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(1 → 3)-β-D-Glucan reduces the damages caused by reactive oxygen species induced in human platelets by lipopolysaccharides

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ABSTRACT

LPS (lipopolysaccharide) induces platelet activation and is a well-known fundamental agent of septic shock and disseminated intravascular coagulation (DIC). Biological activity of (1 → 3)-β-D-glucan is related due to its anti-inflammatory, antioxidant, and antitumor properties. We focus our attention on the (1 → 3)-β-D-glucan (antiplatelet) properties.

The main purpose of our study was to evaluate the influence of (1 → 3)-β-D-glucan from *Saccharomyces cerevisiae* on destructive activity of LPS (from *Escherichia coli* and *Pseudomonas aeruginosa*) on human blood platelets. We assess biochemically *in vitro* if (1 → 3)-β-D-glucan might combat the oxidative stress caused by LPS stroke associated with nitrative and oxidative damages of human platelet biomolecules. We also make an attempt by *in silico* molecular docking to determine the interactions between the molecules of (1 → 3)-β-D-glucan and LPS.

Our conclusion is that protective mechanism of (1 → 3)-β-D-glucan against LPS action on blood platelets is due to as well: its antioxidant properties, as to its interaction with LPS-binding region of TLR4-MD-2 complex.

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1. Introduction

Blood platelets belong to principal haemostatic representatives, but beyond hemostasis and thrombosis they also play a key role in inflammation. As inflammatory cells they seem to be involved in the pathophysiology of sepsis and septic shock (Li et al., 2012). Accumulating evidence strongly suggests the roles of platelets in a variety of inflammatory diseases and in defending the host against invasion by microorganisms (Semple & Freedman, 2010). Platelets participate with lipopolysaccharide (LPS – the major component of the outer membrane of Gram-negative bacteria) in the inflammatory response through production of reactive-oxygen species (ROS) that cause damage to the vascular endothelium and promote multiple organ dysfunctions in sepsis (Tyml, 2011). LPS (endotoxin) that induces platelet activation is a well-known causative agent of septic shock and disseminated intravascular coagulation (DIC) (Ito, Asai, Oshima, & Kobayashi, 1990). Platelets are known to posses Toll-like receptors (TLR) for LPS. In their surfaces expression of TLR2, TLR4 and TLR9 were observed (Aslam et al., 2006; Cognasse et al., 2005). Excessive production of ROS and reactive nitrogen species (RNS) in sepsis modifies biological functions of cells (Huet, Dupic, Harrois, & Duranteau, 2011). Since the sepsis severity is associated with the degree of platelet activation, in our previous studies we investigate the role of systemic generation of RNS and ROS in response of platelets to LPS. We earlier demonstrated that LPS induced an intraplatelet ROS/RNS production in blood platelets and we evaluated its consequences on platelet reactivity (Saluk-Juszczak et al., 2007; Saluk-Juszczak, Wachowicz, & Kaca, 2000). LPS like other agonists (thrombin, platelet-activating factor), stimulates production of NO (Saluk-Juszczak et al., 2007) and other ROS/RNS in platelets and causes lipid peroxidation in these cells (Saluk-Juszczak et al., 2000). Our data have provided evidence that overproduction of ROS in LPS-induced blood platelets occurs mainly superoxide radicals ($O_2^{\bullet -}$). Other reactive oxygen species such as H_2O_2, singlet oxygen, and organic radicals are generated (Saluk-Juszczak et al., 2007), moreover we have described the effects of LPS on the metabolism and level of thiols in platelets, since thiol homeostasis determined critical aspects of cell function and cell response. The obtained results clearly indicated the existence of possible link between oxidative stress induced by LPS in platelets and thiol metabolism (Saluk-Juszczak, Wachowicz, Bald, & Gowacki, 2005). The oxidative/nitrative stress in blood platelets is involved in inflammation and leads to the oxidation of platelet thiols, carbonylation and nitration of platelet proteins and lipid peroxidation (Alexandru, Popov, & Georgescu, 2010; Sabetkar, Low, Naseem, & Bruckdorfer, 2002). After incubation of platelets with LPS a distinct increase in platelet protein

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nitration and 3-nitrotyrosine formation (a biomarker of protein damage induced by peroxynitrite ($ONOO^-$) and other reactive nitrogen species) was observed (Saluk-Juszczak et al., 2000). LPS as a platelet agonist acts directly on blood platelets and *in vitro* induces various steps of platelet activation including adhesion to collagen (Saluk-Juszczak, Wachowicz, Zielinski, & Kaca, 2001; Zielinski, Wachowicz, Saluk-Juszczak, & Kaca, 2002). Adhesion of blood platelets may be involved in pathogenesis of septic shock after damage of endothelial cells caused by LPS or inflammatory cytokines (Casarin, Lopes-Pires, Morganti, Antunes, & Marcondes, 2011). Pro-inflammatory consequences of platelet activation are also associated with the release of important pro-inflammatory mediators: IL-1, RANTES, PDGF, PF4, TGF, lipid mediators (TXA_2, PAF) or sCD40 from activated platelet (Zarbock, Polanowska-Grabowska, & Ley, 2007). Platelets are the richest source of soluble CD40L in circulation (Saluk-Juszczak & Królewska, 2010a, 2010b). The responses of platelets to LPS indicate that endotoxin stimulates the platelet secretory process and release of substances stored in platelet granules (Saluk-Juszczak, Wachowicz, & Kaca, 1999).

The overproduction of ROS and RNS may be responsible for the alteration of the structure of platelet compounds and modifies platelet function, what is associated with uncontrolled development of inflammatory reactions leading to sepsis (Andrades, Ritter, & Dal-Pizzol, 2009), therefore antiplatelet therapy should be taken into account. Therefore we focus our attention on the (1 → 3)-β-D-glucan (antiplatelet) properties.

