PHYSIOLOGICAL CONCENTRATIONS OF CALCIPROTEIN PARTICLES TRIGGER ACTIVATION AND PRO-INFLAMMATORY RESPONSE IN ENDOTHELIAL CELLS AND MONOCYTES

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Abstract. Supraphysiological concentrations of calciprotein particles (CPPs), which are indispensable scavengers of excessive Ca^{2+} and PO_4^{3-} ions in blood, induce pro-inflammatory activation of endothelial cells (ECs) and monocytes. Here, we determined physiological levels of CPPs (10 μg/mL calcium, corresponding to 10% increase in Ca^{2+} in the serum or medium) and investigated whether the pathological effects of calcium stress depend on the calcium delivery form, such as Ca^{2+} ions, albumin- or fetuin-centric calciprotein monomers (CPM-A/CPM-F), and albumin- or fetuin-centric CPPs (CPP-A/CPP-F). The treatment with CPP-A or CPP-F upregulated transcription of pro-inflammatory genes (*VCAM1*, *ICAM1*, *SELE*, *IL6*, *CXCL8*, *CCL2*, *CXCL1*, *MIF*) and promoted release of pro-inflammatory cytokines (IL-6, IL-8, MCP-1/CCL2, and MIP-3α/CCL20) and pro- and anti-thrombotic molecules (PAI-1 and uPAR) in human arterial ECs and monocytes, although these results depended on the type of cell and calcium-containing particles. Free Ca^{2+} ions and CPM-A/CPM-F induced less consistent detrimental effects. Intravenous administration of $CaCl_2$, CPM-A, or CPP-A to Wistar rats increased production of chemokines (CX3CL1, MCP-1/CCL2, CXCL7, CCL11, CCL17), hepatokines (hepassocin, fetuin-A, FGF-21, GDF-15), proteases (MMP-2, MMP-3) and protease inhibitors (PAI-1) into the circulation. We concluded that molecular consequences of calcium overload are largely determined by the form of its delivery and CPPs are efficient inducers of mineral stress at physiological levels.

Keywords: *calciprotein particles, calciprotein monomers, calcium ions, calcium stress, mineral stress, endothelial cells, monocytes, endothelial dysfunction, endothelial activation, systemic inflammatory response*

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INTRODUCTION

Calciprotein particles (CPPs) and calciprotein monomers (CPMs) are formed as a result of interaction of fetuin-A molecules with newly formed calcium phosphate clusters. The formation of CPMs, which neutralize excess $Ca^{(2+)}$and $PO^{(}_{4)}{}^{3-}$ ions, represents one of the main mechanisms of regulation of mineral homeostasis [1-6]. Albumin, which is the most abundant serum protein, is responsible for the excretion of circulating Ca^{2+} ions [5, 7], whereas fetuin-A acts as a mineral chaperone by stabilizing calcium phosphate in the form of colloidal PMPs or promoting their aggregation into corpuscular HRPs [5, 7]. After their formation, HRPs are removed from the bloodstream by endothelial cells (ECs) [8-15], monocytes [13], and liver and spleen macrophages [16-19]. The formation of CPMs and HRCs is an evolutionary mechanism that prevents the supersaturation of blood with $Ca^{(2+)}$and $PO^{(}_{4)}{}^{3-}$ ions (e.g., as a result of bone resorption) and prevents extracellular calcification, a pathologic condition often seen in patients in the late stages of chronic kidney disease [20-22]. At the same time, internalization of HRP by ECs and monocytes/macrophages and its dissolution in lysosomes trigger a chain of pathological processes including increased cytosolic Ca^{2+} concentration, mitochondrial and endoplasmic stress, activation of NF-κB transcription factor signaling pathways and release of pro-inflammatory cytokines (e.g., IL-6, IL-8 and MCP-1/CCL2), which ultimately promotes low-intensity chronic sterile inflammation [8-19, 23-26]. Treatment with the selective tumor necrosis factor (TNF)-α inhibitor infliximab decreased the amount of cPMs and HRP in the serum of patients with autoimmune diseases [27], indicating that anti-inflammatory therapy is effective in suppressing the proinflammatory activation of endothelium and monocytes/macrophages associated with HRP exposure.

Different concentrations of HRP are used in experimental studies: from 25 μg/mL [13, 15] to 100-200 μg/mL calcium [16-18, 25, 28] depending on the type of cells and duration of exposure. Elevated molar concentration of ionized calcium in serum (Ca^{2+}) is a risk factor for myocardial infarction, ischemic stroke and death from circulatory diseases [11, 29, 30]. Myocardial infarction and ischemic stroke are caused by the development of atherosclerosis, which is initiated by endothelial activation and disruption of endothelial integrity [31-35]. The mean interquartile range between risk (upper) and protective (lower) levels of ionized calcium is 0.12 mmol/L (approximately 10% of the mean reference value or 4.8 μg/mL) [11]; thus, the amount of calcium added to cell cultures or administered to experimental animals should not exceed these values to obtain clinically relevant results. Adequate quantification of physiological doses of HRP and CPM should take into account their recalculation with the appropriate mass of ionized calcium (e.g., added as $CaCl_{2)}$ to provide a 10% increase in the ionized calcium content

of the culture medium.

Although the adverse effects of calcium stress are well described in the literature [36-38], it remains unclear whether its pathological effects are determined by the source of calcium (free Ca^{2+} ions, colloidal PMCs, or corpuscular HRP) or solely by the amount of calcium in the microenvironment. It has been previously reported that stimulation of the calcium-sensitive receptor by increasing extracellular Ca^{2+} concentration promotes internalization of HRP, leading to activation of the NLRP3 inflammasome and the IL-1β signaling pathway [39]. The pathologic effects of HRPs depend largely on their crystallinity (amorphous primary HRPs and crystalline secondary HRPs) and density (high-density HRPs that precipitate at ≤ 16,000 *g* centrifugation and low-density HRPs that do not precipitate at these centrifugation parameters) [40]. [40]. Serum high-density HRP levels are positively correlated with high levels of the proinflammatory cytokine eotaxin, whereas low-density HRP levels are negatively correlated with concentrations of another proinflammatory cytokine IL-8 [40]. An increased hydrodynamic radius of HRP, which correlates with impaired renal function and age-dependent vascular remodeling, is associated with circulatory death in patients with peripheral arterial disease [41], as well as with vascular calcification [42] and with overall mortality in patients with terminal chronic renal failure 43]. Exposure to HRP activates vascular wall remodeling, including collagenogenesis, extracellular matrix formation [44] and osteochondrogenic differentiation of vascular smooth muscle cells, which is largely dependent on the particle size distribution, mineral composition and crystallinity of HRP [45]. Recent studies have demonstrated an association between increased HRP concentration and accelerated HRP crystallization with chronic kidney disease [44], ST-segment elevation myocardial infarction [46], and death from circulatory disease in patients with terminal chronic renal failure [47] or type 2 diabetes 48]. Removal of HRP from the blood resulted in decreased chronic inflammation activity, endothelial dysfunction, left ventricular hypertrophy and vascular calcification [49]. Inhibition of HRP crystallization prevented calcification of rat aorta caused by hyperphosphatemia [50].

Quantitative evaluation of MHPs is mainly based on the determination of calcium concentration per unit volume [12, 14, 16]. Artificially synthesized magnesium-protein particles (MPP), which do not contain calcium, did not show significant toxicity when added to EC cultures or intravenously administered to laboratory animals [11]. This indicates that it is the calcium concentration that is the major determinant of the effects of mineral stress. However, the intracellular distribution of calcium can vary depending on the method of its delivery, from a stable and controlled entry of Ca^{2+} ions through the cell membrane [51, 52] to an abrupt and uncontrolled increase in the concentration of Ca^{2+} ions in the cytosol after partial dissolution of HRP in lysosomes [11]. These features of calcium metabolism can significantly modulate the

transcription of EC genes under different types of calcium stress. For this reason, the study of pathophysiology of mineral homeostasis disorders requires an understanding of the molecular response of ECs to different forms of calcium delivery (circulating Ca^{2+} ions, CPM and HRP).

In the present study, we examined whether the form of calcium delivery determines the response of ECs and monocytes to physiologically relevant mineral stress, which was achieved by adding 10 μg/mL calcium (an amount sufficient for a 10% increase in ionized calcium levels) to the culture medium or by intravenously administering this concentration of calcium to Wistar rats. We found that incubation of primary human arterial ECs with albumin-derived HRP (HRP-A) initiated their proinflammatory activation, which was manifested by increased release of proinflammatory cytokines (IL-6, IL-8, MCP-1/CCL2, MIP-3α, PAI-1, uPAR) and confirmed by increased expression of genes encoding pro-inflammatory cell adhesion molecules (*VCAM1*, *ICAM1*, *SELE*) and pro-inflammatory cytokines (*IL6*, *CXCL8*, *CCL2* and *CXCL1*). Incubation with fetalized HRCs (HRC-F) also promoted the release of IL-6, IL-8 and MCP-1/CCL2 and increased the expression of genes encoding the above cell adhesion molecules (*VCAM1*, *ICAM1*, *SELE* and *SELP*) and pro-inflammatory cytokines (*IL6*, *CXCL1* and *MIF*). Similarly, incubation of monocytes with CHR-A under pulsatile flow conditions promoted the release of IL-6, IL-8, MIP-1α/1β, MIP-3α, CXCL1, CXCL5, PAI-1, uPAR, NGAL and MMP-9. Addition of free Ca^{2+} ions and albumin CPMs (CPM-A) caused only minor changes in gene expression and cytokine release by primary arterial ECs and monocytes. Intravenous administration of excess Ca^{2+} ions (as $CaCl_2$), CPM-A, or CHR-A to Wistar line rats induced a systemic inflammatory response that included increases in cytokines, hepatokines, and proteases. We hypothesize that the pathologic effects of HRP *in vitro* are determined by local calcium stress after their dissolution in lysosomes, whereas the inflammatory response to intravenous bolus injection of calcium is less dependent on the form of calcium delivery. Nevertheless, even physiologic doses of HRP induced proinflammatory activation of ECs and monocytes, as well as systemic inflammatory response *in vivo*.

