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Original article

Stimulation of neutrophil oxidative burst by calcium phosphate particles with adsorbed mucin

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Abstract: *Objective* — Mucin can promote formation of gallstones via precipitation with calcium phosphate. The proinflammatory effect of mucin-coated particles is still unclear, and our aim was to study the role of mucin sorption in activation of neutrophil respiratory burst.
Material and Methods — Polydisperse calcium phosphate nanowires (CP) were prepared from hot gelatin solution and according to scanning electron microscopy (SEM) had the length 1-10 μm and thickness 50-450 nm. CP were incubated in mucin or human serum albumin (HSA) giving CP-Muc and CP-HSA. Their hemolytic activity towards human erythrocytes was assayed, and neutrophil lucigenin- and luminol- chemiluminescence (Luc-CL and Lum-CL) response to CP, CP-HSA and CP-Muc was measured. Cytokine RNA was detected in neutrophils by means of reverse transcription with subsequent real-time PCR. Cytokines (IL-1β, IL-6, IL-8, IL-10) were assessed in cell medium by ELISA.
Results and Conclusion — Hemolytic activity of CP was 3.0±0.5%, mucin sorption (0.019 mg/mg) reduced it to 0.24±0.04% ($p<0.05$) as well as HSA. CP and CP-HSA stimulated neutrophil Lum-CL and Luc-Cl by 2-3 times vs. spontaneous values while for CP-Muc the effect was 10-fold and higher. No increased cytokine gene expression or cytokine secretion was detected after 1h incubation of neutrophils with samples. Obviously, sorption of mucin but not that of HSA stimulated generation of reactive oxygen and halogen species with no increase in cytokine production. Thus, the mucin-coated CP has the potential to contribute to gallstone-associated cholecystitis via oxidative damage of mucosa and epithelium.

Keywords: calcium phosphate, mucin, neutrophils, chemiluminescence.

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Introduction

Exogenous nano- and microparticles of calcium phosphate can be inhaled by people, ingested with food additives or toothpaste [1] while co-precipitation of Ca^{2+} and PO_4^{3+} in the small intestine [2] or gallbladder [3] are considered as sources of endogenous particles. Mucin, a major component of mucosa, is not only absorbed by these particles but is also actively incorporated [2, 4]. Mucins are high molecular-weight glycoproteins (120-1000 kDa) with sialylated oligosaccharides. Adsorption of mucin by hydroxyapatite is related mainly to Ca-dependent electrostatic interactions between calcium ions and COO- of mucin [5]. The possible role of inflammatory neutrophil-derived factors in gallstone pathogenesis, including reactive oxygen and NETs, is extensively studied [6]. Earlier it was shown that crystals coated with plasma proteins can stimulate greater chemiluminescent response of polymorphonuclear leukocytes than untreated particles [7], but little is known about effects of adsorbed mucin. Mucin is considered rather as a coating preventing excessive activation of neutrophils induced by bacteria [8] or polymer surfaces [9]. It can scavenge hydroxyl radicals, which are formed in respiratory burst of neutrophils [10]. The aim of the present work was to investigate the chemiluminescence (CL) of neutrophils activated with calcium phosphate particles coated with mucin. We also assessed cytokine RNA expression and their secretion by neutrophils incubated with the particles. Polydisperse calcium phosphate nanowires (CP) were studied as calcium phosphate nano- and microparticles varying in the length and thickness. Both lucigenin and luminol were used to amplify neutrophil CL via reaction with superoxide anion-radical and hypochlorite, correspondingly, as respiratory burst products. Hemolytic activity was also assayed to control possibility of direct cell membrane damage [11, 12].

Material and Methods

Synthesis of polydisperse calcium phosphate nanowires (CP)

CP were synthesized according to slightly modified procedure described in the literature [13]. Briefly, 5.98 g $CaCl_2 \cdot 2H_2O$ (EP grade) and 4.8 g urea (USP grade) were dissolved in water solution (1.9 L) of gelatin (1 gL) at 80 ℃. Then a water solution (100 mL) of 6.24 g $NaH_2PO_4 \cdot 2H_2O$ (purum) was added dropwise within an hour and obtained mixture was gently refluxed for 3 days. Finally, the precipitate was centrifuged, washed twice with isopropyl alcohol (IPA) and vacuum desiccated at 60 ℃.

SEM

CP powder was sonicated in isopropyl alcohol for 10 min, deposited on clean Si-wafer and examined with a Zeiss Merlin microscope equipped with Gemini II Electron Optics (Zeiss, Oberkochen, Germany). SEM imaging was performed at 1-5 kV accelerating voltage and 100-300 pA probe current.