Several experimental studies have demonstrated biological properties of (1 → 3)-β-D-glucan, especially its anti-inflammatory, antioxidant, and antitumor effects (Akramiene, Kondrotas, Didziapetriene, & Kevelaitis, 2007; Pelizon, Kaneno, Soares, Meira, & Sartori, 2005). (1 → 3)-β-D-Glucan derived from the yeast cell walls of *Saccharomyces cerevisiae* is classified as a biological response modifier (BRM) of cellular and humoral immunity because of its ability to stimulate the immune system against infections caused by various pathogens: bacterial, viral, mycotic and microparasitic infections, as well as against malignant cell growth (Laroche & Michaud, 2007). It is supposed that protective and immunomodulative activity of (1 → 3)-β-D-glucan may be partially attributed to its ability to free radical scavenging (Kogan, Stasko, Bauerova, & Palonka, 2005). For this reason we studied the antioxidative activity of (1 → 3)-β-D-glucan against oxidative and nitrative damages of platelet biomolecules caused by two strong physiological oxidants: H_2O_2 and $ONOO^-$ generating *in vivo* in vascular system during oxidative stress. (1 → 3)-β-D-Glucan seems to affect platelet functions (enzymatic cascade of arachidonic acid, platelet aggregation and secretion) induced by physiological agonists (thrombin, ADP, collagen) involved in inflammatory process (Saluk-Juszczak, Krolewska, & Wachowicz, 2010a; Saluk-Juszczak, Krolewska, & Wachowicz, 2010b). Moreover, (1 → 3)-β-D-glucan has powerful protective effects against the impairment of plasma protein and lipid molecules caused by the strong biological oxidants and inflammatory mediators (Saluk-Juszczak, Krolewska, & Wachowicz, 2011). At present, the role of (1 → 3)-β-D-glucan in the platelet activation and oxidative/nitrative stress induced by LPS is unknown.

Therefore, the main purpose of present study is to assess biochemically *in vitro* if the natural occurring polysaccharide, (1 → 3)-β-D-glucan, might combat the oxidative stress caused by LPS stroke associated with nitrative and oxidative damages of human platelet biomolecules. It is unclear, whether (1 → 3)-β-D-glucan can interact with LPS or with its specific Toll-like receptors present on platelet surface. In this study we also make an attempt by *in silico* molecular docking to determine the interactions between the molecules of (1 → 3)-β-D-glucan and LPS, and their impact on the binding of LPS to the TLR receptor as an additional mechanism of antioxidant activity.

2. Materials and methods

2.1. Materials

(1 → 3)-β-D-glucan of *S. cerevisiae*, LPS from *Escherichia coli* and from *Pseudomonas aeruginosa*, rabbit anti-DNP antibody, cytochrome c, thrombin, thiobarbituric acid (TBA) and Bicinchoninic Acid Kit for Protein Determination were purchased from Sigma (St Louis, MO, USA). Biotynylated anti-goat/mouse/rabbit antibody and streptavidin–biotinylated horseradish peroxidase were from DAKO (Gdynia, Poland). Goat anti-nitrotyrosine polyclonal antibodies were from Oxis (Portland, USA). All other reagents were of analytical grade and were provided by commercial suppliers.

Peroxynitrite was synthesized according to the method of Pryor & Squadrito (1995). Freeze fractionation (−70 °C) of the peroxynitrite solution formed a yellow top layer, which was retained for further studies. The top layer typical contained 80–100 mM peroxynitrite as determined spectrophotometrically at 302 nm in 0.1 M NaOH ($\varepsilon_{302\,nm} = 1679\,M^{-1}\,cm^{-1}$).

2.2. Isolation of blood platelets

Human blood was obtained from young (20–25 years) healthy, non-smoking men who denied taking any medication known to interfere with platelet function for at least 14 days previous and had fasted for at least 12 h. Blood was collected into ACD solution (1.36% citric acid/2.5% sodium citrate/2% dextrose; 5:1 v/v). Blood platelets were isolated by differential centrifugation of blood as described by Wachowicz and Kustron (1992). The final platelet concentration was about 4×10^8 platelets/ml. The platelets were counted by the photometric method according to Walkowiak, Michalak, Koziolkiewicz, and Cierniewski (1989). The entire platelet washing procedure was performed in plastic tubes and carried out at room temperature. Washed human platelet suspensions in the modified Tyrode's Ca^{2+}/Mg^{2+} free buffer (127 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM HEPES, 5.6 mM glucose, pH 7.4) were pre-incubated (5 min, 37 °C) with (1 → 3)-β-D-glucan at final concentration of 2 μg/ml, and then were treated with LPS (0.15 or 1.5 μg/ml, 5 min, 37 °C) (a positive control was platelets treated with $ONOO^-$ (0.1 mM, 2 min, 37 °C); with H_2O_2 (2 mM, 5 min, 37 °C) or with thrombin (6 U/ml, 5 min, 37 °C). TBARS and $O_2^{\bullet -}$ levels were measured. To study the level of carbonyl groups and nitrotyrosine in platelets proteins samples of blood platelets were dissolved in lysis buffer (2% Triton-X-100, 100 mM EDTA and 100 mM Tris–HCl; pH 7.4)

The study was performed under the guidelines of the Helsinki Declaration for Human Research and approved by the committee on the Ethics of Research in Human Experimentation at the University of Łódź (KBBN-UŁ/II/21/2011).

2.3. Thiobarbituric acid reactive substances estimation

Samples (control platelets; platelets incubated with thrombin (a positive control) (6 U/ml, 5 min, 37 °C); platelets incubated with (1 → 3)-β-D-glucan (2 μg/ml, 5 min, 37 °C); platelets treated with LPS (0.15 or 1.5 μg/ml, 5 min, 37 °C) or platelets incubated with (1 → 3)-β-D-glucan and by LPS) were mixed with an equal volume of 15% (w/v) cold trichloroacetic acid in 0.25 M HCl and with an equal volume of 0.37% (w/v) thiobarbituric acid in 0.25 M HCl. Then samples were immersed in a boiling water bath for 10 min. After cooling and centrifugation absorbance at 535 nm was measured and results were expressed as nmoles of TBARS per mg of platelet proteins (Wachowicz, 1984).

2.4. $O_2^{\bullet-}$ generation

Samples (control platelets; platelets incubated with thrombin (positive control) (6 U/ml, 5 min, 37 °C); platelets incubated with (1 → 3)-β-D-glucan (2 μg/ml, 5 min, 37 °C); platelets treated with LPS (0.15 or 1.5 μg/ml, 5 min, 37 °C) or incubatedd with (1 → 3)-β-D-glucan and by LPS) were prepared. Generation of superoxide anion radicals ($O_2^{\bullet-}$) was measured by cytochrome c reduction. For that an equal volume of cytochrome c (160 μM) prepared in Ca^{2+}/Mg^{2+} free Tyrode's buffer was added to suspension of platelets. After incubation, the platelets were sediment by centrifugation at 2000 × *g* for 5 min and the supernatants were transferred to cuvettes. Reduction of cytochrome c was measured spectrophotometrically at 550 nm. To calculate the molar concentration of $O_2^{\bullet-}$ an extinction coefficient for cytochrome c of 18,700 M^{-1} cm^{-1} was used (Jahn & Hansch, 1990).