MATERIALS AND METHODS

Synthesis and quantitative analysis of CPM and HRP. To prepare the mixture for the synthesis of CPM and HRC, 340 mg of bovine serum albumin (BSA, "Sigma-Aldrich", USA) or 8 mg of bovine serum fetuin-A (BSF, "Sigma-Aldrich") was dissolved in 4 mL of saline solution followed by the addition of 2 mL of Na_2HPO_4 (24 mmol/L, "Sigma-Aldrich") and 2 mL of $CaCl_2$ (40 mmol/L, "Sigma-Aldrich"). The mixture was resuspended after addition of each reagent. The

final concentrations of the components in the mixture were 42 mg/mL for BSA and 1 mg/mL for BSF (equivalent to median human serum levels [11]), 10 mmol/L for $CaCl_2$ (3.2 mg calcium), and 6 mmol/L for Na_2HPO_4. The suspension was then aliquoted into 8 microtubes (1 ml per tube), which were placed in a preheated (37° C) heating block (Thermite, "DNA-Technology", Russia) and incubated for 10 min. After this procedure, the mixture contained three calcium sources: free Ca^{2+} ions, CPMs (CPM-A or CPM-F, fetuin CPMs) and HRP (HRP-A or HRP-F).

The resulting suspension was dispensed into four ultracentrifuge tubes (2 ml per tube, Beckman Coulter, USA) and centrifuged at 200,000 *g* (OPTIMA MAX-XP, Beckman Coulter) for 1 h to precipitate HRC-A/HRC-F, which were then resuspended in sterile deionized water at a dilution of 1 : 200 and visualized by scanning electron microscopy (S-3400N, "Hitachi", Japan) at an accelerating voltage of 10 or 30 kV. HRP from atherosclerotic plaques and human serum were incubated in culture vials (Wuxi NEST Biotechnology, PRC) for 6 weeks after adding 3 mL of plaque homogenate or 3 mL of serum, 1 mmol/L $CaCl_2$, and 1 mmol/L Na_2HPO_4 to 7 mL of Eagle's medium, modified Dulbecco's medium (DMEM, PanEco, Russia) containing 10% fetal bovine serum (FBS, Capricorn Scientific, Germany), 1% solution of L-glutamine, penicillin and streptomycin (Thermo Fisher Scientific, USA) and 0.4% amphotericin B (Thermo Fisher Scientific). The plaques were homogenized according to a previously described technique [8]. After incubation for 6 weeks, HRP was precipitated and visualized by scanning electron microscopy according to previously described methodology [8]. The supernatant with CPM-A/CPM-F and free Ca^{2+} ions was transferred to centrifuge concentrators with a molecular weight threshold of 30 kDa (Guangzhou Jet Bio-Filtration, PRC) and centrifuged at 1800 *g* for 25 min to separate CPM-A/CPM-F (retentate) and free Ca^{2+} ions (filtrate).

Calcium concentration in CHR-A/HRC-F, CPM-A/CPM-F and as free Ca^{2+} ions was measured by colorimetric analysis using orthocresolphthaleincomplexone and diethanolamine (CalciScore, "AppScience Products", Russia) after dilution of the sample 1 : 30, 1 : 10 and 1 : 10, respectively. Albumin concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific) after 1 : 200 dilution of retentate containing CPM (filtrate containing free Ca^{2+} ions was not diluted). Spectrophotometry (Multiskan Sky, "Thermo Fisher Scientific") was performed at 575 nm (calcium) and 562 nm (albumin). All procedures were performed under sterile conditions.

Dose estimation. The amount of calcium required for a 10% increase in ionized calcium in the medium was estimated by adding 5, 10, 15, or 20 μg of calcium (in the form of $CaCl_2$) dissolved in an aqueous solution of BSA (300 mg/ml, average concentration of albumin in retentate) or BSF (28 mg/ml, average concentration of fetuin-A in retentate) per 1 mL of serum-free culture medium (EndoLife, "AppScience Products") or by adding 10, 15, 20, or 40 μg of

calcium dissolved in an aqueous solution of BSA (300 μg/mL) per 1 mL of rat serum. The mixture was resuspended and incubated for 1 h, after which the ionized calcium Ca^{2+} concentration was measured (Konelab 70i, Thermo Fisher Scientific). EndoLife medium and rat serum without added $CaCl_2$ were used as negative controls. According to our previous study, a 10% increase in ionized calcium (0.10 to 0.14 mmol/L (4.0 to 5.6 μg/mL); mean 0.12 mmol/L (4.8 μg/mL) for human serum) corresponds to the interquartile range between the upper (risk) and lower (protective) quartiles.

Cell Cultivation. Primary human coronary artery ECs (HCAEC, Cell Applications, USA) and human internal thoracic artery ECs (HITAEC, Cell Applications) were cultured in T-75 vials according to the manufacturer's protocol in EndoBoost medium (EB1, AppScience Products) using 0.25% trypsin-EDTA solution (PanEco) and 10% FBS trypsin inhibition. Immediately before the experiments, EndoBoost medium was replaced with serum-free EndoLife medium, and in the interval, cell cultures were washed twice with warm (37° C) Dulbecco's phosphate-salt buffer solution without Ca^{2+} and Mg^{2+} (FSBD, "BioLot", Russia) to remove residual serum components. HCAECs and HITAECs were cultured in parallel in flow chambers (Ibidi, Germany) or 6-well plates (Wuxi NEST Biotechnology) until confluency was achieved.

Monocytes were obtained from the blood of 5 healthy volunteers (authors of the study) by sequential isolation of peripheral blood mononuclear cells in Ficoll gradient (1077 g/cm^3, PanEco) followed by positive magnetic separation of $CD14^+$ cells using EasySep Magnet kit (STEMCELL Technologies, USA) and monocyte isolation kit (STEMCELL Technologies) according to the manufacturer's instructions under sterile conditions. The number of monocytes was determined using Countess II automatic cell counter (Thermo Fisher Scientific) and cell counting chambers (Thermo Fisher Scientific).

Internalization analysis. For the internalization assay of CPM and CHR-A ECs, CPM-A and CHR-A labeled with fluoresceinisothiocyanate-labeled BSA (FITC-BSA, Thermo Fisher Scientific) were used either during synthesis (by adding 750 μg of FITC-BSA at a concentration of 5 μg/μl), or after synthesis by incubation of precipitated HRP-A with 125(25 μl) FITC-BSA for 1 h at 4° C and incubation of 500 μl retentate (HRP-A) with 250 μg (50 μl) FITC-BSA for 1 h at 4° C after stirring. The synthesis of KPM-A and KHR-A was carried out in the dark less than 24 h before the experiment. After labeling, precipitated CHR-A was resuspended in FBSD, centrifuged at 13,000 *g* (Microfuge 20R, Beckman Coulter) for 10 min to remove unbound FITC-BSA and further resuspended in 400 μl of FBSD.

Laminar flow was generated using the Ibidi Pump System Quad ("Ibidi") with four separate flow modules (one chamber per module) and the Perfusion Set Yellow/Green ("Ibidi").

Prior to the experiment, HCAECs and HITAECs were cultured to confluency in flow chambers (350,000 cells per chamber) and exposed to laminar flow (15 dyne/$cm^{2)}$ in serum-free EndoLife medium for 24 h. FITC-labeled CPM-A and HRP-A were then added to the system (10 μg calcium per 1 mL of medium; 150 μg calcium per cell). A total of three consecutive experiments were performed: 1) using FITC-labeled CPM-A and CHR-A during their synthesis; 2) using FITC-labeled CPM-A and CHR-A after their synthesis; and 3) using unlabeled CPM-A and CHR-A. ECs were incubated with KPM-A and KHR-A for 1 h; nuclei were stained with Hoechst 33342 dye (2 μg/mL, Thermo Fisher Scientific) for 5 min. After thorough washing, FITC-labeled KPM-A and HRP-A were visualized using confocal microscopy (LSM 700, Carl Zeiss, Germany).

