture Collection, Rockville, MD), was grown adherent to Petri dishes with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS), 25 mM HEPES, 2 mM glutamine, 100 μ/mL penicillin and 100 mg/mL streptomycin at 37°C in a 5% CO₂ atmosphere.

Antiproliferative activity

J774A.1 cells (5x10⁴/well) were plated on 96-well plates and allowed to adhere for 4 h. Thereafter, the medium was replaced with fresh medium alone
or containing serial dilutions of guanidine compounds (0.01-1 mM), and incubation was performed for 24, 48 and 72 h. Cell viability was assessed using the MTT assay as previously reported [23,24]. Briefly, 25 µL of MTT (5 mg/mL) were added and cells were incubated for an additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilised with 100 µL of a solution containing 50% (v:v) N,N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340-DASIT) equipped with a 620 nm filter. Macrophage viability in response to treatment with tested guanidine compound was calculated as:

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\% \text{ dead cells} = 100 \times \left(\frac{\text{OD treated}}{\text{OD control}}\right) \times 100
\]

Nitrite determination

J774A.1 macrophages were seeded in 24 well plates (2.5 x 10^4/well) and allowed to adhere for 4h. Thereafter LPS (1 µg/ml) was added for 24 h in order to induce iNOS expression. The medium was then replaced with fresh medium and cells were treated with guanidino compounds or L-NAME, a well known inhibitor of NO release used as a reference drug (0.1-10 mM), and LPS for further 24 h. Some experiments were made in normal medium, others in presence of an L-ARG supplementation (10mM).

NO generation was measured as nitrite content (NO_2^-, µM), index of NO released by cells in the culture medium 24 h after LPS stimulation, as previously reported [25]. NO_2^- amounts were measured by Griess reaction. Briefly, 100 µL of cell culture medium were mixed with 100 µL of Griess reagent – equal volumes of 1% (w:v) sulphanilamide in 5% (v:v) phosphoric acid and 0.1% (w:v) naphthylenediamine-HCl and incubated at room temperature for 10 min, and then the absorbance was measured at 550 nm in a microplate reader Titertek (Dasit, Cornaredo, Milan, Italy). The amount of NO_2^-, as µM concentration, in the samples was calculated from a sodium nitrite standard curve.

NO determination and Western blot analysis for iNOS expression in LPS treated J774A.1 co-treated with MG and GN

J774A.1 macrophages were seeded in P60 plates (1.8 x 10^6/P60) and allowed to adhere for 4h. Thereafter, the medium was replaced with fresh medium and cells were treated with MG or GN (0.01-1 mM) alone or in combination for 1 h and then co-exposed to LPS (1 µg/ml) for further 24 h.

NO generation was measured as previously indicated. The expression of iNOS protein was assessed by Western blot analysis. After nitrite determination cells were scraped off, washed with ice-cold phosphate-buffered saline (PBS), and centrifuged at 5,000 g for 10 min at 4°C. The cell pellet was lysed in a buffer containing 20 mM Tris hydrogen chloride (HCl; pH 7.5), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 mM sodium fluoride, 150 mM sodium chloride, 10 mg/ml trypsin inhibitor, and 1% Tween-20.

Protein concentration was estimated by the BioRad protein assay using bovine serum albumin as standard. Equal amounts of protein (50 µg) were dissolved in Laemmli’s sample buffer, boiled, and run on a SDS polyacrylamide gel electrophoresis (SDS-PAGE) minigel (8% polyacrylamide) and then transferred to hybond polyvinylidene difluoride membrane for 40 min. at 5 mA cm^-2 into 0.45 µm. Membranes were blocked for 40 min in PBS and 5% (w/v) nonfat milk and subsequently probed overnight at 4°C with mouse monoclonal anti-iNOS (BD Laboratories), or anti-tubulin (Santa Cruz Biotechnologies) in PBS, 5% w/v non fat milk, and 0.1% Tween-20.