2.5. Detection of protein carbonyl groups in human blood platelets by an ELISA method

Samples (control platelets; platelets with H_2O_2 (2 mM, 5 min, 37 °C) (a positive control); platelets incubated with (1 → 3)-β-D-glucan (2 μg/ml, 5 min, 37 °C); platelets treated with LPS (0.15 or 1.5 μg/ml, 5 min, 37 °C) or platelets after treated with (1 → 3)-β-D-glucan and LPS) were prepared and then the platelet lysates were created by adding into platelet samples of equal volume of lysis buffer: 0.1 M Tris/HCl pH 7,4; 100 mM EDTA; 2% Triton X-100; 2% SDS. Detection of carbonyl groups by an ELISA method was carried out according to a method described previously by Buss, Chan, Sluis, Domigan, and Winterbourn (1997), modified by Alamdari et al. (2005). Concentration of protein in supernatants of platelet lysates were estimated by BCA method. 200 μl of diluted standards, platelet protein samples (5 μg protein per 1 ml PBS) and PBS as blank were added into wells. The micro plates were incubated overnight at 4 °C where proteins were non-specifically adsorbed to an ELISA plate. The wells were washed 3 times with 300 μl PBS, reacted with dinitrophenylhydrazine (DNPH; 0.05 mM, 200 μl, pH 6.2), incubated for 45 min at room temperature in the dark, washed 5 times with 300 μl PBS: ethanol (1:1, v/v) and last time with 300 μl PBS, all in accordance with Alamdari et al. (2005). The anti-dinitrophenylhydrazine (anti-DNPH) antibodies were used to detection of carbonyl groups according to Alamdari et al. (2005) procedure. Hydrogen peroxide oxidized fibrinogen (10 nmol of carbonyl groups/mg of fibrinogen) was prepared for use in the standard curve. The linearity of the ELISA method was confirmed by the construction of a standard curve (nmol carbonyl groups/mg of fibrinogen). The amount of carbonyl groups present in fibrinogen after treatment with H_2O_2 (2 mM) was determined spectrophotometrically as described by Levine et al. (1990).

2.6. Nitrotyrosine in the proteins of human blood platelets determined by a competition ELISA method

Samples (control platelets; platelets with $ONOO^-$ (0.1 mM, 2 min, 37 °C) (a positive control); platelets incubated with (1 → 3)-β-D-glucan (2 μg/ml, 5 min, 37 °C); platelets treated with LPS (0.15 or 1.5 μg/ml, 5 min, 37 °C) or platelets after incubation with (1 → 3)-β-D-glucan treated with LPS) were prepared and then the platelet lysates were used. Detection of nitrotyrosine-containing proteins by a competition ELISA (C-ELISA) method was carried out according to the modified procedure of Khan et al. (1998). The assay was performed in 96-well plates coated with 50 μl of nitro-fibrinogen (at the concentration of 1 μg/ml and 3–6 mol nitrotyrosine/mol protein) which had been blocked with skim milk to prevent non-specific binding. A standard curve was constructed by incubating in the wells serial dilutions of nitro-fibrinogen in 50 mM Tris/HCl buffer (pH = 9.0) containing 0.05% Tween (TBST) with polyclonal anti-nitrotyrosine goat IgG (1:30,000) for 2 h at 37 °C, followed by washing the plate with TBST. Sequential incubations were then performed with biotinylated anti-goat IgG and avidin–biotin horseradish peroxidase complex. After further washing, color development was initiated by the addition of substrate (*o*-phenylenediamine dihydrochloride) and was allowed to develop for up to 20 min at room temperature and terminated by addition of stopping reagent (40% H_2SO_4). Antibody binding was determined from the absorbance at 490 nm. The concentrations of nitrated proteins that inhibited anti-nitrotyrosine antibody binding were estimated from the standard curve and are expressed as nitrofibrinogen (nitro-Fg) equivalents. The amount of nitrotyrosine present in fibrinogen after treatment with peroxynitrite (at a final concentration of 1 mM) was determined spectrophotometrically (at pH 11.5, $\varepsilon_{430\,nm}$ = 4400 M^{-1} cm^{-1}).

2.7. Ligand docking

Docking of (1 → 3)-β-D-glucan (downloaded from http://pubchem.ncbi.nlm.nih.gov/) and *E. coli* LPS (Park et al., 2009) was simulated *in silco* using Autodock Vina 1.0 (Trott & Olson, 2010) (http://vina.scripps.edu/). For TLR4-MD-2 complex grid box of 68 × 68 × 68 points was used with a spacing 1.0 Å and the grid box center was put on *x* = 1.046 y= 20.198 and z= -27.653 whereas for LPS as receptor grid box of 50 × 50 × 50 with center put on *x* = 4.447 *y* = 68.662 and *z* = 53.972 was used. Gasteiger charges were assigned to rigid receptors and flexible ligands molecules. Modeling was performed with extracellular domain of unliganded human TLR4-MD-2 complex processed by Swiss-PdbViewer (Guex & Peitsch, 1997) (http://spdbv.vital-it.ch/) from 3FXI Crystal structure of human TLR4-human MD-2-*E.coli* LPS Ra complex (Park et al., 2009). Analysis of three-dimensional structure of docked ligand and receptor molecules was performed with Autodock Tools v 1.5.6rc1 (Sotriffer et al., 2000) (http://autodock.scripps.edu) and with Swiss-PdbViewer. The most favorable binding mode was presented.

2.8. Data analysis

The statistical analysis was performed by several tests. To eliminate uncertain data, the Q-Dixon test was performed. All values in this study were expressed as mean ± SD. Results were analyzed under the account of normality with Shapiro–Wilk test and equality of variance with Levene test. The statistically significant differences were assessed by the paired Student's *t* test. *P* levels < 0.05 were considered significant.