To study the colocalization of lysosomes and FITC-labeled CPMs and CHRs, FITC-labeled CPM-A, CPM-F, CHR-A, and CHR-F were used as described above. FITC-labeled KPM-A, KPM-F, KHR-A, and KHR-F (10 μg calcium per 1 mL of medium, 4 μg calcium per well) were added to confluent cultures of HCAEC and HITAEC in 8-well chambers ("Ibidi") for 3 hr. The culture medium was then replaced with fresh medium containing the pH sensor LysoTracker Red (1 μmol/L, Thermo Fisher Scientific) and incubated for 1 h. Free FITC-BSA (60 μg) was used as a control; nuclei were stained with Hoechst 33342 for 10 min. After thorough washing, FITC-labeled CPM-A, CPM-F, CPM-A and CPM-F were visualized using confocal microscopy.

Addition of free Ca^{2+} ions, CPM and HRC to ECs and monocytes. To investigate the response of ECs to the same concentrations of calcium in different forms, FSBD (control), free $Ca^{(2+)}$ ions (in the form of $CaCl_{2)}$, CPM (CPM-A or CPM-F), or HRC (HRC-A or HRC-F) were added to confluent cultures of HCAECs and HITAECs for 24 h (10 μg calcium per 1 mL of medium; 20 μg calcium per well of a 6-well plate; n= 18 wells per group). To offset potential protective effects of proteins, BSA (12 μg; average mass of albumin in added CPM-A) or BSF (0.33 μg; average mass of fetuin-A in CPM-F) was also added to all wells in the respective experiments. EndoBoost medium was replaced with serum-free EndoLife medium immediately before the start of the experiment. After incubation for 24 h, cells were visualized by phase-contrast microscopy. After removal of medium, cells were washed with cold (4° C) FBSD and lysed in TRIzol reagent (Thermo Fisher Scientific) for RNA isolation, according to the manufacturer's protocol. The culture medium was centrifuged at 2000 *g* (MiniSpin Plus, "Eppendorf", USA) to remove cell detritus, transferred to new tubes and frozen at− 80° C.

To evaluate the cytotoxicity of different forms of calcium stress, microplate colorimetric analysis using water-soluble tetrazolium salt (WST-8) and annexin V/propidium iodide staining followed by flow cytometry were performed. To assess the cellular metabolic rate, HCAECs and

HITAECs were cultured in 96-well plates (Wuxi NEST Biotechnology) until confluency in EndoBoost medium, followed by its replacement with serum-free EndoLife medium and addition of FSBD (control), free Ca^{2+} ions (in the form of $CaCl_2$), CPM (CPM-A or CPM-F) or HRC (HRC-A or HRC-F) (10 μg calcium per 1 ml of medium; 2 μg calcium per well of a 96-well plate; *n*= 12 wells per group). After incubation for 24 h, the medium was replaced with 100 μl of fresh serum-free EndoLife medium and 10 μl of WST-8 reagent (Wuhan Servicebio Technology, PRC) was added for 2 h. The reaction products were detected by spectrophotometry at 450 nm.

For annexin V/propidium iodide staining, HCAECs and HITAECs were cultured in 6-well plates (Wuxi NEST Biotechnology) confluency in EndoBoost medium followed by its replacement with serum-free EndoLife medium and addition of FSBD 24 h (control), free Ca^{2+} ions (in the form of $CaCl_2$), CPM (CPM-A or CPM-F) or HRC (HRC-A or HRC-F) (10 μg calcium per 1 ml of medium, 20 μg calcium per well of a 6-well plate). ECs were then separated from the culture plate using Accutase solution (Capricorn Scientific) and analyzed using an annexin V/propidium iodide staining kit (ab14085, Abcam, UK), according to the manufacturer's protocol. Flow cytometry was performed using a CytoFlex instrument and CytExpert software (Beckman Coulter).

To analyze monocyte responses, 350,000 cells in each chamber were incubated in serum-free EndoLife medium with equal concentrations of free Ca^{2+} ions ($CaCl_{2)}$, CPM-A, or HRP-A (10 μg calcium per 1 ml medium; 150 μg calcium per chamber; *n*= 5 donors/experiment per group) in a pulsed-flow system using the aforementioned perfusion kit for 24 h. FSBD was used as a control; BSA (87 mg, mean mass of albumin in added CPM-A) was also added to all chambers to offset potential protective effects of albumin. The four experimental groups (FSBD, free Ca^{2+} ions, CPM-A, and CHR-A) were distributed in four modules of the pulsed flow system. The experiments were performed under sterile conditions. After 24 h of incubation, the culture medium was centrifuged at 220 *g* (5804R, "Eppendorf") to precipitate monocytes and at 2000 *g* to remove cell detritus, followed by freezing at -80° C.

Gene expression analysis. Gene expression in ECs after addition of free Ca^{2+} ions, CPM-A/CPM-F, or CPM-A/CPM-F was analyzed by quantitative polymerase chain reaction after reverse transcription (RT-PCR). cDNA was synthesized using the M-MuLV-RH First Strand cDNA Synthesis Kit (R01-250, "Evrogen", Russia) and M-MuLV-RH reverse transcriptase (R03-50, "Evrogen"). RT-PCR was performed using primers (500 nmol/L each, "Evrogen", Table P1 of the Supplementary Material), 20 ngcDNA and BioMaster HS-qPCR Lo-ROX SYBR Master Mix kit (MHR031-2040, "Biolabmix", Russia) according to the manufacturer's protocol. Quantitative analysis of mRNA levels (*VCAM1*, *ICAM1*, *SELE*, *SELP*,

IL6, *CXCL8*, *CCL2*, *CXCL1*, *MIF*, *NOS3*, *SNAI1*, *SNAI2*, *TWIST1*, and *ZEB1* genes) was performed using the method $2^{-\Delta\Delta Ct}$ and normalized relative to the average expression of three housekeeping genes (*GAPDH*, *ACTB*, and *B2M*) as well as the FSBD group (control).

Administration of free Ca^{2+} ions, CPM and CHR to Wistar rats. The animal study protocol was approved by the Local Ethical Committee of the Federal State Budgetary Scientific Institution "Research Institute for Complex Problems of Cardiovascular Diseases" (protocol code: 042/2023; approval date: April 04, 2023). Animal experiments were conducted in accordance with the European Convention for the Protection of Vertebrate Animals (Strasbourg, 1986) and Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Male rats of the Wistar line (body weight ~300 g; blood volume ~20 ml, representing approximately 6.5% of body weight) were injected with FSBD (control), free $Ca^{(2+)}$ ions ($CaCl_2$), KPM-A, or KHR-A (10 μg calcium per 1 ml rat blood; 200 μg calcium per rat; n= 5 rats per group, total n= 20) via the tail vein. BSA (120 mg, average weight of albumin added to KPM-A) was added to all injections to control for a possible immune response to foreign albumin. After 1 h, all rats were euthanized by intraperitoneal injection of sodium pentobarbital solution (100 mg/kg body weight). Serum was isolated by centrifugation of blood at 1700 *g* for 15 min.

Dot-blotting and enzyme-linked immunosorbent assay (ELISA). Protein levels in the culture medium were measured by dot-blotting and ELISA. Dot-blotting was performed using Proteome Profiler Human XL Cytokine Array Kit (ARY022B, R&D Systems, USA) and Proteome Profiler Rat XL Cytokine Array (ARY030, R&D Systems) according to the manufacturer's instructions; proteins were visualized using Odyssey XF chemiluminescence detection system (LI-COR Biosciences, USA). To increase the sensitivity of dot-blotting, culture medium samples were concentrated using a HyperVAC-LITE vacuum centrifuge concentrator (Gyrozen, Republic of Korea) before measurements. Rat serum was analyzed without preconcentration. Culture medium samples were concentrated to the same level between experimental groups (for monocytes, 7-fold, from 14 mL to 2 mL; for ECs, , from 3 mL to 1 mL). For dot-blotting, 1 mL each of concentrated medium or unconcentrated rat serum was used. The content of IL-6, IL-8 and MCP-1/CCL2 was determined by ELISA using appropriate kits (A-8768, A-8762 and A-8782, Vector-Best, Russia) according to the manufacturer's protocols. Colorimetric determination of ELISA results was performed by spectrophotometry at a wavelength of 450 nm. 100 μl of unconcentrated culture medium per sample was used for ELISA.

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, USA). Data for OT-CPCR are presented as mean values ± standard deviation. Four

independent groups were compared by one-factor analysis of variance (OVA) followed by correction for multiple comparisons using Dunnett's criterion. ELISA results are presented as median, 25th and 75th percentiles and range. Four independent groups were compared using the Kraskell-Wallis test followed by correction for multiple comparisons by Dunn's criterion. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

Physiological significance of CPM and CHR synthesis under conditions of mineral stress. To study the effects of different forms of calcium delivery to ECs and monocytes, a reaction mixture containing a physiological concentration of BSA or BSF, physiological solution (NaCl), and supraphysiological levels of Na_2HPO_4 and $CaCl_2$ was synthesized to simultaneously produce CPM-A/CPM-F and CHR-A/HRC-F. Ultracentrifugation was used to isolate HRC, followed by ultrafiltration to separate CPM (yellow retentate) from free ions and salts (clear filtrate). Thus, calcium was represented as (i) free Ca^{2+} ions, (ii) CPM (colloidal form), and (iii) CHR (particulate form). Both BSF and BSA were chosen for the synthesis of CPM (CPM-A) and CHR (CHR-A) because low serum albumin levels have previously been shown to be an independent risk factor for coronary heart disease and ischemic stroke (in combination with elevated serum Ca^{2+} levels) [11]. Low serum albumin levels correlated with a greater propensity for calcification (expressed by HRP precipitation), and albumin concentration correlated positively with the molar concentration of total calcium [11]. Since fetuin-A plays a key role as a mineral chaperone and regulates the formation of CPM and HRP in human blood, CPM-F and HRP-F synthesized using the described protocol were also used in most experiments.

