Time related inhibition by methylguanidine in LPS-stimulated J774A.1 macrophages

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Abstract

Methylguanidine (MG) is a nitrogen compound deriving from protein catabolism that accumulates in Chronic kidney disease (CKD). 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. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and their respective metabolites, nitric oxide and prostaglandins, are a crucial step both in the activation of immunoresponsive cells and in the mechanism of citotoxicity, NO mediated. It has been previously reported the ability of MG to inhibit iNOS activity and expression both in vitro and in vivo. The aim of this study is to evaluate if MG could interfere with macrophagic immunoresponses also modulating INOS and COX-2 at different time of incubation in J774A.1 stimulated with Lipopolysahharide from E.coli. Our results demonstrated that MG exerted inhibitory effect on iNOS and COX-2. These effects are related to incubation time thus highlighting the detrimental effect of immune system by MG in uremic conditions.

KEY WORDS: METHYLGUANIDINE, UREMIA, COX-2, MACROPHAGES
Introduction

Guanidino compounds are a large group of structural metabolites of L-arginine. Among them are well-known uremic retention solutes as methylguanidine (MG). More than 50 years ago, Mason et al. [1] first suggested that MG was an uremic toxin and subsequent studies showed that MG is elevated in uraemia and that it is toxic [2, 3]. Chronic kidney disease (CKD) progression leads to the dysfunction of multiple organs with clinical features constituting the uremic syndrome that, despite pharmacological and dialytic treatment remains associated with multiple complications [4-6]. This syndrome is attributed to the progressive retention of a large number of compounds called uremic toxins, as MG, that under normal conditions are excreted by healthy kidneys but accumulates in CKD patients and are harmful effects in various physiological functions [4, 5]. The development of CKD is associated with a significant increase in all-cause mortality [7, 8]. 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 [9, 10]. 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 to cardiovascular disease. We have previously reported the pro-apoptotic effect of MG on hydrogen peroxide-treated astrocytes [11] and its ability to inhibit, both in vitro and in vivo, the inflammatory response [12-15]. In this study we report the effect of MG on important pro-inflammatory enzymes as the inducible isoform of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression and activity incubating J774A.1 macrophage with MG at different experimental time both before and after Lipopolysaccharide from E.coli (LPS) stimulation.

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 u/mL penicillin and 100 mg/mL streptomycin at 37 °C in a 5% CO₂ atmosphere.

Antiproliferative activity

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 MG or L-NAME (0.1-10 mM), and incubation was performed for 24, 48 and 72 h. Cell viability was assessed using the MTT assay as previously reported [16, 17]. 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 MG and GN, was calculated as: % dead cells = 100 x [(OD treated/OD control) x100]

Nitrite and PGE₂ determination

Macrophages J774A.1 were seeded in P60 plates (1.8 x 10⁶/P60) and allowed to adhere for 4h. Thereafter, the medium was replaced with fresh medium and cells were treated with MG or L-NAME (0.1-10 mM) alone for 30 min or 18 h and then co-exposed to LPS (1 μg/ml) for further 24 h. In another set of experiments MG was added 24h after LPS stimulation. NO production was measured as nitrite (NO₂⁻, μM), index of NO released by cells, in the culture medium 24 h after LPS stimulation, as previously reported [18]. NO₂⁻ amounts were measured by Griess reaction. Briefly, 100 IL of cell culture medium were mixed with 100 IL of Griess reagent - equal volumes of 1% (w:v) sulphanilamide in 5% (v:v) phosphoric acid and 0.1% (w:v)
naphtylethylenediamine-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\textsubscript{2}, as µM concentration, in the samples was calculated by a sodium nitrite standard curve.

PGE\textsubscript{2} concentration in macrophage culture medium, after MG and LPS treatment as previously described, was assessed by an Enzyme-Linked Immuno Sorbent Assay (ELISA) assay by using a commercial kit, for murine PGE\textsubscript{2}, according to manufacturer's instruction (Cayman Chemicals). Results are expressed as percentage of inhibition vs J774A.1 treated with LPS alone.

**Western blot analysis for iNOS and COX-2 expression**

iNOS and COX-2 protein expression was assessed by Western blot analysis. After nitrite and PGE\textsubscript{2} 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 Bio-Rad 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\textsuperscript{-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, anti-COX-2 antibody (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 XOmait 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 as previously reported [19,20], and expressed as % inhibition vs J774A.1 treated with LPS alone.