3. Results

We have shown that (1 → 3)-β-D-glucan at the concentration of 2 μg/ml decreased lipid peroxidation expressed as the amount of TBARS formed in human blood platelets after their activation by LPS. The level of TBARS produced in the presence of both tested LPSs (from *E. coli* and *P. aeruginosa*) was about 1.5–2 fold lower than in platelets stimulated by thrombin (strong platelet agonist). The level of TBARS in platelets treated with *E. coli* LPS was significantly lower ($p < 0.005$ for LPSs at concentration of 0.15 μg/ml; $p < 0.05$ for LPSs at concentration of 1.5 μg/ml) than in platelets treated with LPS from *P. aeruginosa*. The stronger protecting effect of (1 → 3)-β-D-glucan was observed at lower concentration of both used LPSs (0.15 μg/ml) (Fig. 1).

(1 → 3)-β-D-Glucan at the concentration of 2 μg/ml also distinctly reduced (about 20%) the level of $O_2^{\bullet-}$ formed in platelets after LPS stimulation. The inhibition was stronger when the lower concentration of both used LPSs were used (Fig. 2).

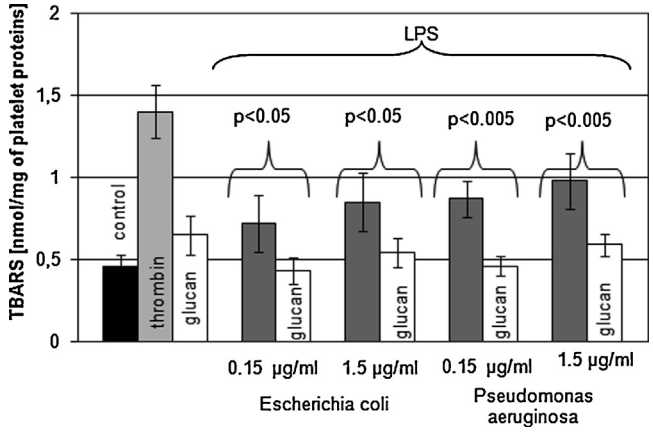

Fig. 1. The effects of (1 → 3)-β-D-glucan on the level of TBARS in platelets treated with LPS of *E. coli* and *P. aeruginosa* (0.15 and 1.5 μg/ml). The results are measured and expressed as nmol TBARS/mg of platelet proteins. The results are expressed as means ± SD; $n = 8$. The effects were statistically significant according to the paired Student's t test (LPS and (1 → 3)-β-D-glucan - treated platelets *vs.* LPS-treated platelets), $p < 0.05$ (*E. coli*) and $p < 0.005$ (*P. aeruginosa*).

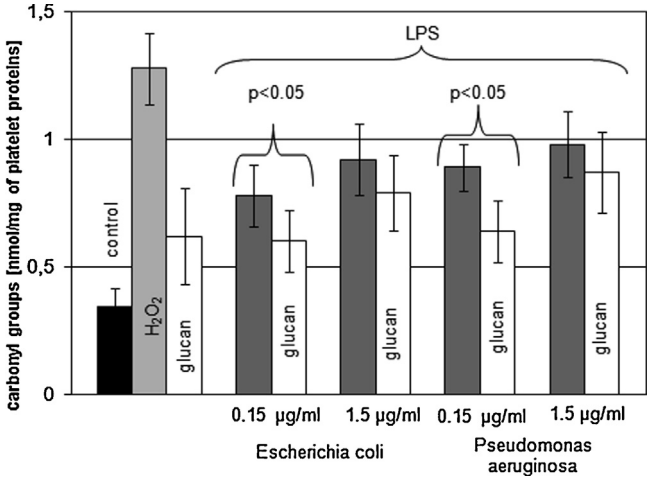

Fig. 3. The effects of (1 → 3)-β-D-glucan on carbonyl groups formation in platelets treated with LPS of *E. coli* and *P. aeruginosa* (0.15 and 1.5 μg/ml). The results are measured and expressed as nmol carbonyl groups/mg of platelet proteins. The results are representative of four independent experiments done in triplicate, and are expressed as means ± SD. The effects were statistically significant for LPS concentration of 0.15 μg/ml, according to the paired Student's t test (LPS and (1 → 3)-β-D-glucan - treated platelets *vs.* LPS-treated platelets), $p < 0.05$.

The results from another set of experiments demonstrated that (1 → 3)-β-D-glucan may partially prevent the oxidative stress in blood platelets caused by LPS, leading to an increase of carbonyl groups in proteins determined by ELISA method. The level of protein carbonylation induced by LPS was only 20–30% lower than amount of carbonyl groups formed by very strong oxidant H_2O_2. The stimulatory effect was dose-dependent for both used LPSs and was stronger for LPS from *P. aeruginosa*. (1 → 3)-β-D-Glucan at the concentration of 2 μg/ml significantly inhibited ($p < 0.05$) protein oxidation, however it was not able to reduce the formation of carbonyl groups after the powerful action of LPS (*P. aeruginosa* 1.5 μg/ml) (Fig. 3).

Our *in vitro* experiments showed that LPSs from *E. coli* and *P. aeruginosa* are responsible for tyrosine nitration in human blood platelet proteins, however the level of 3-NT after LPS treatment was about 2-fold lower than in platelets treated with strong nitrative agents - $ONOO^-$ (a positive control). As assessed by the c-ELISA test, the increase generated 3-nitrotyrosine was dependent on the LPS dose. (1 → 3)-β-D-Glucan at the concentration of 2 μg/ml was found to protect platelet proteins against tyrosine nitration induced by LPS. By the generation of 3-NT the protectory effect of (1 → 3)-β-D-glucan was stronger when of lower LPS concentration (0.15 μg/ml) was used. The comparison of inhibitory effects of (1 → 3)-β-D-glucan on stimulatory action of different LPSs indicates that suppression of 3-NT formation is inversely related to potency of LPS (Fig. 4).

Bioinformatic analysis by Vina docking demonstrated (only the conformation with lowest energy for each compound corresponding to the best bound structure prediction are showed) that both (1 → 3)-β-D-glucan and *E. coli* LPS bound to the same region of human TLR4-MD-2 complex (Fig. 5A and B). The results also revealed higher affinity of (1 → 3)-β-D-glucan to TLR4-MD-2 complex than to *E. coli* LPS (−7.5 *vs.* −6.4 kcal/mol, respectively). In addition, we also determined the interaction between (1 → 3)-β-D-glucan and *E. coli* LPS. We observed that (1 → 3)-β-D-glucan bound with affinity of -5.9 kcal/mol to LPS molecule in the cavity between its lipid and sugar parts (Fig. 5C).