ζ-potential measurement

The ζ-potential of the particles and mucin was measured using Zetasizer (Nano ZS, Malvern, UK) and estimated using Smoluchowski's equation.

Sorption of mucin and human serum albumin (HSA) by CP

Mucin from porcine stomach (Sigma) or HSA (Sigma) were thoroughly dissolved in 0.15 M NaCl. CP was suspended in 0.15 M NaCl (20 mg/mL) and mixed with equal volume of protein solution (20 mg/mL), incubated at 37 ºC for 30 min under vortexing. The control samples were mixed with 0.15 M NaCl instead of protein solution. Then the CP, CP with sorbed mucin (CP-Muc) or with sorbed albumin (CP-HSA) were separated by centrifugation and the supernatants were further analyzed for protein sorption evaluation. The pellets were washed twice with 0.15 M NaCl and then resuspended in sterile saline.

Protein sorption evaluation

The unbound portion of protein was assayed in supernatants by absorbance at 214 nm and by analytical size exclusion chromatography in the Biofox 17 SEC 8×300 mm column (Bio-Works, Uppsala, Sweden) in a solution of 0.15 M NaCl [14]. Smartline chromatographic system (Knauer, Berlin, Germany) has been used. Preliminarily, the column was calibrated using solutions of purified mucin with different concentrations (0.01-1.00 mg mL^{-1}). A total of 0.02 mL of the mucin solution were used for the chromatography analysis at the elution rate of 0.5 mL min^{-1}. Absorbance of the eluted solutions was measured using the UV detector at wavelengths 214 nm and 260 nm.

Blood cells isolation

Human erythrocytes and neutrophils were isolated from normal blood of 4 volunteers, on the basis of their informed consent. Erythrocytes were sedimented by centrifugation of each blood collected with EDTA as anticoagulant, at 400g and then washed with 0.15 M NaCl. Another blood volume was layered over the double gradient of Histopaque 1.077/1.119 gL and after centrifugation for 45 min neutrophils were collected and washed with Krebs-Ringer solution.

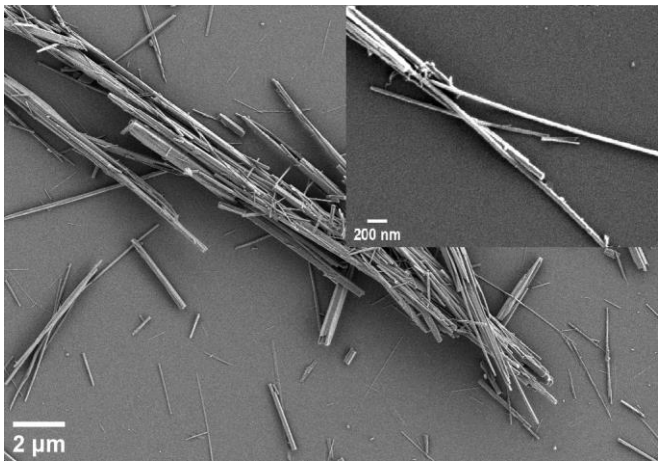

Figure 1. SEM images of CP. CP, polydisperse calcium phosphate nanowires.

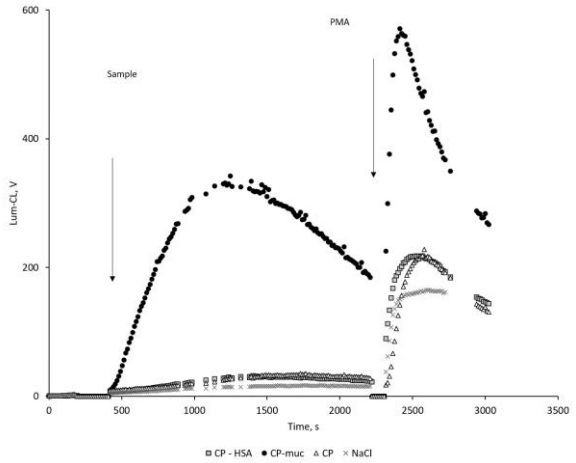

Figure 2. Time-course of neutrophil's Lum-CL stimulated with CP, CP-HSA, CP-Muc and NaCl, and subsequent PMA addition. Lum-CL, luminol-dependent chemiluminescence; CP, polydisperse calcium phosphate nanowires; CP-HAS, CP with sorbed human serum albumin; CP-M, CP with sorbed mucin; PMA, phorbol-12-myristate-13-acetate.