**Results**

**MG and L-NAME don’t affect J774A.1 macrophage viability**

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

**MG inhibits NO and PGE\textsubscript{2} release from LPS-stimulated J774A.1 macrophages**

To asses time-dependently effect of MG on NO production we measured nitrite release, a stable end-product of NO, in cellular medium of murine macrophage cell line J774A.1 stimulated with MG (0.1-10 mM) alone or in combination with LPS (1μg/ml). A marked increase of nitrite production in cellular medium was observed at 24h after LPS (44.21 ± 0.8 μM; P < 0.001, vs control). When MG was added to J774A.1 macrophages both 30 min and 18h before and 24 h after LPS a significant and
concentration-dependent decrease of nitrite production in cell medium was observed (Figure 1A). Interestingly the inhibitory effect observed with MG at the lowest concentration tested (0.1 mM) resulted stronger respect to that observed with L-NAME, a well known NO inhibitor, tested at the same concentration (Figure 1B).

In the same experimental conditions MG (0.1-10 mM) inhibited also PGE$_2$ release. In particular the inhibitory effect resulted stronger when MG was added 30 min or 18 h before LPS rather than when added after LPS (Figure 2).

**MG inhibits iNOS and COX-2 expression in LPS-stimulated macrophage.**

LPS treatment induces in J774A.1 macrophages iNOS and COX-2 expression (Figure 3, 4). When MG was added to J774A.1 macrophages 30 min or 18 h before LPS we observed a significant and concentration-related inhibition in iNOS expression. In particular we observed a stronger effect when MG was added 30 min before respect to 18 h (Figure 3).
As shown in Figure 4, similarly to the effect observed on iNOS, also COX-2 expression resulted inhibited only when MG was added before LPS but, on reverse respect to iNOS, the inhibition was more pronounced when MG was added 18 h before respect to 30 min.
phlogogenic agents, cytokines and lipopolysaccharide [29, 30]. LPS enhances NO formation, following the induction of iNOS, that has been implicated in the pathogenesis of shock and inflammation [31]. In fact, during inflammation, NO is mainly produced by iNOS.

Our evidences indicate that MG inhibits NO and PGE₂ release both when added before and when added after LPS, thus indicating that the observed effect at this time was due respectively to an inhibition of the iNOS and COX-2 activity. Furthermore the effects on NO inhibition was comparable to those observed with L-NAME, a well known NO inhibitor. MG only when added before LPS, also inhibits iNOS expression and this effect was more pronounced when MG was added 30 min before LPS respect to 18h indicating that to exerts iNOS inhibition MG requires brief time.

During inflammation another important pro-inflammatory enzyme is COX-2 and its expression was also influenced by NO and iNOS [32]. COX-2 is induced by LPS, certain serum factors, cytokines and growth factors, and is a predominant COX at sites of inflammation. Even though to a lesser extent respect to iNOS, also COX-2 resulted inhibited by MG in LPS-stimulated macrophages. Interestingly COX-2 expression resulted inhibited by MG mostly after a 18h pre-treatment respect to 30 min incubation, indicating that to strongly inhibit COX-2 expression MG requires a longer time respect to iNOS inhibition thus contributing to a reduced response to LPS-induced inflammation. Our results are in according with previous studies demonstrating, in in vivo experimental model, the inhibitory effect of MG on iNOS and COX-2 expression/activity [25, 12-15].

Uremia-related immune dysfunction result from a complex interaction between the innate and adaptive immune systems, in which immune activation (hypercytokinemia and acute-phase response) and immune suppression (impairment of response to infections and poor development of adaptive immunity) coexist. Therefore, accelerated tissue degeneration (as a consequence of chronic inflammation) and increased rate of sepsis (because of a poorly orchestrated immune response) represent one of the most important targets for interventions aiming to reduce mortality in CKD patients. Our results further highlight the importance of removing uremic toxins in CKD patients, considering that the alteration of mediators primarily involved in immune response, iNOS and COX-2, resulted influenced by the time of exposure to MG.

Recently, classification of normal and pathologic concentrations of uremic toxins has been extended and updated [33-34]. Their removal is largely hampered by their physicochemical properties and alternative removal techniques, such as strategies to modify intestinal generation or absorption, are considered [35-36]. 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 [37].

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References


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