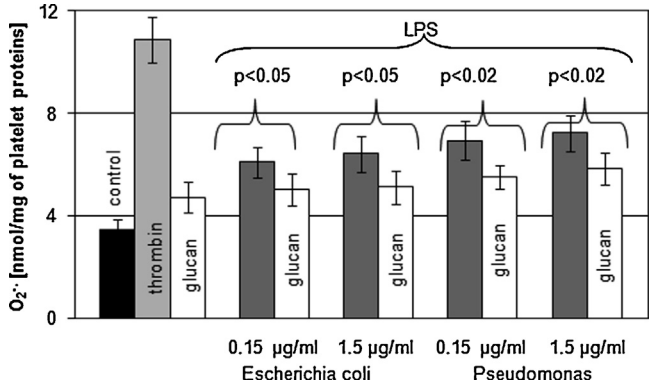

Fig. 2. The effects of (1 → 3)-β-D-glucan on the generation of superoxide anion radicals in platelets treated with LPS of *E. coli* and *P. aeruginosa* (0.15 and 1.5 μg/ml). The results are measured and expressed as nmol O_2^-/mg of platelet proteins. The results are expressed as means ± SD; $n = 6$. The effects were statistically significant according to the paired Student's t test (LPS and (1 → 3)-β-D-glucan - treated platelets *vs.* LPS-treated platelets), $p < 0.05$ (*E. coli*) and $p < 0.02$ (*P. aeruginosa*).

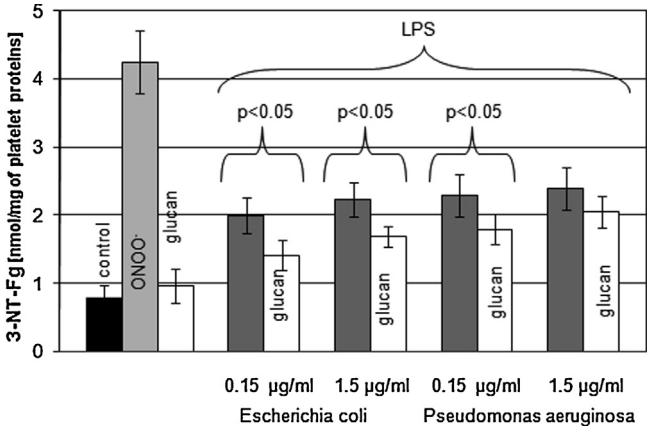

Fig. 4. The effects of (1 → 3)-β-D-glucan on nitration of tyrosine residues in proteins of platelets treated with LPS of *E. coli* and *P. aeruginosa* (0.15 and 1.5 μg/ml), measured by C-ELISA method. The results are representative of three independent experiments done in triplicate, and are expressed as means ± SD. The effects were statistically significant for LPS concentration of 0.15 μg/ml, according to the paired Student's t test (LPS and (1 → 3)-β-D-glucan - treated platelets *vs.* LPS-treated platelets), $p < 0.05$ (*E. coli*) and $p < 0.05$ (*P. aeruginosa*, 0.15 μg/ml).

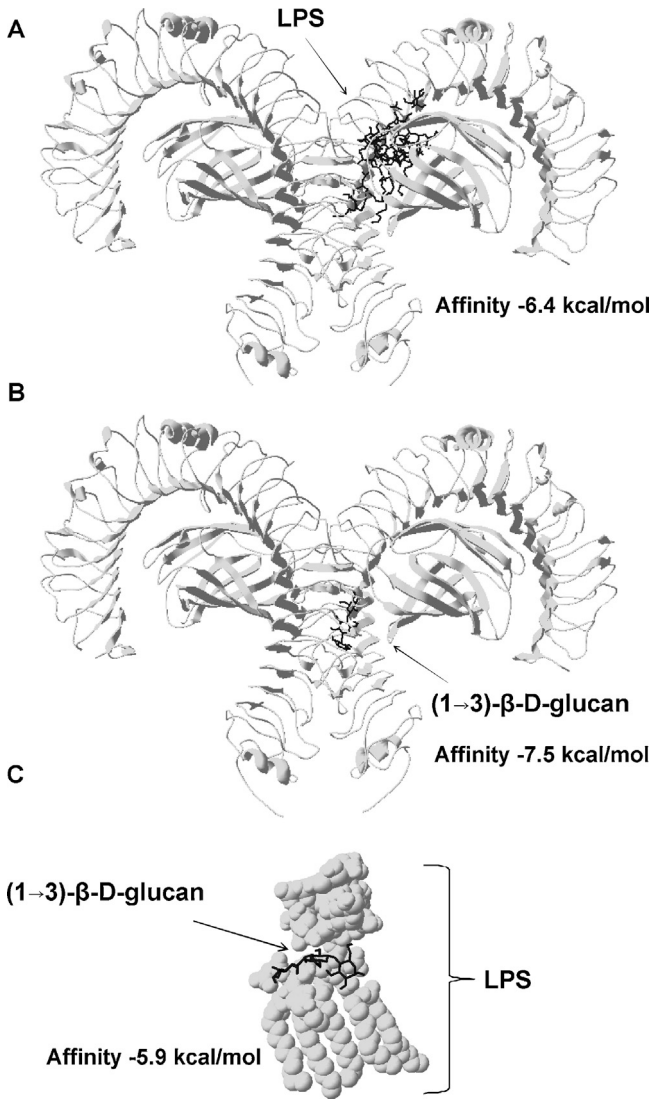

Fig. 5. 3D structures of bioinformatic analysis results. The crystal structure human TLR4-human MD-2-*E.coli* LPS Ra complex (PDB Id:3FXI) was taken from the RCSB PDB databank. 3D structures of (1 → 3)-β-D-glucan was obtained from the PubChem website. Software used to docking was Autodock Vina 1.0 together with Autodock Tools v 1.5.6rc1. Visualization of docking results (conformation with highest affinity) was performed using Swiss-PdbViewer. A – interaction between LPS and TLR4-MD-2 complex, B – interaction between (1 → 3)-β-D-glucan and TLR4-MD-2 complex, C – interaction between LPS and (1 → 3)-β-D-glucan. The affinity of ligand binding is written below.