Blots were then incubated with horseradish peroxidase conjugated goat antimouse immunoglobulin (Ig)G (1:5,000) for 1h at room temperature. Immunoreactive bands were visualized using electro-chemiluminescence assay (ECL) detection system according to the manufacturer’s instructions and exposed to Kodak X-Omat film. The protein bands of iNOS and COX-2 on XOMAT films were quantified by scanning densitometry (Imaging Densitometer GS-700 BIO-RAD U.S.A.).
Data are normalized with tubulin expression, used as reference protein, and expressed as arbitrary densitometric units as previously reported [26,27].

Data analysis

Data are expressed as mean±standard error mean (s.e.m.) values of at least three independent experiments. Statistical analysis was performed by analysis of variance test, and multiple comparisons were made by Bonferroni’s test. A P-value less than 0.05 was considered significant.

Results

Guanidino compounds don’t affect J774A.1 macrophage viability

To elucidate the influence of tested guanidine compounds on cell viability of J774A.1 macrophages under our experimental conditions, and also for longer period, cells were treated with MG, GN, CRT and CRTN (0.01-1 mM) for 24, 48 and 72 h. Our data indicated that J774A.1 macrophages cell viability was not significantly affected by guanidine compound treatment (% viability >92% for the highest tested concentrations of both compounds).

Guanidino compounds reduced NO release and acts as a false substrate for iNOS

To assess if tested guanidino compounds influence NO production we measured nitrite release, a stable end-product of NO, in cellular medium of the murine macrophage cell line J774A.1 stimulated with LPS (1μg/ml). In normal medium NO released by J774A.1 macrophages treated with LPS alone was 7.8±2.4 mM NO$_2^-$.

Treatment with MG (0.1, 1 and 10 mM) or GND (0.1, 1 and 10 mM) significantly inhibited NO release by 93.3±3.4%, 43.3±5.4% and 9.64±14.0% for MG (P<0.001; Figure 1) and 80.8±9.2%, 34.1±9.7% and 10.2±14.0% for GND (P<0.001; Figure 1) respectively. The addition of CRT or CRTN did not produce any significant variation on NO release (Figure 1).

Moreover MG and GN inhibitory effects were reverted by adding, to the culture medium, L-ARG (10mM) supplementation. These results show that both MG and GND are able to inhibit, as false substrate, NO production (Figure 2).

MG and GN synergistically act in inhibiting NO and iNOS expression in LPS-stimulated macrophage

Our results showed that, among tested guanidine compounds, GN and MG exerted the stronger effect in inhibiting NO release. Thus, as possible in vitro, in order to simulate the uremic condition where more
than one toxin is present at the same time, we co-exposed J774A.1 to GN and MG simultaneously [15]. Interestingly, when J774A.1 macrophages were co-exposed to GN and MG, NO release was significantly reduced respect to GN and MG alone (Figure 3 A,B).

Under the same experimental conditions we also observed a significant induction in iNOS expression in macrophages treated with LPS alone (P<0.001 vs control). When GN or MG (0.1-10 mM) were added to J774A.1 macrophages, 1h before and simultaneously with LPS, a significant and concentration-dependent inhibition in iNOS (Figure 4) expression was observed at the highest concentrations tested.

Under the same experimental conditions when GN and MG were co-added to J774A.1 macrophages a further significant inhibition in iNOS expression were observed also at the lower concentration (Figure 5).
Fig. 5 Effect of a synergistic action of MG (1 mM panel A and 1.0 mM panel B) and GN (0.1, 1.0) INOS expression in J774A.1 macrophages co-incubated for 24 h with LPS (1 μg / mL). *** denotes P<0.001 vs LPS; ** denotes P<0.01 vs LPS; * denotes P<0.05.