Scanning electron microscopy of HRP-A showed their spongy structure and irregular shape, which differed from the spherical and needle shape of primary and secondary HRPs obtained from blood (Fig. 1). HRP-F had a spherical shape and spongy structure, which made them similar to primary HRCs isolated from atherosclerotic plaques and serum [11]. These observations are consistent with earlier data comparing albumin, fetuin, plaque, and serum HRPs [8] and can be explained by different interactions of acidic serum proteins during the formation of HRPs in blood.

Fig. 1. Scanning electron microscopy micrographs of albumin CHRs (CHR-A), fetuin CHRs (CHR-F), CHRs from atherosclerotic plaques (CHR-B), and serum (CHR-S). Secondary electron mode; accelerating voltage - 10 kV (HRC-A) or 30 kV (other HRC types); magnification

×30,000; scale bar - 1 μm

The formation of HRP and CPM consumed 30% and 20% of calcium, respectively, whereas 50% of calcium remained in solution as free Ca^{2+} ions. This distribution corresponds to the physiological ratio between ionized calcium ($Ca^{2+)}$ and calcium bound to proteins and phosphorus in human serum (1 : 1). HRP contained 11 to 17% of total albumin, whereas 83 to 89% of albumin remained in the retained solution, retaining the ability to bind Ca^{2+}. The effectiveness of ultrafiltration was confirmed by the absence of BSA or BSF in the filtrate. These data confirm the physiological significance of the developed procedure for artificial synthesis of CPM and CHR under conditions of mineral stress.

Physiologic concentrations of CHR cause proinflammatory activation of ECs and monocytes. A dose-response curve was constructed to determine the amount of calcium required to provide a physiological increase in the molar concentration of ionized calcium. The addition of 10 μg calcium per 1 ml of serum-free culture medium (Fig. 2, *a*) or rat serum (Fig. 2, *b*) was sufficient to produce a 10% increase in ionized calcium concentration (interquartile range between risk and protective levels in the population). Thus, a calcium concentration of 10 μg/mL was chosen as the optimal concentration for modeling clinically relevant mineral stress. Further experiments included four groups: 1) control (FSBD); 2) free $Ca^{(2+}$) ions added as $CaCl_2$; 3) CPM (CPM-A or CPM-F); 4) HRC (HRC-A or HRC-F).

Fig. 2. Increase in the concentration of ionized calcium ($Ca^{2+)}$ in culture medium (*a*) and rat serum (*b*) upon addition of increasing amounts of $CaCl_2$. Abscissa axis, concentration of added calcium; ordinate axis, increase in molar Ca^{2+} concentration relative to control medium or serum without added calcium. A 10% increase in Ca^{2+} concentration (blue dashed line) was achieved by adding 10 μg calcium per 1 mL of medium or serum (red circle)

To test the internalization ability of CPM and CHR-A and CHR-A labeled with fluorescein isothiocyanate (FITC-BSA) either during their synthesis or after incubation of precipitated CHR-A and retentate (CHR-A) with FITC-BSA were used to test the internalization ability of CPM-A and CHR-A under pulsed-flow cultivation. Intense green fluorescence was observed in the EC as early as 1 h after the addition of FITC-labeled KPM-A and KHR-A to the pulsed-flow system (Fig. 3, *a*). KPM-A and CHR-A incubated with FITC-BSA after synthesis showed significantly higher fluorescence levels compared to the samples labeled during

synthesis (Fig. 3, *a*). Detection of FITC-labeled CPMs/HRCs in lysosomes stained with pH-sensor LysoTracker Red confirmed the internalization of CPM-A, CPM-F, CPM-A and CPM-F by HCAECs and HITAECs after 4 h incubation, while free FITC-BSA did not penetrate the cells (Fig. 3, *b*).

Fig. 3. Internalization of FITC-labeled CPM (FITC-CPM) and HRC (FITC-HRC) by HCAEC and HITAEC. *a* - Comparison of FITC signal intensity internalized by ECs after incubation with unlabeled CPM and HRC (left), CPM and HRC labeled with FITC-BSA during their synthesis (center), and CPM and HRC labeled after their synthesis (right). Nuclei were stained with Hoechst 33342. Confocal microscopy; magnification ×630; scale bar, 5 μm. *b* - Lysosomes stained with LysoTracker Red in ECs incubated with CPM (FITC-CPM-A and FITC-CPM-F) or HRC (FITC-CPM-A and FITC-CPM-F) for 4 h. Left, free FITC-BSA; center, KPM-A and KPM-F labeled during synthesis; right, HRC-A and HRC-F labeled after synthesis. Yellow arrows indicate HRCs within cells. Nuclei were stained with Hoechst 33342. Confocal microscopy; magnification ×200; scale bar, 50 μm

To compare the pathologic effects of different forms of calcium delivery, Ca^{2+}, CPM-A/CPM-F or CHR-A/CPM-F (10 μg/mL) were added to HCAEC and HITAEC. Using light-field and phase-contrast microscopy, pathological changes in EC morphology (loss of intercellular contacts, typical elongated shape of ECs and their detachment from the culture plastic) were observed after incubation with KHR-A/KHR-F, but not with Ca^{2+} or KPM-A/KPM-F (Fig. 4).

Figure 4. Light-field microscopy (KPM-A/HRC-A, top) and phase-contrast microscopy (KPM-F/HRC-F, bottom) of HCAECs (left) and HITAECs (right) incubated with FSBD (control), free Ca^{2+} ions, KPM (KPM-A, top; CPM-F, bottom) or HRC (HRC-A, top; HRC-F, bottom) (10 μg calcium per 1 ml serum-free culture medium) for 24 h; magnification ×200; scale bar 100 μm

To assess the cytotoxicity of various forms of calcium stress, we performed colorimetric analysis of cell viability and metabolic activity using the water-soluble tetrazolium salt WST-8. After 24-hour incubation with HRP-A or HRP-F, the intensity of metabolic activity of HCAEC and HITAEC cells was significantly decreased (Fig. 5, *a*). Flow cytometric analysis of cell death after staining with annexin V and propidium iodide showed that a significant proportion of ECs underwent apoptosis after 24 h exposure to HRP-A or HRP-F (Fig. 5, *b*).

Fig. 5. Cytotoxicity test after incubation of HCAECs (left panels) and HITAECs (right panels) with FSBD (control), free $Ca^{(2+)}$ ions ($Ca^{(2+)}$-A or $Ca(^{2+})$-F), CPM (CPM-A or CPM-F), or CHR (CHR-A or CHR-F) (10 μg calcium per 1 mL serum-free culture medium) for 24 h. *a* - WST-8 colorimetric test (evaluation of the reduction of WST-8 by intracellular dehydrogenases to water-soluble orange-yellow formazan, whose maximum absorbance is observed at 450 nm). *b* - Test with annexin V and propidium iodide (lower left quadrant Q2-LL - normal cells; lower right quadrant Q2-LR - cells in early apoptosis; upper right quadrant Q2-UR - cells in late apoptosis; upper left quadrant Q2-UL - in necrosis). Upper panel - statistical analysis of the content of intact cells and cells in a state of late apoptosis. Lower panel - representative flow cytometric plots. The values of the probability to reject the correct null hypothesis (*p*) for intergroup comparison are indicated above the graphs

By means of RT-PCR, a significant increase in the expression of genes encoding pro-inflammatory cell adhesion molecules (*VCAM1*, *ICAM1* and *SELE*) and pro-inflammatory cytokines (*IL6*, *CXCL8*, *CCL2* and *CXCL1*) was shown in HCAECs incubated with HRC-A (Table 1). Exposure to HRC-F induced a similar response, including increased expression of *VCAM1*, *SELP*, *IL6* and *MIF* genes, as well as a trend toward increased expression of *ICAM1* and *SELE* genes (Table 2). A similar gene expression profile was observed for HITAEC, including induction of *VCAM1*, *IL6* and *CXCL8* gene expression upon exposure to HRC-A (Table 1), while incubation with HRC-F increased the expression of *SELE*, *SELP*, *CXCL1* and *MIF* genes (Table 2). These results indicated the development of proinflammatory endothelial activation and indicated an increase in proinflammatory cytokines in the culture medium. In contrast, free Ca^{2+} ions and CPM-A/CPM-F caused random rather than systematic changes that were not associated with endothelial dysfunction.