4. Discussion

The main purpose of our study was to evaluate the influence of (1 → 3)-β-D-glucan from *S. cerevisiae* on destructive activity of LPS (from *E. coli* and *P. aeruginosa*) on human blood platelets. LPS is a potent activator of reactive oxygen species generation in human and the main cause of sepsis developed by Gram-negative bacteria (Peters, Unger, Brunner, & Kirkpatrick, 2003). However, there is little known about the intraplatelet ROS production in sepsis and its consequences on platelet reactivity. Our previous studies indicates that LPS is responsible for platelet activation and overproduction of ROS similar to respiratory burst of immune cells (Saluk-Juszczak et al., 2000, 2005, 2007). Blood platelets beyond to their haemostatic role can function as inflammatory cells in innate immunity. Recently evidence accumulating confirms that platelet activation may be significant in the development of inflammation (Pitchford, 2007). During platelet activation ROS are formed as a by side product of metabolism (Monteiro et al., 2012). They are produced in the receptor-mediated signaling pathways due to arachidonic acid metabolism mediated by cyclooxygenase or lipooxygenase, the glutathione cycle, metabolism of phosphoinositoles and due to activity of xanthine oxidase and NADPH oxidase (Jahn & Hansch, 1990; Wachowicz, Olas, Zbikowska, & Buczynski, 2002). Intraplatelet ROS generation is involved in the regulation of platelet activities (Krotz, Sohn, & Pohl, 2004).

Gram-negative bacteria are among the most serious pathogens for hospitalized patients (Martin & Yost, 2011). *E. coli* is responsible for about 60–80% of all nosocomial infections of the urinary tract, as well as nosocomial pneumonia and infections of the abdomen. These conditions particularly affect immunocompromised people: surgery, after chemotherapy, as well as children and the elderly (Jacobsen, Stickler, Mobley, & Shirtliff, 2008). *P. aeruginosa* belongs to one of the most important and most dangerous microorganisms that cause nosocomial infection (Barie, 2000). It is an opportunistic bacteria that causes infection only in patients with weakened immune systems. Infections caused by *P. aeruginosa* may occure in patients with neutropenia, immunosuppression, severe burns, AIDS and cystic fibrosis. The patients with intra-abdominal surgery, endocarditis, meningitis, urinary tract infections, bone and joint infections are subject to certain risk for *P. aeruginosa* infections.

People with impaired immunity may develop bacteremia with subsequent sepsis (Breidenstein, Fuente-Nunez, & Hancock, 2011). Treatment of *P. aeruginosa* infections is difficult because of the high resistance of the bacteria to most antibiotics (Barsic et al., 1997). In addition, antibiotics can result in an increased release of LPS from the cell surface. Antibiotics are responsible for the death of the bacteria but do not inactivate the LPS activity. One cell of Gram-negative bacteria contains several millions of LPS molecules as the main components of the outer layer of the cell wall. Death and lysis of bacterial cell lead to the release of LPS molecules into the bloodstream, which can further enhance endotoxin effect, leading to sepsis (Wilson et al., 2002). Therefore, the main problem is to find the drugs able to neutralize the toxic effects of LPS, which would reduce the mortality caused by sepsis. In our experiments we used (1 → 3)-β-D-glucan, the polysaccharide well known to increase human resistance to viral, bacterial and parasitic infections and to possess therapeutic properties associated with anti-infective and antitumorigenic action (Laroche & Michaud, 2007). The antioxidative properties of (1 → 3)-β-D-glucan were extensively investigated and it is postulated that its protective and immunomodulative activity may partially be attributed to its ability to exert free radical scavenging properties (Kogan et al., 2005; Saluk-Juszczak et al., 2010a, 2010b, 2011). Our previous results demonstrated that (1 → 3)-β-D-glucan from *S. cerevisiae* distinctly reduced free radical generation in blood platelets as well as the oxidation and nitration of platelet proteins induced by $ONOO^-$ (0.1 mM) and H_2O_2 (2 mM) (Saluk-Juszczak et al., 2010a, 2011). The presented experiments have been designed to investigate the role of (1 → 3)-β-D-glucan in the defence of blood platelets against LPS action by measurement of well-known markers of platelet activation associated with arachidoniate metabolism and oxidative stress such as TBARS level, $O_2^{\bullet -}$ generation, protein carbonyl groups formation and tyrosine residue nitration together with hypothetical role of D-glucan in interaction with platelets Toll-like receptor which is known to bind LPS and start signal transfer leading to ROS/RNS generation.

Our previous findings (Saluk-Juszczak et al., 2010a, 2010b, 2011) clearly confirmed that (1 → 3)-β-D-glucan from *S. cerevisiae* has antioxidative and antiplatelets properties *in vitro* at the concentrations that may correspond to its physiological levels in blood. The recommended, preventive oral dosage of (1 → 3)-β-D-glucan is normally about 2 mg per 1 kg of body mass, daily (Babineau et al., 1994). In our earlier studies we used (1 → 3)-β-D-glucan

at the concentration of 1; 2 and 4 μg/ml and we showed the concentration-dependence of the protective effect of this compound. However the results from our previous study showed the similar effect of (1 → 3)-β-D-glucan for all tested concentrations. For this reason, in the present study to determine the effect of glucan on oxidative stress caused by the action of LPS, we have used only one dose of (1 → 3)-β-D-glucan (2 μg/ml).

The platelet membranes contain a high amount of polyunsturated fatty acids in the phospholipids that can be the potential site of oxidative stress. Oxidative damage of blood platelet membrane may lead to its altered structure and function contributing to changes in signal transduction. The peroxidation of membrane lipids changes fluidity of membranes, their permeability and exposition of receptors (Miller, Mrowicka, Saluk-Juszczak, & Majsterek, 2011). The determination of lipid peroxidation products relies on different methods. The most widely used marker of lipid peroxidation is the level of malonyldialdehyde (MDA). The commonly used assay for MDA bases on the reaction with thiobarbituric acid and formation of TBARS. On the other hand, the TBARS determination (MDA) in activated platelets is used as a marker of endogenous arachidonate metabolism by thromboxane A_2 synthesis (Abd El-Aal, 2012). The responses of platelets to LPS suggest that endotoxin, similar to thrombin (a strong platelets agonist), stimulates an enzymatic cascade of platelet arachidonate *via* cyclooxygenase and produces thromboxane A_2 (TXA_2) concomitant with malonyldialdehyde (MDA). Our data support the suggestion that (1 → 3)-β-D-glucan may contribute to the maintenance of redox balance in LPS-treated platelets to restore the platelet response.

