Glucose initially inhibits and later stimulates blood ROS generation*

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Received 14 November 2012; revised 16 December 2012; accepted 25 December 2012

ABSTRACT

Background: Glucose is the main substrate for the generation of NADPH, the cofactor of the oxidative burst enzyme NADPH-oxidase of blood neutrophils. Changes in blood glucose are thus expected to modify the generation of reactive oxygen species (ROS). The new blood ROS generation assay (BRGA) quantifies ROS changes induced by blood glucose concentrations as they are found in diabetes mellitus. Material and Methods: Citrated or EDTA blood of 6 healthy donors were analyzed in the BRGA: 10 µl sample in black polystyrene F-microwells (Brand®781608) were incubated in triplicate with 125 µl Hanks’ balanced salt solution, 40 µl 0 - 200 mM glucose in 0.9% NaCl (final added conc.: 0 - 41 mM; final basal glucose conc.: about 4 mM), 10 µl 5 mM luminol, and 10 µl zymosan A (final conc.: 1.9 µg/ml) in 0.9% NaCl. The plates were measured within 0 - 250 min (37°C) in a photons-multiplyer microtiter plate luminometer (LUmo) with an integration time of 1 s. Results: Up to about 30 min reaction time the mean ROS generation was 50% inhibited by about 1 mM added glucose (= approx. IC50). At ≥80 min reaction time (possibly necessary for full phosphorylation of glucose to glucose-6-phosphate (G6P), the substrate metabolized by G6P-dehydrogenase to generate NADPH, the cofactor of the NADPH-oxidase) the mean ROS generation approximately doubled at about 1 mM added glucose (= approx. SC200) in citrated blood. Discussion: Elevated glucose concentrations not only increase systemic thrombin generation, they can also diminish cellular fibrinolysis and increase systemic inflammation, resulting in a chronic pro-thrombotic state. The fascinating importance of NADPH-oxidases not only in phagocytes but also in the beta cells of pancreas points towards a new pathogenesis explication of diabetes mellitus type 1: whatever stimulus (e.g. a pancreas-tropic virus) could activate the beta cell’s autodestructive NADPH-oxidase.

Keywords: Reactive Oxygen Species; ROS; Neutrophils; Phagocytes; Blood ROS Generation Assay; BRGA; NADPH-Oxidase; Glucose

1. INTRODUCTION

The generation of reactive oxygen species (ROS) in blood is a very important biomarker for both innate immunology [1-3] and cellular fibrinolysis [4]. The active oxidative burst enzyme NADPH-oxidase consists of the subunits gp91PHOX/p22 PHOX in the plasma membrane, of the subunits p67 PHOX/p47 PHOX in the cytosol, and of the regulator Rac2; the cofactor of the enzyme is NADPH, generated by glucose metabolism [5,6]. Therefore, changes of the conc. of blood glucose, the precursor of glucose-6-phosphate, could well influence blood ROS generation. The aim of the present work was to find out if blood glucose concentrations that would be typical for diabetes mellitus modulate blood ROS generation.

2. MATERIAL AND METHODS

Citrated blood (4.5 ml venous blood drawn into polypropylene monovettes containing 0.5 ml 106 mM sodium citrate; Sarstedt, Nümbrecht, Germany) or EDTA blood (2.6 ml venous blood drawn into polypropylene monovettes containing 1.6 mg/ml K3-ethylene diamine tetra acetate (EDTA); Sarstedt) of 6 healthy donors that gave written consent after explanation were analyzed in the blood ROS generation assay (BRGA) [7,8]: 10 µl sample in black high quality polystyrene F-microwells (Brand, Wertheim, Germany; article nr. 781608) were incubated in triplicate with 125 µl Hanks’ balanced salt solution (HBSS; modified without phenol red; SAFC Biosciences-Sigma, Deisenhofen, Germany; article nr. 55037C-1000ML), 40 µl 0 - 200 mM glucose in 0.9% NaCl (final added concentration: 0 - 41 mM; final basal glucose concentration: about 4 mM), 10 µl 5 mM luminol sodium salt (Sigma; article nr. A4685-1G; 1 g dissolved in 25.1
ml resulted in a 200 mM stem solution) in 0.9% NaCl, and 10 µl 36 µg/ml zymosan A (ZyA; Sigma, Deisenhofen, Germany; article nr. Z-4250-1G, lot nr. 27H0495), final conc.: 1.9 µg/ml. The plates were measured within 0 - 250 min (37°C) in a photons-multiplyer microtiter plate luminometer (LUmo; anthos, Krefeld, Germany) with an integration time of 1 s. The approximate 200% stimulatory (approx. SC200) or 50% inhibitory (approx. IC50) conc. of added glucose on oxidative blood burst were determined.