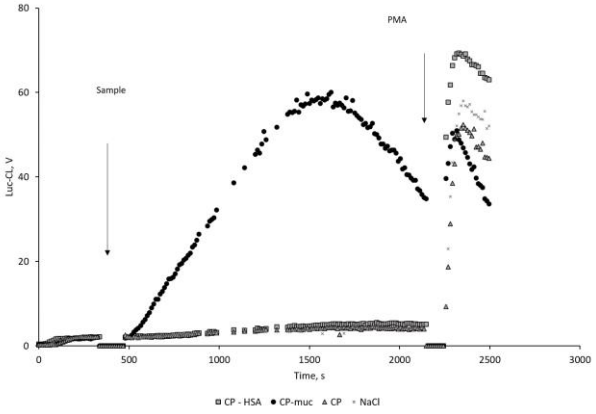

Figure 3. Time-course of neutrophil's Luc-CL stimulated with CP, CP-HAS, CP-Muc and NaCl, and subsequent PMA addition. Luc-CL, lucigenin-dependent chemiluminescence; CP, polydisperse calcium phosphate nanowires; CP-HAS, CP with sorbed human serum albumin; CP-M, CP with sorbed mucin; PMA, phorbol-12-myristate-13-acetate.

Hemolytic activity assay

40 ul of CP, CP-HSA or CP-Muc suspension (10 mg/mL) was mixed with 40 ul of erythrocytes and 0.15 M NaCl was added so that final concentration of particles was 1.67 mg/mL. The mixtures were incubated for 2 hr. at 37 °C. The erythrocytes and particles were separated by centrifugation and supernatants were collected for further hemoglobin (Hb) assay by absorbance at 540 nm. The total hemolysis was induced by distilled H_2O; to assay spontaneous hemolysis 0.15 M NaCl was added to erythrocytes instead of particles. The results were expressed in %: HA=(Hbx-Hbsp)/(Hbt-Hbsp) × 100%, where HA-hemolytic activity (%); Hbx, Hbt, Hbsp – hemoglobin concentration (mg/mL) in supernatants of the probes with particles, H_2O or NaCl, correspondingly.

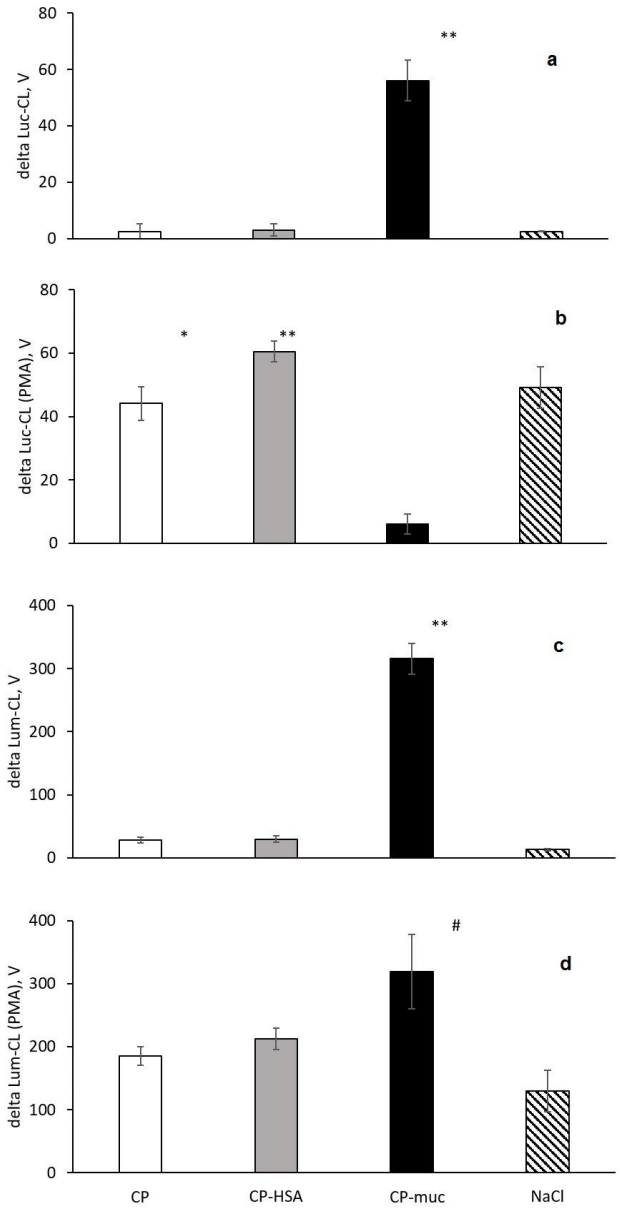

Figure 4. Amplitude of neutrophils' Luc-CL (a, b) and Lum-CL (c, d)-response stimulated with CP, CP-HSA, CP-Muc and NaCl (a, c) and to subsequent PMA addition (b, d).

