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Glibenclamide reduces proinflammatory cytokines in an ex vivo model of human endotoxinaemia under hypoxaemic conditions

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ABSTRACT

Aims: In vivo application of the K_{ATP}-channel blocker glibenclamide can reverse endotoxin-induced hypotension, vascular hyporeactivity and shock in experimental animals. The hypothesis of the present study is, that the drug effects might not only be based on direct inhibition of K_{ATP}-channels of vascular smooth muscle cells, but might also reflect reduction of shock-induced excess proinflammatory cytokines and procoagulatory molecules produced in the blood monocytes.
Main methods: Human whole blood (normoxaemic or hypoxaemic) supplemented ex vivo with 100 ng/ml LPS was used to assess glibenclamide (3–100 μM) effects on IL-1beta, IL-6, TNF-alpha, tissue factor, and plasminogen-activator-inhibitor-2 (PAI-2). Co-incubations with monocytes and erythrocytes and cytosolic calcium measurements were performed to reveal their purinergic intercellular interaction.
Key findings: In heparinized blood, glibenclamide reduced LPS-induced release of IL-1beta and TNF-alpha, tissue factor and PAI-2 mRNA in a concentration-dependent manner. When samples were subjected to strong hypoxemia using 95% N_2/5% CO_2, these parameters became even more sensitive to the drug. No drug effect was observable in citrated blood or in isolated monocytes. IL-1beta mRNA inhibition by glibenclamide appeared to be dependent on P2X7-receptor activation of monocytes by ATP-releasing erythrocytes during hypoxia. Cytosolic calcium values as well as the duration of calcium transients elicited by P2X7-receptor stimulation in isolated monocytes were strongly increased during hypoxia, both of which could be abolished by glibenclamide.
Significance: We conclude that the anti-inflammatory effect of glibenclamide is mainly based on the reduction of calcium entry by drug-induced depolarization of hypoxic monocytes. Thus, glibenclamide possesses a potentially beneficial shock-specific anti-inflammatory action.

Introduction

Septic shock caused by bacteraemia is a leading cause of death in critically ill patients. Lethality rates range between 37 and 47%, and are still rising (Dombrovskiy et al. 2007). During septic shock, progressive systemic organ failure may develop due to an overwhelming inflammatory response (Jackson et al. 2008). This response can be triggered by several components of Gram-negative bacteria, especially endotoxic lipopolysaccharide (LPS) (Dunn et al. 1986). LPS not only induces hypotension but also vascular hyporeactivity toward counter-regulatory humoral factors such as norepinephrine. This hyporeactivity is largely due to the overproduction of nitric oxide (NO), a highly potent vasodilator, by inducible nitric oxide synthase (iNOS) from endothelial cells (Thiemermann 1997).

An additional contribution to vascular hyporeactivity during septic shock has been ascribed to the inflammatory cytokine tumor necrosis factor (TNF)-alpha, which is mainly produced by LPS-activated monocytes. The proinflammatory cytokines IL-1-beta, IL-6 and the chemokine IL-8 have also been reported to be involved (Dinarello 2000). TNF-alpha upregulates iNOS and cyclooxygenase 2 (COX-2) in different cell types, thereby mediating a generalized production of the potent vasodilators NO and prostaglandin E_2 (PGE_2) (Lazarov et al. 2000), respectively. Regulation of vascular tone by smooth muscle cells involves ATP-sensitive potassium channels (K_{ATP}-channels), which act to modulate the membrane potential and cytosolic calcium (Nelson et al. 1990): opening of K_{ATP}-channels due

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to tissue hypoxia and/or insufficient perfusion causes hyperpolarization, reduced calcium entry through L-type calcium channels, and finally an arteriolar vasodilation (Ishizaka et al. 1999; Taggart and Wray 1998).

Recently it has been shown that K_{ATP}-channels preferentially open during sepsis and are an important underlying cause of hypotension and vascular hyporeactivity (Chen et al. 2000). The effects of the K_{ATP}-channel blocker glibenclamide, a sulfonylurea drug, have been previously investigated in rats treated with LPS. This demonstrated a significant increase in blood pressure following post-treatment with glibenclamide in hypotonic rats that had been subjected to LPS-infusion, and was largely due to an increase in systemic vascular resistance (Gardiner et al. 1999). In addition, glibenclamide has also been shown to reverse endotoxin-induced hypotension in pigs (Vanelli et al. 1995).

Pore-forming Kir6.2 subunits of ATP-sensitive potassium channels are found in human monocytes, and can be blocked using glibenclamide (Gros et al. 1999; Schmid et al. 2007). Specifically, it could be demonstrated that glibenclamide downregulates stasis- and LPS-induced tissue factor-dependent procoagulatory conditions of monocytes. This meant that since particular hypoxaemia- and LPS effects can be modified by glibenclamide, then perhaps additional effects, including those on cytokine release, might also be induced by this compound. Hence, this led us to the hypothesis that glibenclamide might reduce the release of proinflammatory cytokines from leucocytes, thereby reversing LPS-induced hypotension indirectly. In the present study, we report on the effects of glibenclamide on IL-1-beta, TNF-alpha, IL-6, IFN-gamma, as well as tissue factor and plasminogen-activator inhibitor-2 (PAI-2) in a whole blood model of human endotoxinaemia. The influence of glibenclamide on purinergic signaling between erythrocytes and monocytes specifically during hypoxia/acidosis was also investigated, and the findings are reported herein.