Table 1: Relative gene expression (ΔCt; fold change; *p* value) in HCAEC and HITAEC incubated with FSBD (control), free Ca^{2+} ions, CPM-A, or CHR-A (10 μg calcium per 1 ml serum-free culture medium) for 24 h

Gene	Metric	HCAEC				HITAEC			
		FSBD	Ca^{2+}	KPM-A	HRC-A	FSBD	Ca^{2+}	KPM-A	HRC-A
VCAM1	ΔCt	0,0003 ± 0,0006	0,0001 ± 0,0001	0,0002 ± 0,0001	0,0015 ± 0,0010	0,0003 ± 0,0002	0,0011 ± 0,0011	0,0006 ± 0,0004	0,0010 ± 0,0008
	Multiples.	1	0,52	0,76	**6,00**	1	**3,67**	2,00	**3,33**

	p	1,00	0,904	0,985	**0,001**	1,00	**0,009**	0,463	**0,029**
ICAM1	ΔCt	0,0148 ± 0,0066	0,0372 ± 0,0210	0,0118 ± 0,0034	0,1169 ± 0,0837	0,0338 ± 0,0213	0,0503 ± 0,0339	0,0404 ± 0,0244	0,0432 ± 0,0201
	Multiples.	1	2,52	0,80	**7,90**	1	1,49	1,20	1,28
	p	1,00	0,320	0,994	**0,001**	1,00	0,148	0,782	0,559
SELE	ΔCt	0,0056 ± 0,0026	0,0089 ± 0,0036	0,0020 ± 0,0009	0,0134 ± 0,0065	0,0595 ± 0,0351	0,1049 ± 0,1387	0,0909 ± 0,1203	0,0943 ± 0,0872
	Multiples.	1	**1,59**	**0,36**	**2,39**	1	1,76	1,53	1,58
	p	1,00	**0,041**	**0,026**	**0,001**	1,00	0,415	0,687	0,619
SELP	ΔCt	0,0077 ± 0,0067	0,0027 ± 0,0008	0,0015 ± 0,0009	0,0026 ± 0,0023	0,0009 ± 0,0006	0,0054 ± 0,0058	0,0056 ± 0,0055	0,0025 ± 0,0030
	Multiples.	1	**0,35**	**0,19**	**0,34**	1	**6,00**	**6,22**	2,78
	p	1,00	**0,001**	**0,001**	**0,001**	1,00	**0,008**	**0,005**	0,567
IL6	ΔCt	0,0182 ± 0,0131	0,0058 ± 0,0023	0,0072 ± 0,0045	0,1197 ± 0,0837	0,0085 ± 0,0043	0,0132 ± 0,0155	0,0197 ± 0,0214	0,0247 ± 0,0272
	Multiples.	1	0,32	0,40	**6,58**	1	1,55	2,32	**2,91**
	p	1,00	0,729	0,796	**0,001**	1,00	0,803	0,196	**0,035**
CXCL8	ΔCt	0,0371 ± 0,0260	0,0441 ± 0,0152	0,0250 ± 0,0105	2,1412 ± 1,5287	0,1396 ± 0,0561	0,1801 ± 0,2005	0,1825 ± 0,1871	0,3279 ± 0,3681
	Multiples.	1	1,19	0,67	**57,71**	1	1,29	1,31	**2,35**
	p	1,00	0,999	0,999	**0,001**	1,00	0,914	0,901	**0,045**
CCL2	ΔCt	0,7514 ± 0,6502	0,4398 ± 0,4293	0,6965 ± 0,6669	1,3616 ± 1,0636	0,8908 ± 0,4072	1,2866 ± 1,5286	1,4987 ± 1,6929	1,6162 ± 1,9876
	Multiples.	1	0,59	0,93	**1,81**	1	1,44	1,68	1,81
	p	1,00	0,448	0,992	**0,042**	1,00	0,777	0,494	0,353
CXCL1	ΔCt	0,1267 ± 0,0562	0,0436 ± 0,0408	0,0444 ± 0,0343	0,3486 ± 0,1551	0,0647 ± 0,0279	0,1520 ± 0,1842	0,0944 ± 0,0885	0,0983 ± 0,1052
	Multiples.	1	**0,34**	**0,35**	**2,75**	1	2,35	1,46	1,52
	p	1,00	**0,017**	**0,018**	**0,001**	1,00	0,069	0,782	0,715
MIF	ΔCt	0,3853 ± 0,1660	0,2309 ± 0,1040	0,2731 ± 0,0839	0,4170 ± 0,2857	0,2753 ± 0,1576	0,7919 ± 0,9280	0,5270 ± 0,4019	0,4618 ± 0,4146
	Multiples.	1	**0,60**	0,71	1,08	1	**2,88**	1,91	1,68
	p	1,00	**0,031**	0,155	0,910	1,00	**0,018**	0,386	0,618
NOS3	ΔCt	0,0094 ± 0,0063	0,0069 ± 0,0036	0,0069 ± 0,0033	0,0091 ± 0,0087	0,0031 ± 0,0018	0,0093 ± 0,0090	0,0119 ± 0,0126	0,0060 ± 0,0037
	Multiples.	1	0,73	0,73	0,97	1	**3,00**	**3,84**	1,94
	p	1,00	0,473	0,475	0,997	1,00	**0,050**	**0,005**	0,547
SNAI1	ΔCt	0,0168 ± 0,0101	0,0100 ± 0,0040	0,0124 ± 0,0065	0,0344 ± 0,0291	0,0049 ± 0,0020	0,0129 ± 0,0110	0,0140 ± 0,0124	0,0094 ± 0,0100

	Multiples.	1	0,60	0,74	**2,05**	1	**2,63**	**2,86**	1,92
	p	1,00	0,487	0,771	**0,009**	1,00	**0,042**	**0,018**	0,371
SNAI2	ΔCt	0,0129 ± 0,0103	0,0038 ± 0,0009	0,0065 ± 0,0046	0,0047 ± 0,0032	0,0009 ± 0,0007	0,0033 ± 0,0022	0,0099 ± 0,0160	0,0030 ± 0,0055
	Multiples.	1	**0,29**	**0,50**	**0,36**	1	3,67	**11,00**	3,33
	p	1,00	**0,001**	**0,005**	**0,001**	1,00	0,784	**0,015**	0,841
TWIST1	ΔCt	0,0015 ± 0,0012	0,0003 ± 0,0002	0,0002 ± 0,0001	0,0009 ± 0,0008	0,0004 ± 0,0004	0,0018 ± 0,0026	0,0037 ± 0,0077	0,0016 ± 0,0016
	Multiples.	1	**0,20**	**0,13**	0,60	1	4,50	9,25	4,00
	p	1,00	**0,001**	**0,001**	0,150	1,00	0,742	0,170	0,874
ZEB1	ΔCt	0,2376 ± 0,1200	0,0779 ± 0,0561	0,1607 ± 0,0596	0,3277 ± 0,2237	0,1438 ± 0,0686	0,3697 ± 0,4382	0,4552 ± 0,4601	0,3559 ± 0,4082
	Multiples.	1	**0,33**	0,68	1,38	1	2,57	**3,17**	2,47
	p	1,00	**0,002**	0,209	0,117	1,00	0,201	**0,049**	0,244

Note. Genes encoding proinflammatory cell adhesion molecules (*VCAM1*, *ICAM1*, *SELE*, *SELP*), proinflammatory cytokines (*IL6*, *CXCL8*, *CCL2*, *CXCL1*, *MIF*), endothelial NO synthase (*NOS3*), and endothelial-mesenchymal transition transcription factors (*SNAI1*, *SNAI2*, *TWIST1*, *ZEB1)* were analyzed. Statistically significant fold change and *p* values are in bold; ΔCt is presented as mean ± standard deviation.

Table 2. Relative gene expression (ΔCt; fold change; *p* value) in HCAECs and HITAECs incubated with FSBD (control), free Ca^{2+} ions, CPM-F, and HRP-F (5 μg calcium per 1 mL serum-free culture medium) for 24 hr

Gene	Metric	HCAEC				HITAEC			
		FSBD	Ca^{2+}	KPM-F	HRC-F	FSBD	Ca^{2+}	KPM-F	HRC-F
VCAM1	ΔCt	0,0020 ± 0,0015	0,0019 ± 0,0007	0,0018 ± 0,0009	0,0042 ± 0,0032	0,0004 ± 0,0003	0,0004 ± 0,0002	0,0005 ± 0,0003	0,0006 ± 0,0005
	Multiples.	1	0,95	0,90	**2,1**	1	0,95	1,28	1,54
	p	1,00	0,998	0,984	**0,008**	1,00	0,995	0,628	0,113
ICAM1	ΔCt	0,0540 ± 0,0510	0,0165 ± 0,0133	0,0208 ± 0,0266	0,1011 ± 0,1201	0,0646 ± 0,0282	0,0906 ± 0,0503	0,0820 ± 0,0306	0,0885 ± 0,0519
	Multiples.	1	0,31	0,39	1,87	1	1,40	1,27	1,37
	p	1,00	0,229	0,319	0,098	1,00	0,159	0,454	0,213
SELE	ΔCt	0,0048 ± 0,0037	0,0082 ± 0,0107	0,0028 ± 0,0019	0,0091 ± 0,0053	0,0018 ± 0,0007	0,0084 ± 0,0055	0,0069 ± 0,0032	0,0058 ± 0,0061
	Multiples.	1	1,71	0,58	1,90	1	**4,67**	**3,83**	**3,22**
	p	1,00	0,441	0,879	0,375	1,00	**0,001**	**0,003**	**0,023**