During blood platelet activation stimulated by an agonist, a signal is transmitted into the platelet that produces different signaling molecules involved in biochemical events, including reactive oxygen/nitrogen species. ROS/RNS may behave as signaling molecules—second messengers—and may regulate platelet functions (Krotz et al., 2004). We investigated the effects of (1 → 3)-β-D-glucan on $O_2^{\bullet -}$ production in blood platelets and presented evidence that (1 → 3)-β-D-glucan inhibits $O_2^{\bullet -}$ generation in stimulated by LPS cells. We may suppose that its antioxidative properties may be involved in the anti-platelet action of this polysaccharide. Agonist-induce platelet stimulation leading to the release of endogenous arachidonic acid from membrane phospholipids and its immediate metabolism by cyclooxygenase, causes also the activation of phosphoinositides 3-kinase (PI 3-K). PI 3-K is involved in receptor-mediated ROS production in platelets and supports the importance of ROS signaling in platelet functions (Gregg, de Carvalho, & Kovacic, 2004). The enzymatic pathway of arachidonic acid also is associated with $O_2^{\bullet -}$ generation. $O_2^{\bullet -}$ production in activated platelets is mainly dependent on NADPH oxidase or xanthine oxidase activity (Jahn & Hansch, 1990; Wachowicz et al., 2002). The suppressed production of both, MDA and superoxide anion observed in the presence of (1 → 3)-β-D-glucan suggests that (1 → 3)-β-D-glucan may affect platelet activation partly by inhibition of arachidonic acid pathway. This finding may be particularly important in the case of *P. aeruginosa* infections associated with a potent cytotoxin production, in relation to phospholipase A_2 activity (Sato & Frank, 2004).

ExoU is the virulence factor that enhances the bacteria drug-resistance and can induce haemostatic abnormalities. The inflammatory ability of ExoU is responsible for induce vascular hyperpermeability, platelet activation, and thrombus formation during *P. aeruginosa* pneumosepsis (Machado et al., 2011).

Inflammatory conditions support oxidative and nitrative modifications in some amino acids (Cannizzo, Clement, Sahu, Follo, & Santambrogio, 2011). The contribution of inducible nitric oxide synthase (iNOS) to oxidative/nitrative stress is well-documented in inflammation (Yang, Taboada, & Liao, 2009). Formation of nitric oxide and its derivative reactive nitrogen species during endotoxemia has been implicated in the pathogenesis of the associated cardiovascular dysfunction. The nitrative stress can promote nitrosative post-translational modifications of proteins that in turn may alter their activity and contribute to dysregulation (Sun & Murphy, 2010). However, concomitant formation of superoxide radicals may cause lowering of nitric oxide and reduce S-nitrosylation of protein due to simultaneously generate the potent tyrosine-nitrating agent peroxynitrite (Reiter, Teng, & Beckman, 2000). Protein tyrosine residues appear to be main targets for nitration, and the presence of 3-NT in proteins, as an usual modification is a good marker of oxidative/nitrative abnormalities (Forde & Fitzgerald, 1997). In current study we assessed that tyrosine nitration in blood platelets treated *in vitro* by LPS occurred. Quantification of nitrotyrosine was performed using immunoenzymatic assay. The levels of 3-NT were significantly elevated in platelets under inflammation conditions (treated with LPSs) and correlated with the LPS concentrations. We used the c-ELISA method to assay 3-nitrotyrosine level to monitor the changes in nitrosative protein oxidation during platelet activation by LPS in the presence of (1 → 3)-β-D-glucan. The results c-ELISA analysis established that (1 → 3)-β-D-glucan is effective in reducing of blood platelet protein nitration caused by both LPSs from *E. coli* and *P. aeruginosa*.

Amino acid modifications may be responsible for the loss of some biochemical and physiological protein functions and lead to the pathological consequences. Proteins constitute one of the major targets of ROS, and oxidation of proteins can be responsible for their conversion to forms more susceptible to degradation by proteinases (Droge, 2002). Among amino acid modifications by ROS, the formation of carbonyls as a result of oxidation of arginine, lysine, threonine, or proline is well known. Protein carbonylation is the result of secondary reactions of amino groups of lysine residues with reducing sugars or their oxidation (glycation/glycoxidation reactions) and also as reactions of lysine, cysteines, or histidine with α- and β-unsaturated aldehydes formed during the peroxidation of polyunsaturated fatty acids (Uchida & Stadtman, 1993). Protein carbonyl formation is an index of oxidative stress and it has been well established that protein carbonylation is increased significantly during sepsis (Barreiro et al., 2005). In the current study, we measured carbonyl formation in blood platelet proteins detected by their reaction with 2,4-dinitrophenylhydrazine (DNPH) and the conversion to hydrazones. Carbonyl formation is widely used as an important marker of protein oxidation (Dalle-Donne, Rossi, Giustarini, Milzani, & Colombo, 2003). We showed a significant rise in protein carbonyls level in the blood platelets in response to LPS. Our results are in general agreement with previous studies confirming significant increase of ROS generation in blood cells during inflammatory conditions (Rutkowski, Pancewicz, Rutkowski, & Rutkowska, 2007). The increased protein carbonylation was significantly diminished when platelets were preincubated *in vitro* with (1 → 3)-β-D-glucan. It indicates that (1 → 3)-β-D-glucan could play an important role in the maintenance of redox balance in LPS-treated platelets to restore the normal cell response.

In this study, we present for the first time the possible direct or indirect interaction of (1 → 3)-β-D-glucan with LPS and TLR receptor, that lead to reduced toxic effects of endotoxin on blood cells. We would like to elucidate the molecular mechanisms responsible for protective action of (1 → 3)-β-D-glucan against LPS molecule. The research, regarding potential interactions between (1 → 3)-β-D-glucan and LPS and TLR receptor, may also partly explain the phenomenon connected with the development of sepsis. The knowledge about sepsis is still limited and the treatment of infections induced by LSP of Gram-negative bacteria remains a significant problem in medicine. Due to the supported activity of LPS, even after the death of the bacterial cell, it is necessary to

find the mediator binding and neutralizing the adverse effect of LPS. (1 → 3)-β-D-Glucan as well known antibacterial agent with its effectiveness and application has been confirmed in various bacterial infections. However, so far there are no data describing the interaction of (1 → 3)-β-D-glucan and LPS molecules.

