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ATP-sensitive potassium channels expressed by human monocytes play a role in stasis-induced thrombogenesis via tissue factor pathway

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Abstract

Blood stasis is one of the key risk factors in deep vein thrombosis. Localized blood oxygen and glucose depletion are main characteristics observed during stasis. However, the causal chain leading to clot formation is still obscure. According to our hypothesis, energy depletion causes opening of K_{ATP} channels present on monocytes, facilitating influx of calcium and triggering tissue factor-(TF)-dependent procoagulatory activity and eventually clot formation. Using Reverse-Transcript-PCR (RT-PCR) in magnetically enriched human monocytes, mRNA transcription of the K_{ATP}-channel subunits Kir6.1 and Kir6.2 could be confirmed. Membrane potential and cytosolic calcium were recorded by time-resolved flow cytometry. The specific K_{ATP}-channel opener pinacidil caused a glibenclamide-sensitive hyperpolarization of monocytes and a prolongation of cytosolic calcium transients triggered by purinergic stimulation. TF-initiated whole blood clotting time (TiFaCT) was accelerated comparing 2 and 8 h of simulated in vitro blood stasis using blood of male healthy volunteers. Both with and without activation of the monocytes with 100 ng/ml LPS, the K_{ATP}-channel blocker glibenclamide resulted in a significantly ($p<0.001$) prolonged clotting time after 8 h of stasis compared to vehicle control and LPS, respectively. In the course of stasis, flow cytometry showed an increase in monocytes expressing TF (0.1% and 1.3% after 2 and 8 h, respectively). LPS (100 ng/ml) increased the amount of TF expression significantly to 36%, whereas 30 μM glibenclamide partly reversed this increase down to 24%. Phosphatidylserine-exposure (PSE) on monocytes increased strongly during stasis by 11.2 times, a process which glibenclamide attenuated by 23%. LPS increased PSE further by 65%, which glibenclamide reduced by 50%. In conclusion, presence of integral subunits of K_{ATP}-channels is demonstrated in human monocytes. These channels are able to enhance Ca^{2+}-dependent intracellular signalling and can increase TF-activity and phosphatidylserine exposure thereby accelerating clot formation during stasis by monocytes.
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Introduction

Virchow's (1856) triad describes three major causes for the development of venous thrombosis. Firstly lesions of the vessel wall, secondly a changed composition of the blood, and thirdly blood stasis. The first two facts are well understood. However, the mechanisms by which blood stasis promotes blood clotting still remain partly unclear.

Oxygen partial pressures (pO_2) within venous valve pockets were shown to decrease significantly after 2 h of ineffective blood flow and this was accompanied by thrombus formation (Hamer et al., 1981). In a murine model of a whole body oxygen deprivation, an accumulation of mononuclear phagocytes, mainly monocytes, expressing tissue factor (TF) within fibrin deposits was reported (Lawson et al., 1997). In a study of hypobaric hypoxia, rats were held in an atmosphere representing an altitude of 5500 m above sea level (Nakanishi et al., 1998). TF-producing macrophages were found, triggering thrombus formation on the heart valves. Therefore, hypoxaemia per se seems to activate monocytes and promote thrombus development (the so-called "procoagulatory state").

Thus, an impact of low levels of pO_2 on blood clotting is evident. Activated monocytes are able to promote clotting by expressing and de-encrypting TF (Butenas et al., 2005).

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Therefore, it is important to understand how monocytes react on blood stasis and how this condition triggers TF activity. In this paper we investigate the potential influence of the metabolic sensor adenosine-5′-tri-phosphate sensitive potassium channel (K_{ATP}-channel) on this process.

K_{ATP}-channels were described for the first time by Noma (1983). Recently, binding assays using a fluorescent K_{ATP}-ligand provided evidence for a K_{ATP}-channel on bovine monocytes (Löhrke et al., 1997). This led to our hypothesis that K_{ATP}-channels are present on human monocytes, too. In most tissues, K_{ATP}-channels are closed under normal metabolic conditions and increase their open probability when metabolic stress decreases the ratio of intracellular ATP/ADP concentrations and there is a concomitant acidification (Babenko et al., 1998). A deprivation of oxygen causes a K_{ATP}-channel-mediated change of membrane potential towards the potassium equilibrium. In case of monocytes it would cause a hyperpolarization (Löhrke et al., 1997). As a consequence, we surmise an increased dragging force and accumulation of ionized calcium, which was previously identified as an activator of already expressed, but a yet inactive tissue factor on the surface of cells (Wolberg et al., 1999). This process was termed "de-encryption" of tissue factor and is mostly due to increased phosphatidylserine exposure.