SELP	ΔCt	0,0119 ± 0,0131	0,0060 ± 0,0060	0,0089 ± 0,0079	0,0318 ± 0,0276	0,0050 ± 0,0023	0,0032 ± 0,0016	0,0035 ± 0,0017	0,0078 ± 0,0053
	Multiples.	1	0,50	0,75	**2,67**	1	0,64	0,70	**1,56**
	p	1,00	0,534	0,895	**0,002**	1,00	0,227	0,374	**0,025**
IL6	ΔCt	0,0072 ± 0,0029	0,0100 ± 0,0097	0,0063 ± 0,0046	0,0233 ± 0,0188	0,0044 ± 0,0016	0,0045 ± 0,0045	0,0031 ± 0,0012	0,0064 ± 0,0042
	Multiples.	1	1,39	0,88	**3,24**	1	1,02	0,70	1,45
	p	1,00	0,783	0,988	**0,001**	1,00	0,999	0,463	0,191
CXCL8	ΔCt	0,1330 ± 0,0572	0,2582 ± 0,1501	0,1892 ± 0,0573	0,1632 ± 0,0898	0,0294 ± 0,0175	0,0636 ± 0,0714	0,0366 ± 0,0185	0,0522 ± 0,0411
	Multiples.	1	**1,94**	1,42	1,23	1	2,16	1,24	1,78
	p	1,00	**0,001**	0,203	0,668	1,00	0,053	0,924	0,272
CCL2	ΔCt	1,1073 ± 0,3168	2,8331 ± 1,9144	1,1255 ± 0,3747	1,3772 ± 0,8673	0,3096 ± 0,0759	0,5079 ± 0,3852	0,4625 ± 0,1941	0,5145 ± 0,3698
	Multiples.	1	**2,56**	1,02	1,24	1	1,64	1,49	1,66
	p	1,00	**0,001**	0,999	0,795	1,00	0,105	0,265	0,090
CXCL1	ΔCt	0,3756 ± 0,1000	0,6205 ± 0,3781	0,4173 ± 0,1658	0,5612 ± 0,3499	0,0543 ± 0,0156	0,1053 ± 0,0885	0,0831 ± 0,0437	0,1477 ± 0,1538
	Multiples.	1	**1,65**	1,11	1,49	1	1,94	1,53	**2,72**
	p	1,00	**0,026**	0,942	0,117	1,00	0,235	0,668	**0,009**
MIF	ΔCt	2,8076 ± 0,9811	2,6168 ± 1,6847	2,2889 ± 0,8340	5,5963 ± 3,2599	0,2999 ± 0,0856	0,4214 ± 0,3609	0,3627 ± 0,1458	0,6572 ± 0,5581
	Multiples.	1	0,93	0,82	**1,99**	1	1,41	1,21	**2,19**
	p	1,00	0,983	0,763	**0,001**	1,00	0,583	0,904	**0,007**
NOS3	ΔCt	0,1599 ± 0,0597	0,1075 ± 0,0592	0,1686 ± 0,0643	0,2636 ± 0,0972	0,0176 ± 0,0056	0,0187 ± 0,0174	0,0170 ± 0,0060	0,0347 ± 0,0382
	Multiples.	1	0,67	1,05	**1,65**	1	1,06	0,97	**1,97**
	p	1,00	0,082	0,969	**0,001**	1,00	0,997	0,999	**0,050**
SNAI1	ΔCt	0,0595 ± 0,0214	0,0513 ± 0,0284	0,0650 ± 0,0257	0,0943 ± 0,0377	0,0017 ± 0,0008	0,0020 ± 0,0017	0,0015 ± 0,0008	0,0031 ± 0,0024
	Multiples.	1	0,86	1,09	**1,58**	1	1,18	0,88	**1,82**
	p	1,00	0,728	0,892	**0,002**	1,00	0,937	0,969	**0,031**
SNAI2	ΔCt	0,0162 ± 0,0088	0,0097 ± 0,0056	0,0196 ± 0,0100	0,0521 ± 0,0311	0,0003 ± 0,0003	0,0005 ± 0,0006	0,0002 ± 0,0001	0,0003 ± 0,0003
	Multiples.	1	0,60	1,21	**3,22**	1	1,57	0,63	1,01
	p	1,00	0,596	0,901	**0,001**	1,00	0,451	0,856	0,999
TWIST1	ΔCt	0,0019 ± 0,0010	0,0016 ± 0,0009	0,0022 ± 0,0011	0,0029 ± 0,0014	0,00013 ± 0,0001	0,0002 ± 0,0001	0,0001 ± 0,0001	0,0003 ± 0,0003
	Multiples.	1	0,84	1,16	1,53	1	1,54	0,85	2,31

	p	1,00	0,757	0,794	0,086	1,00	0,992	0,981	0,094
ZEB1	ΔCt	0,3158 ± 0,2627	0,2493 ± 0,0424	0,2738 ± 0,0783	0,2246 ± 0,2089	0,0468 ± 0,0208	0,0734 ± 0,0970	0,0227 ± 0,0064	0,0589 ± 0,0651
	Multiples.	1	0,16	0,23	0,71	1	1,57	0,49	1,26
	p	1,00	0,294	0,323	0,276	1,00	0,403	0,477	0,875

Note. Descriptions of the genes analyzed and data presentation are given in the note to Table 1.

Incubation with CHR-A significantly increased the expression of inducible endothelial proinflammatory cytokines (IL-6, IL-8, and MCP-1/CCL2) in culture medium from HCAECs and HITAECs (Figure 6), whereas addition of free Ca^{2+} ions and CPM-A did not induce a stable pathologic response at the protein level, confirming the results of gene expression analysis (Figure 6). Incubation with HRP-F also increased the expression of the mentioned cytokines in HCAECs and HITAECs, whereas free Ca^{2+} ions and CPM-F induced a cytokine response only in HITAECs (Fig. 7).

Fig. 6. Evaluation of IL-6 (top panel), IL-8 (middle panel), and MCP-1/CCL2 (bottom panel) levels in unconcentrated serum-free culture medium from HCAECs and HITAECs incubated with FSBD (control; black), free $Ca^{(2+)}$ ions (blue), CPM-A (green), and CHR-A (red) (10 μg calcium per 1 mL of medium) for 24 h (ELISA). The probability values to reject the correct null hypothesis (*p*) for intergroup comparison are indicated above the graphs

Fig. 7. Evaluation of IL-6 (top panel), IL-8 (middle panel), and MCP-1/CCL2 (bottom panel) levels in unconcentrated serum-free culture medium from HCAECs and HITAECs incubated with FSBD (control; black), free $Ca^{(2+)}$ ions (blue), CPM-F (green), and HRP-F (red) (10 μg calcium per 1 mL medium) for 24 h (ELISA). The probability values to reject the correct null hypothesis (*p*) for intergroup comparison are indicated above the graphs

To further investigate the release of cytokines under calcium stress, semiquantitative analysis by dot-blotting of serum-free culture medium from HCAECs and HITAECs incubated with Ca^{2+}, CPM-A, and HRC-A was performed. Incubation with CHR-A resulted in increased release of PAI-1 (serpin E1), CXCL1, MCP-1/CCL2, IL-8, MIF, soluble forms of CD105 and CD147 in HCAEC, and stimulated synthesis of ST2 and RANTES/CCL5 in HITAEC (Figure 8 and Appendix Table P1). Levels of the soluble form of uPAR and MIP-3α/CCL20 were also

elevated in culture medium from both EC lines after incubation with HRC-A (Figure 8 and Table 1). Thus, exposure to HRC-A induced the release of 11 cytokines (Figure 8 and Appendix Table P1). The expression of six of these proinflammatory molecules (CXCL1, MCP-1/CCL2, MIF, uPAR, sCD147, and ST2) was also upregulated in the culture medium after incubation of ECs with KPM-A; the expression of five proteins (CXCL1, sCD147, ST2, PDGF-AA, and RANTES/CCL5) was upregulated after addition of $CaCl_2$ (Figure 8 and Appendix Table P1). The most pronounced increases were observed for the soluble form of CD147 (EMMPRIN/basigin; 11,42 change in HCAEC after exposure to CHR-A) and MIP-3α/CCL20 (12,52 change in HITAEC after exposure to CHR-A).