** p<0.001; * p=0.004; # p=0.019 vs. CP.

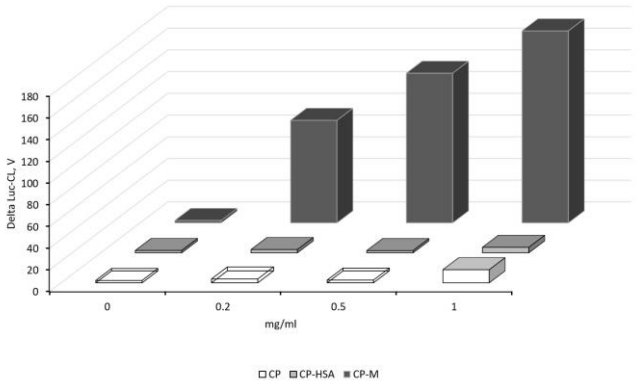

Figure 5. Amplitude of neutrophils' Luc-CL stimulated with CP, CP-HSA, CP-M at various concentrations. Luc-CL, lucigenin-dependent chemiluminescence; CP, polydisperse calcium phosphate nanowires; CP-HAS, CP with sorbed human serum albumin; CP-M, CP with sorbed mucin.

Chemiluminescent assay (CL)

CL was measured with the use of luminometer Lum1200 (DiSoft, Moscow) in 0.5mL of Krebs-Ringer solution (pH7.4) with 0.2 mM luminol (Lum-CL) or lucigenin (Luc-CL), 2% of autologous blood serum, 0.5-0.7x10^6 neutrophils and 1 mg/mL particles. Spontaneous CL was measured before particles addition, then CP, CP-HSA or CP-Muc was added and CL was registered until maximum values were reached; the CL amplitude (V) was calculated as difference between maximum and spontaneous values. If necessary, 100 ng/mL phorbol-12-myristate-13-acetate (PMA) as a second neutrophil activator was added after maximum was reached.

Cytokine RNA expression

Cytokine RNA was detected by means of reverse transcription with subsequent real-time PCR (RT^2-PCR) with fluorescent hydrolysis probes. Total nucleic acids were isolated from 50 µl of control intact and experimental neutrophils after their treatment with CP, CP-HSA or CP-Muc for 1 hr. at 37 °C, by using Proba-NK kit (DNA-technology, Russia). Then the reverse transcription was performed using Reverta-L kit (AmpliSens, Russia). RT^2-PCR to detect mRNA of human interleukins with specific primers and probes was performed as previously described [15].

Cytokine ELISA assay

Neutrophils (2x10^6 cells/mL) were incubated with CP, CP-HSA, CP-Muc (1 mg/mL) or with 0.15 M NaCl in Krebs-Ringer solution with 2% blood serum for 1 hr. at 37 °C. Supernatants after centrifugation were collected and stored at -60 °C until analysis. Cytokines (IL-1β, IL-6, IL-8, IL-10) were assayed with use of ELISA kits (OOO Cytokine, Russia) according to manufacturer instructions.

Statistics

Statistical significance levels were calculated with the Student's t-test using Statistics 12 (StatSoft). Data are represented as mean value ± standard deviation.

Table 1. RNA expression in neutrophils incubated with CP, CP-Muc and NaCl (delta Ct, %)

	IL-6	IL-8	IL-10
NaCl	100	100	100
CP	108	98	102
CP-Muc	108	98	103

CP, polydisperse calcium phosphate nanowires; CP-Muc, CP with sorbed mucin.

Table 2. Concentration of interleukins in cell media after incubation of neutrophils with CP, CP-M and NaCl (pg/mL)

	IL-1β	IL-6	IL-8	IL-10
NaCl	1.6±0.1	4.9±0.1	22.8±2.8	2.7±0.7
CP	0	6.0±1.2	18.6±4.4	3.5±2.3
CP-Muc	0	4.0±1.7	22.1±5.0	1.8±0.8

CP, polydisperse calcium phosphate nanowires; CP-Muc, CP with sorbed mucin.

Results

According to SEM images (*Figure* 1) CP had the length 1 to 10 µm and thickness 50 to 450 nm. Small fraction of nanorods with thickness of about 0.5 µm was present, but each nanowire was a bundle of thinner nanowires with thickness of about 30 nm. These particles adsorbed mucin in the range of 0.023±0.003 mg/mg and this sorption resulted in increase of net negative charge from -0.74±0.58 mV to -9.2±0.7 mV.

