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Research paper

The inhibition of inducible nitric oxide synthase and oxidative stress by agmatine attenuates vascular dysfunction in rat acute endotoxemic model

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ABSTRACT

Vascular dysfunction leading to hypotension is a major complication in patients with septic shock. Inducible nitric oxide synthase (iNOS) together with oxidative stress play an important role in development of vascular dysfunction in sepsis. Searching for an endogenous, safe and yet effective remedy was the chief goal for this study. The current study investigated the effect of agmatine (AGM), an endogenous metabolite of L-arginine, on sepsis-induced vascular dysfunction induced by lipopolysaccharides (LPS) in rats. AGM pretreatment (10 mg/kg, i.v.) 1 h before LPS (5 mg/kg, i.v.) prevented the LPS-induced mortality and elevations in serum creatine kinase-MB isoenzyme (CK-MB) activity, lactate dehydrogenase (LDH) activity, C-reactive protein (CRP) level and total nitrite/nitrate (NOx) level after 24 h from LPS injection. The elevation in aortic lipid peroxidation illustrated by increased malondialdehyde (MDA) content and the decrease in aortic glutathione (GSH) and superoxide dismutase (SOD) were also ameliorated by AGM. Additionally, AGM prevented LPS-induced elevation in mRNA expression of iNOS, while endothelial NOS (eNOS) mRNA was not affected. Furthermore AGM prevented the impaired aortic contraction to KCl and phenylephrine (PE) and endothelium-dependent relaxation to acetylcholine (ACh) without affecting endothelium-independent relaxation to sodium nitroprusside (SNP). **In conclusion**: AGM may represent a potential endogenous therapeutic candidate for sepsis-induced vascular dysfunction through its inhibiting effect on iNOS expression and oxidative stress.

1. Introduction

Sepsis is a serious life threatening manifestation of severe systemic infection. It imposes a medico-economic burden on healthcare systems. The mortality rates associated with sepsis exceeds those of HIV/AIDS, breast cancer and prostate cancer combined (Chaudhry and Duggal, 2014).

Sepsis is manifested by multi-organ dysfunction, most commonly affecting respiratory and cardiovascular systems. This multi-organ dysfunction provoked by sepsis can cause hypovolemia, a decrease in vascular tone and myocardial depression (De Backer et al., 2014).

The increased release of nitric oxide (NO) is observable in sepsis-induced cardiovascular dysfunction and it is believed to be due to the increase in inducible nitric oxide synthase (iNOS) mRNA expression as a part of the body's self defensive mechanism against endotoxemia. The iNOS expression increases throughout the body in tissues such as endothelial cells, platelets, macrophages and cardiac myocytes (Chuaiphichai et al., 2016; Titheradge, 1999).

Lipid peroxidation and the decrease in superoxide dismutase (SOD) caused by oxidative stress can also account for the deleterious cytotoxic effects of sepsis (Li et al., 2016). The role of mitochondrial dysfunction in sepsis relies not only on reactive oxygen species (ROS) but also on reactive nitrogen species (RNS) that are formed due to excessive NO production (Galley, 2011).

The systemic inflammatory response associated with sepsis can be triggered by infection with bacteria such as *Escherichia coli (E.Coli)* (Opal et al., 2003) or the bacterial prime endotoxin lipopolysaccharide (LPS) (Ramachandran, 2014). LPS is the main constituent of gram-negative bacterial cell wall. Injecting the experimental animals with LPS is widely used as a simple and reproducible model for sepsis (Poli-de-Figueiredo et al., 2008).

Targeting the excessive release of NO and oxidative stress with a natural endogenous compound was the burgeoning goal behind this study.

Agmatine (AGM), a polycationic amine, is the endogenous metabolite of L-arginine, formed by the action of arginine decarboxylase

Abbreviations: AGM, agmatine; CRP, C-reactive protein; CK-MB, creatine kinase-MB isoenzyme; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; LPS, lipopolysaccharides; MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; NOx, total nitrite/nitrate

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enzyme (Tabor and Tabor, 1984). The versatile pharmacological targets of AGM (imidazoline receptors, NO release and α_2-receptors among many other targets) make it a reliable compound for treating wide spectrum of disorders such as diabetes mellitus, insulin resistance, endothelial dysfunction, neurodegenerative disorders, addiction, neuropathic pain and many inflammatory disorders (Chang et al., 2010; El-Agamy et al., 2014; El-Awady and Suddek, 2014; Gilad et al., 1996; Keynan et al., 2010; Piletz et al., 2013; Sharawy et al., 2016). In addition, AGM has a potent antioxidant effect shown in its ability to decrease lipid peroxidation and increase SOD activity and reduced glutathione (GSH) content (El-Agamy et al., 2014; Freitas et al., 2014). AGM consumption for long term is believed to be safe (Gilad and Gilad, 2013, 2014; Keynan et al., 2010).

Since AGM has a potential inhibitory effect on iNOS and oxidative stress, therefore this study investigates its protective effect in LPS-induced vascular dysfunction in rats.

2. Materials and methods

2.1. Materials

AGM sulphate, pentobarbital sodium and LPS (E. coli *O55:B5*) were purchased from Sigma Chemical Co. (Saint Louis, Mo, USA). AGM (1-Amino-4-guanidinobutane sulfate salt) was available as white to off-white powder with purity ≥97%, it is soluble in water (50 mg/ml). AGM was dissolved in saline directly before use. LPS was available as white powder, purified by phenol extraction, and is soluble in water (5 mg/ml). All chemicals and reagents used in this study were procured from approved chemical suppliers with the finest analytical grade.

