European Journal of Pharmacology 807 (2017) 174–181

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular pharmacology

Protective role of cGMP in early sepsis

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ARTICLE INFO

Keywords:
Sepsis
Guanylate cyclase
Nitric oxide
Sildenafil
Phosphodiesterase

ABSTRACT

Septic shock, which is triggered by microbial products, is mainly characterised by inadequate tissue perfusion, which can lead to multiple organ dysfunction and death. An intense release of vasoconstrictors agents occurs in the early stages of shock, which can lead to ischemic injury. In this scenario, cGMP could play a key role in counterbalancing these agents and preventing tissue damage. Sildenafil, which is a phosphodiesterase-5 inhibitor, increases cGMP in smooth muscle cells and promotes vasodilation. Thus, the purpose of this study was to investigate the effect of treatment with sildenafil in the early stages of sepsis. Male rats were submitted to either cecal ligation and puncture (CLP) or a sham procedure. Eight h after the procedure, the CLP and sham groups were randomly assigned to receive sildenafil (10 mg/kg, gavage) or vehicle, and twelve or twenty-four h later the inflammatory, biochemical and haemodynamic parameters were evaluated. Sepsis significantly increased levels of plasma nitrate/nitrite (NOx), aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, creatine kinase and lactate. Additionally, sepsis led to hypotension, hyporesponsiveness to vasoconstrictor, renal blood flow reduction and also increased lung and kidney myeloperoxidase. Sildenafil increased renal blood flow and reduced the plasma levels of creatinine, lactate and creatine kinase, as well as reducing lung myeloperoxidase. Thus, phosphodiesterase inhibition may be a useful therapeutic strategy if administered at the proper time.

1. Introduction

Sepsis remains a major worldwide healthcare problem, with consistently high mortality rates. In the presence of septic shock and associated multiple organ failure, mortality may approach 30–40% (Investigators et al., 2014; Zhou et al., 2014). Furthermore, apart from the use of fluids, antibiotics, adrenergic pharmacology and organ support, which have been widely used since the 1950s, no targeted biological therapy has decreased sepsis mortality and none is currently considered as part of standard clinical practice (Seeley and Bernard, 2016). However, mortality rates due to sepsis remain high and new treatments are required.

After the discovery of nitric oxide (NO) in 1987, it became clear that large amounts of NO are produced during sepsis, and that NO is involved in cardiovascular collapse (Fernandes and Assreuy, 2008). However, all the nitric oxide synthase (NOS) inhibitors tested so far in clinical settings have caused undesired side effects, such as excessive vasoconstriction, which was the most likely reason for the interruption of a Phase III study with a NOS inhibitor in human sepsis (López et al., 2004).

Cardiovascular hallmarks of septic shock are systemic vasodilatation and hypotension, accompanied by large increase in sympathetic nerve activity and the release of vasoconstrictor hormones (Lukewich and Lomax, 2014; May et al., 2012). An excess of vasoconstrictors can get out of control and cause adverse effects (Dunser and Hasibeder, 2009). In this scenario, the production of NO by constitutive enzymes is essential to preserve a proper blood flow, thereby avoiding excessive tissue ischemia during sepsis.

Thus, if in late sepsis the up-regulation of NOS type II (NOS-2), and consequently high levels of NO and cGMP, mediates cardiovascular collapse (Nardi et al., 2014), in early sepsis, when NO levels are not so high and there is a storm of vasoconstrictors, the vasodilation mediated by NO-sGC-cGMP could be critical to preserve a proper blood perfusion and avoid organ failure. This is especially important in the kidneys, where the release of norepinephrine can induce intense vasoconstriction in the afferent glomerular arterioles, which can result in filtration pressure decreasing, followed by a reduction in the glomerular filtration rate (Schrier, 2004). This can lead to acute renal failure, which occurs in approximately 51% of patients with septic shock and contributes to poor outcomes (Rangel-Frausto et al., 1995).

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http://dx.doi.org/10.1016/j.ejphar.2017.05.012
Received 19 November 2016; Received in revised form 3 May 2017; Accepted 4 May 2017
Available online 05 May 2017
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Interestingly, we have previously shown that in early sepsis there is an impairment in guanylate cyclase activity (Fernandes et al., 2006) and the guanylate cyclase inhibitors lead to a reduction in cGMP levels and an increase in the mortality rate (Fernandes et al., 2009). Since cGMP is the main messenger for NO–mediated arterial vasodilatation, the reduced levels of this cyclic nucleotide may also contribute to renal vasoconstriction during sepsis (Schrier, 2004).

Therefore, we hypothesise that cGMP could be essential to maintaining proper blood perfusion in early sepsis, mainly in the kidneys. Consequently, we evaluated the effect of sildenafil, which is a clinically approved phosphodiesterase-5 that inhibits the breakdown of cGMP, as a strategy to increase the bioavailability of cGMP in early sepsis.

2. Materials and methods

2.1. Animals

Male Wistar rats (200–250 g; 10 weeks old) were housed in a temperature and light-controlled room with free access to water and food. All the procedures were in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and the Guide of the Brazilian National Council of Animal Experimentation. The procedures were approved by the University's Institutional Ethics Committee (Protocol number 019/2013).