Discussion

Uraemia-related immune dysfunction result from a complex interaction between the innate and adaptive immune system response, in which immune activation (hypercytokinemia and acute-phase response) and immune suppression (impairment of response to infections and poor development of adaptive immunity) coexist. On the one hand, as a consequence of tissue damage, the innate immune system is triggered, and although inflammation in principle is an essential response to eliminate aggressors and it can be considered a double-edged sword when the initial reaction is not limited [8]. During inflammation, vasodilatation, vascular permeability, movement of inflammatory cells, and activation of cells of the immune system are increased. In addition, acute-phase reactants can be produced, as well as complement components, fever, and activation of systemic immunity. Therefore, to avoid tissue damage, inflammatory responses must be well organized and controlled by inflammatory mediators. Interestingly, the main causes of death in patients with CKD are related to infectious and cardiovascular diseases, both being pathologic processes closely linked to immune function [4]. Therefore, accelerated tissue damage (as a consequence of chronic inflammation) and increased rate of sepsis (because of a poorly orchestrated immune response) represent the most important targets for interventions aiming to reduce mortality in CKD patients. Sepsis results from the generalized activation of inflammatory cascades following invasion of the blood stream by bacteria, viruses or parasites, with the systemic release of various toxic products. These products include bacterial cell-wall components, such as endotoxin, as LPS, membrane component of gram-negative bacteria, lipoteichoic acid forms gram-positive organisms and various endotoxins [28]. Sepsis induced by gram-negative is accompanied by systemic shock and the initial symptoms of sepsis encompass those usually associated with acute inflammation and, finally multiple organ dysfunction syndrome (MODS) and death [29]. Many of the pathological consequences of gram-negative shock are attributable to the bacterial membrane component, LPS, which induces experimental endotoxemia and has become a valuable experimental model for septicaemia [15]. Factors related to uremic
toxicity, independent from dialysis, as a trigger of immune response in CKD has been highlighted. Reduction of renal function per se, and consequently uremic toxicity, can be responsible for increased plasma concentrations of systemic and vascular inflammatory biomarkers [30-32]. Despite the development of new technologies of renal replacement therapy, it is almost impossible to completely remove the uremic toxins retained by impaired renal function. The uremic toxins consist of heterogeneous substances, including organic compounds and peptides, with proinflammatory effects [11]. As for other immune cells, macrophage function is also inhibited by uremic toxins [11-15]. Our results show that among tested guanidino compounds, like MG and GND, interfere with NO release. In particular, MG and GN could act also as false substrate capable to inhibit iNOS and, with a more complex mechanism, as iNOS expression inhibitor as previously reported [15].

These findings could have significant implications in unravelling the complex mechanism involved in uraemic patient immunodeficiency. In fact, we can hypothesize that guanidino compounds, derived from protein catabolism and accumulated in CKD, could participate to the dysfunctions in immunoresponses in these patients. Furthermore, these effects of guanidino compounds could be increased by the impaired L-ARG concentration, which often occurs in CKD. It is important to point out in uremic condition, where all these products accumulates together, they could act synergistically as iNOS inhibitors. In order to confirm our hypothesis we tested the effect of MG and GN simultaneously added to J774A.1 macrophages. Considering that infections and sepsis are frequent in CKD patients, we evaluated the effects of MG and GN in macrophages in this experimental condition. To mimic, as possible in vitro, the uremic condition we incubated J774A.1 macrophages with GN and MG, 1h before and simultaneously with the pro-inflammatory agents LPS for further 24 h. It is known that LPS exert multiple effects on macrophages, such as regulation of their cellular functions, activation of transcription factors, production of cytokines and other pro-inflammatory mediators [33]. LPS enhances NO formation, following the induction of iNOS, that has been implicated in the pathogenesis of shock and inflammation [34]. In fact, during inflammation, NO is mainly produced by iNOS. Our evidences indicated that MG and GN inhibit NO release and iNOS expression during LPS-induced inflammation in J774A.1 macrophages thus contributing to a reduction in NO release. Furthermore the main finding of this study regard the synergistic effect of MG and GN on the inhibition of inflammatory response in LPS-stimulated macrophages. Uraemia is characterized by the accumulation of various uremic toxins thus their interactions resulted of fundamental importance also to evaluate the increase of their toxic effects. In 2003, the European Uremic Toxin Work Group composed a list of 90 uremic retention solutes known at that time [35]. Recently, classification of normal and pathologic concentrations of uremic toxins has been extended and updated [36]. Their removal is largely hampered by their physicochemical properties and alternative removal techniques, such as strategies to modify intestinal generation or absorption, are considered [37,38]. Thus understanding the effects of uremic toxins, which normally accumulate simultaneously, will help to develop novel therapeutic strategies and the search for pharmacologic strategies blocking responsible physiopathological pathways [39].

Acknowledgments

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