Fig. 8. Cytokine profiling in concentrated () serum-free culture medium from HCAECs (upper panel) and HITAECs (lower panel) incubated with FSBD (control), free Ca^{2+} ions, CPM-A, or CHR-A (10 μg calcium per 1 mL of culture medium) for 24 h (dot-blotting). Green, serpin E1/PAI-1; light brown, CXCL1/GROα; gray, CD105/endoglin; dark blue, MCP-1/CCL2; red, IL-8; purple, MIF; dark brown color - uPAR; golden color - MIP-3α/CCL20; light blue color - CD147/EMMPRIN/basigin; azure color - ST2; pink color - PDGF-AA; dark green color - RANTES/CCL5. Short, medium, and long arrows indicate fold changes of 1.20-1.49, 1.50-1.99, and ≥2-fold, respectively, compared with the control group (FSBD). The amount of hyperexpressed cytokines upon exposure to Ca^{2+}, CPM-A, or HRP-A is indicated at the bottom of the figure

Incubation of monocytes with KHR-A caused an increase in the release of serpin E1/PAI-1, CXCL1, chemokine CXCL5, adiponectin, NGAL, IL-6, CHI3L1, apolipoprotein A-I, uPAR, MIP-3α/CCL20, and MMP-9. In contrast, Ca^{2+} and CPM-A induced only stochastic changes in cytokine release (Figure 9 and Appendix Table P2). Exposure to CHR-A significantly increased the release of the 11 aforementioned cytokines, whereas the effects of Ca^{2+} and CPM-A were limited to the induction of NGAL, CHI3L1, and MMP-9 (Figure 9 and Appendix Table P1). The proteins CXCL1, adiponectin, IL-6, and apolipoprotein A-I were expressed exclusively in the culture medium from monocytes incubated with CHRM-A. Thus, HRP-A was found to induce proinflammatory activation of ECs and monocytes, as evidenced by increased cytokine gene expression and increased secretion of the corresponding proteins. Although free Ca^{2+} ions and CPM-A also stimulated the release of some cytokines by ECs, their proinflammatory effects were less pronounced compared to HRP-A regardless of the cell line type (HCAECs, HITAECs, and monocytes).

Fig. 9. Cytokine profiling in concentrated () serum-free culture medium from human monocytes incubated with FSBD (control), free Ca^{2+} ions, CPM-A, and HRP-A (10 μg calcium per 1 mL of culture medium) for 24 h (dot-blotting). Green color, serpin E1/PAI-1; light brown color, CXCL1/GROα; red color, CXCL5/ENA-78; light blue color, adiponectin; purple color, NGAL/lipocalin-2; dark blue, IL-6; sky blue, chitinase 3-like protein 1; pink, apolipoprotein A-I; brown, uPAR; gold, MIP-3α/CCL20; azure, MMP-9. Short, medium, and long arrows indicate fold changes of 1.20-1.49, 1.50-1.99, and ≥2.00-fold, respectively, compared with the control group (FSBD). The amount of hyperexpressed cytokines upon exposure to Ca^{2+}, CPM-A, or HRP-A is indicated by at the bottom of the figure

Finally, the *in vivo* effects of different forms of calcium stress after intravenous administration of $CaCl_2$, CPM-A, and HRP-A to Wistar line rats (10 μg calcium per 1 mL blood) were investigated. In contrast to the *in vitro* results, all forms of calcium delivery caused an increase in proinflammatory cytokines in rat serum by dot-blotting (22, 30, and 24 cytokines in the case of Ca^{2+}, CPM-A, and CHR-A injections, respectively; Figure 10 and Appendix Table P3). Among the molecules detected were GM-CSF, chemokines: CX3CL1 (fractalkine), MCP-1/CCL2, CXCL7, CCL11 (eotaxin) and CCL17, serpin E1/PAI-1, matrix metalloproteinases (MMP-2, MMP-3), hepatokines: Hepassocin, fetuin-A, FGF-21 and GDF-15, proteins with pleiotropic effects: RAGE/AGER, adiponectin, fibulin-3, galectin-1 and galectin-3 (Fig. 10 and Appendix Table P3). Levels of prolactin, GM-CSF, hepassocin, CNTF, MMP-3, CX3CL1 (fractalkine), FGF-21, fibulin-3, and GDF-15 increased after exposure to all three types of calcium stress (Figure 10 and Appendix Table P3). However, the levels of RAGE/AGER, fetuin-A, MCP-1/CCL2, MMP-9, CCL17, and galectin-3 increased exclusively after injection of CPM-A and CHR-A, whereas the release of HGF, CCL11 (eotaxin), and galectin-1 was stimulated only by CPM-A. PAI-1 content was increased exclusively upon administration of CHR-A (Figure 10 and Appendix Table P3). Meanwhile, expression of uPAR and MIP-3α/CCL20 was increased in all three cell types (HCAECs, HITAECs, and monocytes) but not in rat serum. PAI-1 was the only molecule whose content was increased in all samples (medium from ECs, monocytes and in rat serum) after incubation with HRP-A (Table 3).

Table 3. Specific and total cytokines upregulated in culture medium (ECs or monocytes) or rat serum after incubation with free Ca^{2+} ions, CPM-A and HRP-A (10 μg calcium per 1 ml medium or blood) for 24 h (for cells) or 1 h (for rats) compared to control (FSBD)

Calcium stress inducer	*In vitro* (serum-free culture medium from HCAEC and HITAES)	*In vitro* (serum-free culture medium from monocytes)	*In vivo* (rat blood serum)
Free Ca^{2+} ions (administered with $CaCl_2$)	CXCL1/GROα, CD147/basigin, ST2, PDGF-AA, MIP-3α/CCL20	NGAL, CHI3L1, MMP-9	prolactin, GM- CSF, hepassocin, CNTF, MMP-3, fractalkine, FGF-21, CXCL7, fibulin-3, Cyr61/CCN, GDF-15
KPM-A	MCP-1/CCL2, CXCL1/GROα, MIF, uPAR, CD147/basigin, ST2	MMP-9, NGAL, CHI3L1.	MCP-1/CCL2, MMP-9, prolactin, GM-CSF, RAGE/AGER, hepassocin, fetuin-A, CNTF, MMP-3, HGF, fractalkine, FGF-21, CXCL7, fibulin-3, Cyr61/CCN, CCL11/eotaxin, CCL17/TARC, galectin-1, galectin-3, GDF-15
HRC-A	serpin E1/PAI-1, uPAR, CXCL1/GROα, MIP-3α/CCL20, MCP-1/CCL2, CD105/endoglin, IL-8, MIF, CD147/basigin, ST2, RANTES/CCL5	serpin E1/PAI-1, uPAR, CXCL1/GROα, MIP-3α/CCL20, MMP-9, CXCL5/ENA-78, adiponectin, NGAL, IL-6, CHI3L1, apolipoprotein A-I	serpin E1/PAI-1, MCP-1/CCL2, MMP-9, prolactin, GM-CSF, RAGE/AGER, hepassocin, fetuin-A, CNTF, MMP-3, fractalkine, FGF-21, fibulin-3, CCL17/TARC, galectin-3, GDF-15, Pref-1/DLK1/FA1
CPM-A:	MCP-1/CCL2		

upregulation of expression in ECs and in rats	
HRP-A: increased expression in monocytes and in rats	MMP-9
HRP-A: increased expression in ECs and in monocytes	uPAR, CXCL1/GROα, MIP-3α/CCL20
HRC-A: upregulation of expression in ECs and in rats	MCP-1/CCL2
HRC-A: increased expression in monocytes and in rats	MMP-9
HRP-A: increased expression in ECs, monocytes, and rats	serpin E1/PAI-1

Fig. 10. Cytokine profiling in rat serum after intravenous administration of FSBD (control), free Ca^{2+} ions, CPM-A, and CHR-A (10 µg calcium per 1 ml blood) for 1 h. Upper panel (red box): green, prolactin; dark green, GM-CSF; black, RAGE/AGER; blue, hepassocin/FGL-1; purple, fetuin-A; dark blue, CNTF; gold, MMP-3; pink, HGF; brown, CX3CL1/fractalkine; red, MCP-1/CCL2; azure, MMP-9. Bottom panel (blue frame): green, FGF-21; dark green, CXCL7; black, fibulin-3; blue, Cyr61/CCN1; purple, CCL11/eotaxin; dark blue, serpin E1/PAI-1; gold,

CCL17/TARC; pink, galectin-1; brown, galectin-3; red, GDF-15; azure, Pref-1/DLK1/FA1. Short, medium, and long arrows indicate fold changes of 1.20-1.49, 1.50-1.99, and ≥2.00-fold, respectively, compared with the FSBD group. The amount of hyperexpressed cytokines upon exposure to Ca^{2+}, CPM-A, or HRP-A is indicated at the bottom of the figure

DISCUSSION

Disorders of mineral homeostasis (e.g., pathologic decrease in albumin levels and/or pathologic increase in serum calcium or phosphorus) are an independent risk factor for circulatory diseases [11]. This condition is also characteristic of chronic kidney disease, which contributes to endothelial dysfunction by increasing serum urea and creatinine concentrations [53, 54]. In addition to these biochemical triggers, endothelial dysfunction is exacerbated by HRP internalization by vascular ECs [8-15] and hepatic sinusoidal ECs [17, 18], leading to the development of chronic low-intensity sterile inflammation [13, 14]. HRP internalization is associated with increased levels of proinflammatory cytokines, an imbalance between vasoconstriction and vasodilation (including decreased NO production), and endothelial-mesenchymal transition [55]. In particular, internalization of HRP by ECs and monocytes triggers the release of inducible endothelial cytokines (IL-6, IL-8, MCP-1/CCL2 and the soluble form of ICAM-1) as well as monocyte-derived cytokines (MIP-1α, MIP-3α, CINC-1, CINC-3 and CXCL10) [13]. Since chronic low-intensity sterile inflammation and endothelial dysfunction mutually reinforce each other [56-59], it is rather difficult to objectively determine the contribution to the development of systemic inflammation by ECs and monocytes as a result of exposure to HRP, as well as the specific cytokines they produce.