2.2. Cecal ligation and puncture (CLP)

CLP surgery was performed as previously described (Wichterman et al., 1980) with minor modifications. The rats were anesthetised with ketamine and xylazine (90 and 15 mg/kg, respectively). The cecum lumen was reduced by a non-obstructing ligation right above the ileocecal valve. The cecum was punctured twice with an 18-gauge needle and a small amount of cecal content was squeezed through the punctures. This was placed back in the cavity and the abdominal walls were sutured. The sham-operated rats underwent a similar surgical procedure with cecal exposure but it was neither ligated nor punctured. All the animals received 5 ml/100 g of sterile phosphate-buffered saline (PBS, in mM 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4, and 8.1 $NaHPO_4$; pH 7.4) subcutaneously immediately after the procedure. PBS was administered for fluid resuscitation in order to reproduce clinical haemodynamic support and to induce a hyperdynamic phase circulatory state (Hubbard et al., 2005; Wichterman et al., 1980). Bacteremia in the CLP rat model was confirmed through the analysis of whole blood obtained by cardiac puncture 12 h after the procedure (Table S1).

The rats were allowed to recover from anaesthesia in a pre-warmed fresh cage placed over a heating pad. The animals were monitored until full recovery, which took about 3 h. After recovery from anaesthesia, the animals were kept in a temperature and light-controlled room with free access to water and food until the time of analysis (see Section 2.10).

2.3. Cyclic GMP assay

For the assessment of the plasma cGMP levels, the blood was collected into EDTA tubes (7.5 mM) via a catheter inserted into the right carotid. Isobutyl-methylxanthine was immediately added (0.1 mM) to prevent cGMP breakdown and the blood was centrifuged (1800 *G*, 10 min, 4 °C). Ice-cold ethanol was added to the samples and after five min the precipitation was removed by centrifugation (1800 *G*, 10 min, 4 °C). The supernatant was dried under a stream of nitrogen and then the cGMP content was measured by ELISA (Cayman Chemical, Ann Arbor, USA) according to the manufacturer's instructions. The results were expressed as nM of cGMP.

2.4. Measurement of renal blood flow

The animals were anesthetised intramuscularly with ketamine and xylazine (90 and 15 mg/kg, respectively, supplemented at 50-min intervals) and then a transverse abdominal incision was performed to assess the posterior left subhepatic space, allowing the visualisation of the left kidney. A laser probe (model VP3), connected to a laser Doppler blood flow monitor (moorVMSLDF2, Moor Instruments, England) was carefully placed directly on the kidney, allowing the measurement of the renal blood flow (in arbitrary units). The probe was kept in this position and the surgical incision was covered with gauze sponges soaked in sterile phosphate-buffered saline to protect the kidney from drying. During the experiments, the animals were maintained on a warming pad and were allowed to breathe spontaneously. An interval of 20 min was allowed before the measurement of the basal values.

2.5. Mean arterial pressure (MAP) measurement

The animals were anesthetised as above, and heparinised PE-20 and PE-50 polyethylene catheters were inserted into the left femoral vein for the drug injections, and into the right carotid artery for MAP recording. To prevent clotting, a bolus dose of heparin (300 IU) was injected immediately after vein cannulation. The animals were allowed to breathe spontaneously via a tracheal cannula. The body temperature was monitored by a rectal thermometer and maintained at 36 ± 1 °C by means of a heating table. The blood pressure and heart rate data were recorded with a catheter pressure transducer coupled to a Powerlab 4/30 (AD Instruments Pty Ltd., Castle Hill, Australia) running proprietary LabChart 8® software.

After stabilisation, the animals received intravenous injections containing either phenylephrine (3, 10 and 30 nmol/kg) or angiotensin II (3, 10 and 30 pmol/kg). The doses were injected in a total volume of 250 µl (including washing of the catheter). An interval of eight min was allowed for MAP stabilisation between each administration. The change in MAP (in mmHg) was calculated and compared between the groups. At the end of the experiment the animals were killed by a pentobarbital overdose.

2.6. Organ Bath

The animals were euthanised using an overdose of sodium pentobarbital (100 mg/kg, i.p.). The thoracic aorta was cleaned of surrounding fat and mounted in organ baths under a basal tension of 1 g. After 60 min of equilibration, the rings were exposed to 60 mM potassium chloride (KCl) twice. The last contraction was taken as the reference value for tension development. Following this, the rings were pre-contracted with phenylephrine (10^{-6} M), and the relaxant response to acetylcholine (10^{-5} M) was measured to determine the endothelial integrity. The ability of acetylcholine to induce at least 80% relaxation in phenylephrine-contracted preparations was used to confirm the integrity of the endothelium. Only endothelium-intact aortic rings were used in this study. Thus, the contraction response curves for phenylephrine (10^{-9} to 3×10^{-5} M) or KCl (10–90 mM) were constructed. The tension was recorded via isometric force transducers (Panlab, model TRI 201) coupled to a Powerlab 4/30 (AD Instruments Pty Ltd., Castle Hill, Australia) running proprietary LabChart 8® software.

2.7. Leukocyte and platelet counts; urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate and creatine kinase assays

The total leukocyte and platelet counts were determined by Cell Dyn 1400 (Abbott Diagnostics, Abbott Park, Illinois, USA). The levels of urea nitrogen, creatinine, AST, ALT, lactate and creatine kinase were measured using commercially available clinical assay kits (Gold Analisa Diagnostica, Minas Gerais, MG, Brazil).

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