Methods

Blood donors

Venous blood was drawn from 10 healthy, male volunteers in the age group between 20 and 40 years after signing written consent. They had to confirm not having taken any drugs, especially no "non-steroidal antiphlogistic drugs (NSAD)" at least 2 weeks prior to phlebotomy. The investigation was approved by the local ethics review committee (EK-Nr. 321/2009) and was therefore assured to be performed according to the principles of the Declaration of Helsinki.

Ex-vivo simulated endotoxinaemia and shock

Blood samples were drawn into commercially available phlebotomy vacuum vials (Vaccuette System, Greiner, Kremsmünster, Austria), containing either 3.8% sodium citrate or sodium heparin. Concentrated glibenclamide stocks in DMSO (Sigma-Aldrich, Schnelldorf, Germany) were prediluted in autologous citrated or heparinized plasma, respectively, in order to avoid haemolysis by concentrated DMSO. 160 µl of drug containing plasma was added to each blood vial by a syringe without opening the tube. Final concentrations of glibenclamide were 0, 3, 10, 30 or 100 µM. Controls contained vehicle only (final concentration of DMSO 0.03%). In some experiments, blood samples were equilibrated for 5 min with either 95% N_2, 5% CO_2 to simulate strong hypoxaemia or 20% O_2, 75% N_2, 5% CO_2 to simulate normoxaemia. After 15 min the tubes were treated with either 100 ng/ml lipopolysaccharide (LPS) or vehicle (H_2O) and incubated horizontally for 2 or 5 h at 37 °C in a gently shaking water bath (SBD50, Heto, VWR, Vienna, Austria) at 120 bpm. The depletion of glucose and pO_2, and the development of lactic acidosis in the tightly sealed blood vials were allowed for a simulation of hypoxemic conditions occurring in vivo during severe septic shock.

Blood gas analysis and measurement of glucose and lactate

Aliquots were obtained through the rubber seals of unopened tubes using a syringe fitted with a needle. Blood glucose concentrations were determined by an enzymatic kit (Roche Diagnostics, Mannheim, Germany). Blood lactate concentration was determined using a Biosen 5030 lactate analyzer (EKF Diagnostics, Magdeburg, Germany). pO_2, pCO_2 as well as pH were measured using a Stat Profile® pHOx® Basic (Nova Biomedical, Waltham, MA, USA) blood gas analyzer.

Measurement of procoagulatory activity in whole blood

The procoagulatory activity on the surface of blood cells was measured by a one-step clotting test, a so-called TiFaCT-test, which was performed as previously described (Schmid et al. 2007).

Surface expression of tissue factor on monocytes by flow cytometry

TF (CD142) expression on the surface of monocytes was quantified by flow cytometry essentially as described (Schmid et al. 2007).

Cytokine release measurements

After citrated or heparinized blood had been subjected to 2 h or 5 h of ex vivo endotoxinaemia, plasma was collected by centrifugation of blood at 800 *g* for 15 min and recentrifugation of the supernatant at 10,000 *g* for 2 min at room temperature. Cytokines and the chemokine IL-8 were measured by enzyme linked immunosorbent assays (ELISAs) using commercially available antibody sets for human IL1-beta, IL-6, TNF-alpha, and IFN-gamma (E-Biosciences, San Diego, CA, USA).

Preparation of mononuclear cells

After citrated or heparinized blood had been subjected to 2 h or 5 h of ex vivo endotoxinaemia peripheral blood mononuclear cells (PBMCs) were prepared by Histopaque (Sigma-Aldrich) density gradient centrifugation as described (Lohrke et al., 1997).

Quantitative real-time RT-PCR

Total RNA of mononuclear cells was isolated using the Peqgold Blood Total RNA Kit (Peqlab, Erlangen, Germany) and adjusted to a concentration of 0.040 µg/µl with DEPC-H_2O after spectrophotometric determination. cDNA synthesis was carried out with 0.4 µg of total RNA using oligo-dT_{18} primers and 200 units of Murine Leukaemia Virus Reverse Transcriptase (Fermentas AG, St. Leon-Rot, Germany) at 42 °C for 60 min. Subsequently cDNA was separated from enzyme, primer and potential PCR inhibitors using silica-membrane DNA-binding columns (Peqlab) and diluted to a concentration of 2 ng/µl with nuclease free H_2O.

For polymerase chain reaction (PCR) intron-spanning custom-synthesized primer sets (Schmid et al. 2009) for the mRNA sequences of human IL-1-beta, IL-6, IL-8, TNF-alpha, IFN-gamma transcripts, tissue factor, PAI-2 and the housekeeping gene GAPDH were used. PCR was carried out on a Light-Cycler® 488 thermal cycler (Roche, Mannheim, Germany) with 10 µl reaction mixes containing 9.6 ng of cDNA and 5.2 µl of 2x Precision™-Mastermix (Primer Design Ltd, Southampton, UK) containing SYBR™-green, dNTPs, buffer and a hot start Taq polymerase completed with 0.2 µM of each primer set. The cycling conditions were 95 °C for 10 min followed by 40 cycles denaturing at 95 °C