Used in our study (1 → 3)-β-D-glucan from *S. cerevisiae* belongs to the class of naturally occurring polymer of (1 → 3)-β-D-linked glucoses. This homopolysaccharide is a polysaccharide composed entirely of glucose residues with glycosidic linkages (1 → 3) between monosaccharide units. The configuration of the sugar (all monosaccharide units) is β-D. The linkage designation shows the carbon atoms involved in the glycosidic bonds. The major linkage in this polymer is β (1 → 3), but the polysaccharide has not only a principal chain ('backbone') but also consist of olygosaccharide chains linked to polysaccharide with glycosidic linkages β (1 → 6).

Toll receptors were first identified in larvae of *Drosophila melanogaster*; receptors with similar structure and activity to the similar receptors found later in mammalian cells are called Toll-like (Toll-like receptor – TLR) (Majewska & Szczepanik, 2006). TLRs are family of membrane proteins which are characterized by an extracellular domain of leucine rich repeats (LRR) and an intracellular toll-IL-1 receptor (TIR) domain (McGettrick & O'Neill, 2010). TLR 4 also designated as CD284 was the first-discovered mammalian homologue of *Drosophila* Toll. For physiological recognition of LPS by TLR4 this receptor must be associated with an extracellular adaptor protein: MD2. To bind LPS to the TLR4-MD-2 requires a complex LPS binding protein (LBP) and CD14 molecule. LBP, a serum glycoprotein, is a lipid transferase which promotes the transfer of LPS from bacterial outer membranes to CD14 which bind LPS to TLR4-MD-2 (Tobias, Soldau, Gegner, Mintz, & Ulevitch, 1995; Wright, Ramos, Tobias, Ulevitch, & Mathison, 1990). TLR4-MD-2 complex is formed before LPS links to two chemically distinct regions, the A and B patches provided by the N-terminal and the central domains of TLR4 (Park et al., 2009). Activation of the TLRs results in the recruitment of TIR domain-containing cytoplasmic protein: MyD88 (Kenny & O'Neill, 2008) which activated cGMP-dependent protein kinase pathway. Blood platelets express components of the LPS receptor-signaling complex, including TLR (TLR4), CD14, MD2, and MyD88. Cognasse et al. (2005) showed that Toll-like receptor expression is significantly increased following activation, in both permeabilized and intact human platelets. Recently, TLR4 play also an important role in LPS-induced thrombocytopenia (Aslam et al., 2006).

LPS consists of three covalently linked regions; lipid A, core oligosaccharide, and an O side chain. Lipid A, which is mainly responsible for the toxicity of LPS, consists of six fatty acyl chains linked to two glucosamine residues. In the crystal structure these lipid chains interact with the hydrophobic pocket in MD-2 (Park et al., 2009).

The *in silico* docking methods has undergone significant changes and improvements over the last years. In present, docking a small molecule to a protein is the first step in explaining the mechanism of some compounds action as well as this methods are new generation tools for new drugs search (Mihasan, 2012). The typical samples for docking programs are multiple ligand conformations, and each conformation is attempted to fit, or dock into a known binding pocket structure. Receptor-ligand interactions are subsequently evaluated using a fast scoring function to estimate binding affinity. AutoDock Vina is one of the accurate and reliable softwares available for drug discovery, molecular docking and virtual screening (Sandeep, Nagasree, Hanisha, & Kumar, 2011). AutoDock Vina combines some advantages of knowledge-based potentials and empirical scoring functions: it extracts empirical information from both the conformational preferences of the receptor–ligand complex and from experimental affinity measurements. Ligands are ranked based on an energy scoring function and, to speed up the score calculation, a grid-based protein–ligand interaction is used (Trott & Olson, 2010).

We observed in our Autodock analysis that LPS bound to the same region as presented in the crystal structure of TLR4–MD-2–LPS complex published by Park et al. (2009). This first step of our *in silico* research confirmed the efficiency of used molecular docking method. (1 → 3)-β-D-Glucan docking showed binding (with higher affinity) to TLR4-MD-2 complex in region where LPS lipid part was bound. In this way, (1 → 3)-β-D-glucan might interfere with the binding of LPS to TLR4-MD-2 complex and protect blood platelets against endotoxin activation. (1 → 3)-β-D-Glucan could also interact directly with unbound LPS molecule but this interaction probably might not play a such important role due to the lower affinity of (1 → 3)-β-D-glucan to LPS in comparison with its affinity to TLR4-MD-2 complex. The role of (1 → 3)-β-D-glucan in competition for a place of binding in Toll like receptors could be really important mechanism of protection against activation of pathways leading to oxidative stress involving LPS.

Ji et al. (2013) study demonstrated inhibition of LPS inflammatory action by other carbohydrate – chitosan. While Qiao et al. (2010) reported that chitosan oligosaccharides are potential inhibitive effector of LPS by binding to TLR4/MD-2 receptor complex.

Our results obtained in the current and previous studies (Saluk-Juszczak et al., 2010a, 2011) showed antioxidative activity of (1 → 3)-β-D-glucan against oxidative and nitrative damages of platelet biomolecules. In the present work, we conclude that protective mechanism of (1 → 3)-β-D-glucan against LPS action on blood platelets is due to as well: its antioxidant properties, as to its interaction with LPS-binding region of TLR4-MD-2 complex. Such dual properties of (1 → 3)-β-D-glucan make this compound an excellent remedy to counteract complications induced by LPS. Modifications of (1 → 3)-β-D-glucan *in silico* could enable design of new antioxidant derivatives with even better properties of TLR blocking, preventing complications initiated by LPS in human organism.

Our future experiment will be carried out in the direction of detailed characterization of (1 → 3)-β-D-glucan interaction with Toll-like receptor (among others by using Biosensor on Biacore system) which should indicate role of (1 → 3)-β-D-glucan against toxic effect of LPS in multiple cells.

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