HBSS consisted of 185.4 mg/l CaCl2·2H2O, 200 mg/l MgSO4·7H2O, 400 mg/l KCl, 60 mg/l KH2PO4, 350 mg/l NaHCO3, 8000 mg/l NaCl, 90 mg/l Na2HPO4, 1000 mg/l glucose, pH 7.0 - 7.4. Expressed in molarity, the concentrations of the HBSS components are: 1.3 mM Ca2+, 0.8 mM Mg2+, 5.8 mM K+, 143 mM Na+, 144 mM Cl−, 1.6 mM SO42−, 0.4 mM H2PO4−, 0.6 mM HPO42−, 4.2 mM HCO3−, and 5.6 mM glucose.

3. RESULTS

Citrated blood in the BRGA had a ROS maximum of about 1600 relative light units per second (RLU/s) obtained after about 60 min (37°C). Half-maximal blood ROS generation was reached at about 35 min or at about 120 min (Figure 1).

EDTA-blood in the BRGA had also a ROS maximum of about 1600 RLU/s after about 80 min (37°C), i.e. 20 min later than citrated blood. Half-maximal blood ROS generation was reached at about 50 min or at about 130 min (Figure 2).

Figures 3-12 describe individual and mean ROS generations in citrated blood:

The basal glucose concentration was about 4 mM (final). At 17 min or 27 min reaction time 1 mM (further) added glucose inhibited about 50% of mean ROS generation (approx. IC50 = 1 mM added glucose) (Figures 3 and 4).

At 38 min or 46 min there appeared neither an IC50 nor a SC200 for mean ROS generation. However, 3 of 6 individual samples had approx. IC50 values of 1 - 2 mM added glucose and the other 3 of 6 individual samples had SC200 values of 1 - 2 mM added glucose (Figures 5 and 6).

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At 80 min, 94 min, or 104 min the mean ROS generation nearly doubled at about 1 mM or at about 21 mM added glucose (Figures 7-9).

At 162 min or 182 min the approx. SC200 of mean ROS generation was 1 mM or 11 mM added glucose (Figures 10 and 11). A similar approx. SC200 of mean ROS generation, however at much lower RLU/s, was found for 250 min (Figure 12).

Figures 13-16 describe individual and mean ROS generations in EDTA blood:

At 27 min reaction time 1 mM added glucose inhibited about 50% of mean ROS generation (approx. IC50 = 1 mM added glucose) (Figure 13).

At 80 min or 113 min there appeared neither an IC50 nor a SC200 for mean ROS generation (Figures 14 and 15).

At 182 min the approx. SC200 for mean ROS generation was 21 mM added glucose (Figure 16).

Thus, citrated blood is better than EDTA-blood to estimate changes of oxidative burst. ROS generation in EDTA-blood responded rather blunted to glucose alterations, the refined changes of ROS generation in citrated blood are demonstrated in Table 1, the reaction times of 38 min and 46 min being in the change phase from inhibition to stimulation.

1 mM glucose is only 20% of the normal plasma concentration of glucose that is about 5 mM.
intrinsic activation of hemostasis by glucose, added glucose maximally enhanced blood ROS generation at more than one concentration [9-11], the first (and most important one) being at about 1 mM and the second one being at 21 mM or 11 mM, depending on the reaction time.