According to our hypotheses, the particular objectives of this study were to identify K_{ATP}-channels on monocytes as well as characterizing their influence on membrane potential and Ca^{2+}-signalling. Furthermore, we aimed to demonstrate the influence of K_{ATP}-channels on procoagulatory state by whole blood clotting tests, TF-expression, and phosphatidylserine exposure and in a simulated ex vivo blood stasis model.

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)" within at least the past 2 weeks. The investigation conforms with the principles outlined in the Declaration of Helsinki.

Measurement of procoagulatory activity of whole blood in an ex vivo stasis model

The blood samples were drawn into commercially available phlebotomy vacuum vials (Vaccuette System, Greiner, Kremsmünster, Austria), which contain 3.8% sodium citrate. Each 5 ml vial of blood was preincubated with 30 μM glibenclamide, 30 μM pinacidil or vehicle (0.1% DMSO). After 15 min the tubes were treated either with 100 ng/ml lipopolysaccharide (LPS) or vehicle (H_2O) and incubated horizontally for 2 or 8 h at 37 °C in a gently shaking water bath (SBD50, Heto, VWR, Vienna, Austria) with a rate of 80 bpm. The depletion of glucose and pO_2, and the development of lactic acidosis in the tightly sealed blood tubes were deliberately allowed and used to simulate the environmental conditions occurring inside localized blood stasis. In some experiments, tubes were opened and allowed to equilibrate with 1 ml of air for another 1 h.

Subsequently 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 essentially according to the literature (Santucci et al., 2000). Clotting time was assessed by addition of 75 μl of a 25 mmol/l $CaCl_2$ solution to 100 μl of samples in rotating plastic caps with a metal ball inside. In the metal ball coagulometer (Amelung KC4 Makro, Lieme, Germany) four specimens were measured simultaneously.

Blood gas analysis and measurement of glucose and lactate

Aliquots were taken out of the tightly sealed tubes by aspiration with a syringe needle through the rubber seal. Blood glucose concentrations were determined by a regular Accu-Check Active® glucose meter (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 Radiometer ABL 700 (Diamond Diagnostics, USA) blood gas analyzer.

Surface expression of tissue factor on monocytes by flow cytometry

TF (CD142) expression on the surface of monocytes was quantified by flow cytometry. Venous blood anticoagulated with sodium-heparin instead of 3.8% sodium citrate was used for ex vivo blood stasis experiments as described above. After 0, 2, and 8 h, 100 μl blood aliquots were taken for antibody staining with either Anti-CD142-PE (clone HIF-1, BD, Heidelberg, Germany) or isotype control (Mouse-IgG1-PE) for 30 min (at room temperature in the dark). Counterstaining was performed using Anti-CD14-PerCP (BD). Erythrocytes were lysed (BD FACS Lysing solution) and remaining leucocytes were washed with PBS and fixed with 3% *para*-formaldehyde PBS. Cellular fluorescence was measured using FACS-Calibur flow cytometer (BD) in the FL2 channel gated to cells exhibiting strong FL3 ($CD14^+$) fluorescence. For each sample, results of isotype controls (IgG1-PE, BD) were subtracted. Three thousand CD14-positive events were acquired and evaluated for each sample.

Exposure of phosphatidylserine on monocytes

Blood aliquots obtained from stasis experiments were incubated for 20 min with both 5 μl of Annexin V-FITC (Alexis Corporation, Lausen, Switzerland) and 5 μl Anti-CD14-PerCP (BD) at room temperature in the dark. Samples were lysed using BD-lysing solution supplemented with 2.5 mM $CaCl_2$ and 2 mM $MgCl_2$, centrifuged at 400 *g* for 5 min and the supernatants discarded. Pelleted leucocytes were washed with annexin binding buffer (ABB: 140 mM NaCl, 10 mM HEPES, 2.5 mM $CaCl_2$, pH = 7.4) and resuspended in 300 μl ABB,

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