2.2. Animals

Male Sprague Dawley rats with average weight (150–200 g) and average age (6–7 weeks) were obtained from Vacsera (Giza, Egypt). All procedures for animal handling were in accordance with the Guidelines described by Animal Ethics Committee of Mansoura University, Mansoura, Egypt.

2.3. Experimental design

Rats were classified into four groups, as follows; **Control group**: rats were injected with saline i.v., **LPS group**: rats were injected with a bolus LPS (5 mg/kg, i.v.), **AGM group**: rats received a single injection of AGM (10 mg/kg, i.v.) and **AGM + LPS group**: rats were injected with AGM 1 h prior to LPS. The doses of LPS (Bayraktar et al., 2015) and AGM (Sharawy et al., 2016; Taksande et al., 2010) were selected depending on previously reported studies in addition to our preliminary experiments using mortality rate and inflammatory markers (lactate dehydrogenase (LDH) activity and C-reactive protein (CRP)) as guide.

Twenty four hours after LPS injection, mortality rate was calculated and animals were anesthetized with pentobarbital sodium (60 mg/kg, i.p.), serum was obtained from the collected blood for measuring the biochemical parameters and the thoracic aorta was gently isolated and divided into three parts; one part was used for determining *in vitro* vascular reactivity, another part was used for preparing aortic homogenate to be used for measurement of oxidative biomarkers freshly on the same day and the last part was frozen quickly in liquid nitrogen and stored at −80 °C for the use in real-time RT-PCR.

2.4. Determination of serum creatine kinase-MB isoenzyme (CK-MB) activity, lactate dehydrogenase (LDH) activity and C-reactive protein (CRP) level

The serum activity of CK-MB and LDH were determined using commercial kits (Stanbio, TX, USA). The CK-MB and LDH activity were measured at 340 nm.

Serum CRP was measured using agglutination test kit (Omega diagnostics LTD, Scotland, UK) according to the instructions enclosed.

2.5. Measurement of serum total nitrite and nitrate (NOx)

The production of serum nitric oxide (NO) was determined by measuring its breakdown products as nitrate and nitrite (NOx). Nitrate was first reduced to nitrite and the total nitrite was measured spectrophotometrically at 540 nm using a commercial kit (R & D systems, USA).

2.6. Determination of aortic malondialdehyde (MDA) concentration, reduced glutathione (GSH) content and Superoxide dismutase (SOD) activity

Isolated aortas were homogenized in PBS buffer as 10% (w/v). The homogenates were centrifuged at 2000 × g/4 °C for 15 min, and supernatant was used for fresh assay of oxidative biomarkers (MDA, GSH and SOD).

Thiobarbituric acid reactive substance was measured as MDA, the end product of lipid peroxidation, at 532 nm. To determine the GSH, TCA-deproteinized serum was used to measure non-protein sulfhydryl compound which is based on the reaction of GSH with Ellman's reagent to give a compound that absorbs at 412 nm. SOD activity was determined by the measurement of the degree of inhibition of the auto oxidation of pyrogallol in alkaline pH by SOD at wavelength 420 nm.

2.7. Preparation of the aortic rings and determining in vitro aortic vascular reactivity

Aortic rings were mounted in an organ bath filled with 10 ml of physiological salt solution (PSS) at 37 °C and bubbled with a mixture of 95% O_2 and 5% CO_2. Rings were allowed to equilibrate under 1 g resting tension for 60 min. Isometric tension generated by the vascular smooth muscle was measured using a force displacement transducer (K30, Hugosachs Elektronik, March, Germany) and recorded with a Powerlab using Chart v4.2 software (ADInstruments Ltd., Oxfordshire, UK).

After the equilibration period, arterial ring responsiveness was assessed by measuring contraction to 80 mM KCl and this response was repeated 3 times. Cumulative dose-response curve was constructed to phenylephrine (PE; 10^{-9}–10^{-5} M).

To assess vasorelaxation, aorta was precontracted with 1 μM PE before measuring relaxation responses to ACh (10^{-9}–10^{-5} M) or SNP (10^{-9}–10^{-5} M).

2.8. Measurement of mRNA expression of eNOS and iNOS using real-time RT–PCR

Aortic tissue was homogenized to extract and purify RNA using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the kits instructions. Then by using Qiagen Quantitect Reverse Transcription Kit (Qiagen), 1 μg of RNA taken from each sample was reverse transcribed into cDNA. Quantitative real time RT-PCR was performed using SYBR Green PCR master mix (Qiagen) as a fluorescent detection dye. The thermocycler used was Rotor Gene Q (Qiagen).

In sample duplicates, the mRNA levels of eNOS and iNOS were measured and normalized relative to the housekeeping gene (β-actin) RNA in the same sample. The primers for eNOS (NM_021838.2), iNOS (NM_012611.3) and β-actin (NM_031144.3) (Invitrogen by Life Technologies, Carlsbad, CA, USA) were as the following: eNOS: (product size: 123-bp), sense 5′- AAAGAACTGGGAAGTGGGCA-3′; antisense 5′- CGGCTCTGTAACTTCCTTGG-3′; iNOS: (product size: 74-bp), sense 5′- CAGCATCCACGCCAAGAAC-3′; antisense 5′- GGCTGGACTTCTCACTCTGC-3′; β-actin: (product size: 96-bp), sense 5′-TGGGTATGGAATCCTGTGG-3′; antisense 5′-GCACTGTGTTGGCATAGAGG-3′. The results were expressed as n-fold change of the relative