4. DISCUSSION

There has already been evidence that glucose can alter blood ROS generation [12-19]: 11 mM glucose reduced the neutrophils’ respiratory burst by 28% ± 5% and 56 mM glucose by 74% ± 7% [12]. 50% decreased ROS generation was detected at 25 mM glucose [13]. Lowering
of blood glucose levels by insulin treatment of diabetic patients improved neutrophil functional activity [20].

The present work was undertaken in the new BRGA because the previous findings were not measured in whole blood that is the physiologic matrix of activated neutrophils. The main cell in blood that is competent for massive oxidative burst explosion is the neutrophil [21-24] that generates about 60 fmoles H₂O₂ per 30 min (21°C), whereas the monocyte generates less than 10% of this amount [21].

An ideal ROS detection technique should [25]:
- Imitate the physiologic ROS generation response to blood pathogens;
- Imitate the pathophysiologic blood ROS generation in autoimmunity;
- Detect globally the most important ROS of blood, that are hydrogen peroxide and singlet oxygen;
- Use a stable pathophysiologic trigger such as hydrophilic zymosan A (ZyA) [26,27];
- Be sensitive enough to detect ROS upon blood stimulation by only 1 - 2 µg/ml ZyA (the approx. concentration of initial severe fungal sepsis [28]);
- Not interfere with cell function (using untoxic concentrations of the light enhancer luminol);
- Be cheap: without taxes one LUmo (microtiter plate luminometer) costs less than €4500;
- Use a close-to-physiologic buffered salt solution [29];
- Be easy to standardize;
- Dilute whole blood only 10 - 20 fold, higher dilutions result in unphysiologic matrix [30];
- Be suitable for routine measurements of hundreds of samples within minutes.

The new BRGA technique with 18 - 20 fold diluted whole blood fulfills all these requirements. This refined blood ROS test demonstrated that at incubation times less than 30 min a blood glucose increase of only about 1 mM resulted in 50% less ROS generation. Incubation times higher than 80 min resulted in 100% increased ROS generation by about 1 mM added glucose. This time is possibly necessary for full phosphorylation in the correct cell compartment of glucose to glucose-6-phosphate (G6P), the substrate metabolized by G6P-dehydrogenase to generate NADPH, the cofactor of the NADPH-oxidase. This means that in the acute phase of blood neutrophil activation, such as the physiologic response to micro- or macro-thrombi, the diabetic patient has a decreased cellular fibrinolysis [6,31].

By contrast, in the chronic phase of neutrophil activation, the diabetic patient suffers of increased systemic inflammation [32,33]. Of particular interest is the diabetic patient in pregnancy, where increased ROS generation can threaten the health of mother and unborn [34]. Interestingly, the healthy placenta produces large quantities of the macrophage derived plasminogen activator inhibitor (PAI)-2, a suppressor of fetal rejection and autoimmunity [35,36]. A physiologic drug “PAI-2” could replace glucocorticoids with dangerous side reactions [37].

In conclusion, the elevated glucose concentration in diabetes mellitus not only increases systemic thrombin generation [9-11,38-40], it might also diminish cellular fibrinolysis and increase systemic inflammation [41,42], resulting in a chronic pro-thrombotic state [43].

Furthermore, the fascinating importance of NADPH-oxidases not only in phagocytes but also in the beta cells of pancreas [44] points towards a totally new pathogenesis explication of diabetes mellitus type 1 [45-47]: whatever stimulus (e.g. a pancreas-tropic virus) such as mumps virus, coxsackie B, cytomegalovirus (CMV), Epstein-Barr virus (EBV) could activate the beta cell’s NADPH-oxidase. The generated ROS could auto-destroy the insulin producing cells [48,49]. Is PAI-2 a new drug against the development of type 1-diabetes mellitus [50]?

5. ACKNOWLEDGEMENTS

REFERENCES