Hemolytic activity of the particles is considered as a measure of cell membrane damage so the study of CP bioactivity started with erythrocytes. CP hemolytic activity was 3.0±0.5% and protein sorption reduced it to spontaneous values: 0.24±0.04% for CP-Muc and 0.55±0.22% for CP-HSA, $p<0.05$ vs. CP.

According to data of Lum-CL (*Figure* 2) and Luc-CL (*Figure* 3) measurement, CP-Muc activated neutrophils to significantly greater extent than CP or CP-HSA. Unbound mucin up to 0.5 mg/mL induced no Luc-CL of neutrophils (data not shown).

Neutrophils' Cl-response was evaluated by amplitude, and data of independent experiments are summarized in *Figure* 4 (a-d).

Neutrophil activation by CP-Muc resulted in CL amplitude increase but did not influence PMA-induced Lum-CL response which could mean that cells remain viable and active. Under the same conditions PMA-induced Luc-CL response after CP-Muc neutrophil stimulation was suppressed compared with responses after CP, CP-HSA and NaCl. Since amplitude is represented as difference between maximum value at PMA stimulation and values before addition of PMA, lack of Luc-Cl values could be interpreted as exhaustion of NADPH-oxidase capacity for further increase of superoxide generation rate.

CL amplitude of neutrophils stimulated by particles depended on their concentration as shown for Luc-CL (*Figure* 5).

Neutrophil activation could result not only in respiratory burst but also in pro- and anti-inflammatory cytokine secretion. The expression level was determined by threshold cycle (Ct) delta (*Table* 1).

No significant difference between the samples was found. These results were confirmed by ELISA assay of interleukin secretion (*Table* 2).

Discussion

One of mucin functions is the protection of the mucosa and epithelium. Not only physical and chemical properties of mucins are important for its protective function but also its immunoregulatory role. Interaction of mucin with some bacteria can reduce inflammatory response via coating them and thus hindering further binding of complement proteins [16]. Sialic acids which are components of mucin oligosaccharide chains could interact with neutrophil receptors (Siglecs) known to dampen innate immune cell activation [17, 18]. Airway mucins suppress the neutrophil oxidative burst through a charge dependent mechanism and bacterial killing [8]. Bound with membranes mucins regulate cell adhesion via lectin-carbohydrate interactions. Some oligosaccharide determinants of mucin such as sialylLe^x (NeuAcα2→3Galβ1→4[Fucα1→3]GlcNac are recognized by neutrophil L-selectin [19].

Soluble mucins are easily sorbed by various surfaces [9] and calcium phosphate [5] or calcium carbonate particles [20] changing their properties. The effects of mucin coating on innate immune functions are still to be studied. Activation of neutrophil respiratory burst is one of the key reactions which characterize bioavailability of the foreign or endogenous particles and their proinflammatory properties. Calcium phosphate nanowires (CP) studied in the present work demonstrated high polydispersity and effects of their size and geometry remains to be investigated [21]. The commercial mucin from pig's stomach was used, with a surface charge of -15±2 mV and hydrodynamic diameter of 30-300 nm [14]. Mucin sorption increased net negative charge of CP which is consistent with data of other authors [5]. Hemolytic activity of CP was no more than 3% and mucin or HSA sorption gave additional protective effect towards erythrocytes. Unexpected enhancement of Lum-CL and Luc-Cl by CP-Muc unlike CP or CP-HSA could be explained by mechanism based rather on conformational effects than specific receptor binding. Mucin deposition on the particles facilitated multiple and cooperative interaction between its oligosaccharide determinants and neutrophil lectins, including L-selectin [22, 23], in the presence of calcium ions of CP. Probably, at least some part of the nanowires was bacterial-sized in length or thickness and that could favor neutrophil lectin clustering. No cytokine RNA expression or cytokine secretion was registered, and earlier the same was shown for dendritic cells activated by mucin, with except for IL-8 [24]. Even though the exact mechanism of the neutrophil activation with mucin-coated CP is unclear yet, its pathogenetic role is highly probable. The precipitation of mucin with calcium and phosphate ions stimulates cholesterol crystallization while activation of neutrophils by such particles induces further mucin production and inflammation [6, 7].

Conclusion

Our results have shown significant proinflammatory reaction of neutrophils towards CP coated with mucin and also gave a new perspective in analysis of proteins bioactivity.

Conflict of interest

The authors have no *conflicts of interest* to declare

Funding

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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