According to recent concepts, nanometer-sized (~9-10 nm) PMCs serve as "building blocks" for submicron (~30-100 nm) primary HRCs (or HRC-I), which then undergo aggregation and transition from amorphous to crystalline form to form micron-sized (100-300 nm) secondary HRCs (or HRC-II) [18, 27, 60, 61]. Primary HRCs (initially amorphous and spherical) mature into spindle-shaped or needle-shaped crystalline secondary HRCs [18, 27, 60, 61]. CPMs and primary HRCs are composed of amorphous calcium phosphate, whereas secondary HRCs are formed by carbonate-hydroxyapatite (bioapatite). HRPs are calcium and phosphorus skewers that can adsorb proteins of the microenvironment (e.g., circulating blood proteins) [18, 27, 60, 61]. Calcium and phosphorus interact with each other in the presence of the mineral chaperone fetuin-A and other acidic serum proteins, resulting in the formation of amorphous calcium phosphate (CPM or primary CHP) or carbonate-hydroxyapatite (secondary CHP) [22, 62-64].

High phosphorus concentrations inhibit nitric oxide production, which impairs vasodilation [65-67], induces EC apoptosis [65, 67], and dose-dependently promotes systemic inflammation by triggering oxidative stress and stimulating the expression of proinflammatory cytokines [68]. High phosphate content is considered to be an independent factor of endothelial dysfunction and chronic low-intensity sterile inflammation [69-71].

Although it has been shown that depletion of serum Ca^{2+} binding capacity leads to HRP deposition and that mineral stress influences the development of endothelial dysfunction and systemic inflammation, it remains unclear whether the effects of calcium stress are due solely to the amount of calcium or also to the form of calcium delivery (free Ca^{2+} ions, CPM and HRP). Typically, CPM and HRC are synthesized artificially by mixing excess calcium and phosphorus ions with proteins in a buffer solution. The above question can be answered by using a holistic approach (adding serum as a protein source) or a reductionist approach (adding any of the major serum proteins, such as albumin or fetuin-A, as a protein source). The holistic approach reproduces the scenario occurring in human serum, whereas the reductionist approach allows to analyze the ability of each serum protein to bind minerals and to exclude possible negative effects of other serum components. To more accurately model mineral stress, the reductionist approach was used in this study to simultaneously synthesize CPM and HRP. BSA was chosen as the protein source for CPM-A and HRC-A because: (i) the lower quartile of serum albumin content is associated with an increased risk of cardiovascular disease and correlates with an increased propensity of serum to precipitate HRP [11]; (ii) serum albumin concentration positively correlates with HRP concentration (measured with labeled bisphosphonate) and total calcium [11]; (iii) albumin is one of the two major ionized calcium skewers along with fetuin-A [7]; (iv) albumin is convenient to use as its distinct yellow color facilitates visual quality control during the experiment. In addition to BSA, BSF was also used for the synthesis of CPM-F and HRP-F, since it is fetuin-A that is the main mineral chaperone supporting the formation of CPM and HRP in the human body [1-4].

When physiological concentrations of BSA were mixed with supraphysiological concentrations of calcium and phosphate, the ratio (%) between ionized, protein-bound (PBC) and phosphate-bound (PB) calcium was 50 : 20 : 30, respectively. This confirmed the physiological significance of the developed model of mineral stress (as the ratio of ionized and bound calcium was 1 : 1) and demonstrated that circulating mineral depots - CPM and CHR are able to maintain their function of calcium binding at physiological level even under pronounced mineral stress. Under conditions of mineral stress, the amount of calcium in the HRC exceeded that in the CPM, underscoring the primary role of HRCs as mineral skevengers regulating ionized calcium levels in human blood. These results are consistent with previously published

data on the ratio of calcium in HRC and CPM (1 : 1) [18, 72]. They suggest that HRP is the final buffer that aggregates excess calcium and phosphorus ions, preventing blood supersaturation of ionized calcium when other mineral buffers are depleted. The relatively low proportion of albumin bound to HRP (~15%), even under supraphysiologic conditions of mineral stress, indicates that HRP formation probably does not affect albumin function in vivo.

To compare the effects of different forms of calcium delivery ($CaCl_2$ as a donor of Ca^{2+} ions, CPM-A/CPM-F and CHR-A/HRC-F), ECs and monocytes were chosen because they are the first cell populations to interact with CHR in humans. In this study, we determined and used a physiologic dose of calcium (10 μg/mL) because this parameter is strictly regulated in the body to prevent arrhythmias and extracellular calcification [73-75]. This dose caused a 10% increase in ionized calcium concentration, which corresponded to the interquartile range of ionized blood calcium in the population (0.10 to 0.14 mmol/L, i.e., 4.0 to 5.6) [11]. Internalization of CPMs is a prerequisite for their pathological effects [8-19]. CPMs have been shown to be internalized by ECs under flow conditions in a similar manner to HRPs [10, 13]. Consistent with our previous data [9-15], incubation of arterial ECs with HRC-A increased the expression of genes encoding pro-inflammatory cell adhesion molecules (*VCAM1*, *ICAM1* and *SELE*) and pro-inflammatory cytokines (*IL6*, *CXCL8*, *CCL2* and *CXCL1*). Incubation of arterial ECs with HRP-F stimulated gene expression of *VCAM1*, *ICAM1*, *SELE*, *SELP*, *IL6*, *MIF*, and *CXCL1*. Such reprogramming is characteristic of dysfunctional ECs [55, 76, 77] and indicates the development of chronic sterile low-intensity inflammation [56-59] and an aging-associated secretory phenotype [78-80].

Incubation of ECs and monocytes with CHR-A or CHR-F (10 μg calcium per 1 mL of culture medium) enhanced the release of proinflammatory cytokines. Among the molecules whose increased expression was observed in arterial ECs and monocytes after exposure to HRP-A were cytokines and chemokines (IL-6, IL-8, MCP-1/CCL2, CXCL1/GROα, MIP-3α/CCL20) and pro- and antithrombotic molecules (PAI-1 and uPAR). Of these, uPAR, MIP-3α/CCL20, and PAI-1 were increased in HCAECs, HITAECs, and monocytes. In summary of the results of the *in vitro* experiments, exposure to HRP induced a proinflammatory response characterized by activation of cytokine release and corresponding changes in gene expression. Although Ca^{2+}, CPM-A and CPM-F also stimulated the production of several cytokines by ECs, this response was less pronounced, indicating limited pathogenic effects of these calcium forms.

Cytokine profiling by ELISA revealed a statistically significant increase in IL-6, IL-8 and MCP-1/CCL2 production in HCAECs and HITAECs after their incubation with HRP-A. However, dot-blot profiling showed an increase in IL-8 and MCP-1/CCL2 exclusively in HCAECs; IL-6 was not detected in the culture medium even after its triplicate concentration. The most likely reason for this discrepancy is the limited sensitivity of semiquantitative

chemiluminescent dot-blotting, as IL-6 levels did not exceed 175 pg/mL (compared with 250-500 pg/mL for IL-8 and 2500-6000 pg/mL for MCP-1/CCL2). This assumption is partially supported by our previous data [13], according to which the concentration of IL-6 after 24-hour cultivation of ECs incubated with HRP reached 300-450 pg/mL and could be detected by dot-blotting.

Previous studies have consistently demonstrated cytotoxic and proinflammatory effects of HRP, predominantly mediated by the NLRP3 inflammasome. These effects are due to calcium and osmotic stress, which results from a dramatic increase in the Ca^{2+} concentration in the cytosol following calcium solubilization in lysosomes [11, 81, 82]. Bafilomycin A1, a specific inhibitor of vacuolar H^+-ATPase (V-ATPase), prevented lysosomal-mediated EC [11] and vascular smooth muscle cell [81] death induced by HRP by preventing lysosome acidification and HRP dissolution. Similar cytoprotective effects have been observed with calcium-sensitive receptor, NLRP3 or caspase-1 inhibitors under conditions of calcium stress [82]. Pharmacologic inhibition of the key lysosomal protease cathepsin B also reduced IL-1β release by macrophages when exposed to HRP [25, 82]. Proteomic profiling revealed increased expression of lysosome-related proteins (especially lysosomal membrane proteins) in arterial ECs [13]. Molecular processes associated with lysosomal calcium solubilization (vacuole acidification, pH regulation, regulation of proteolysis, increased cytosolic Ca^{2+} concentration and mitochondrial outer membrane permeability) were also enhanced in HCAECs and HITAECs incubated with HRP [13]. Proteomic profiling suggested that the lysosomal response to HRP internalization involves the use of the pre-existing protein machinery rather than regulation at the transcriptional, post-transcriptional and translational levels [15]. Although some studies have reported alkalization of lysosomes and decreased hydrolase activity upon HRP solubilization due to excess Ca^{2+} [26, 28], in this study, HRP-A and HRP-F were detected in EC lysosomes using a standard pH sensor (LysoTracker Red). The summarized data on the lysosome-specific distribution and cytotoxic and proapoptotic effects of CHR-A and CHR-F suggest that their pathogenic profile is similar to that of CHR isolated from atherosclerotic plaques and serum [8], as well as to CHR-P and CHR-C (crystalline secondary CHR) [9-13, 15].

Intravenous administration of Ca^{2+}, CPM-A, or CHR-A to Wistar rats lacking other cardiovascular risk factors also induced a systemic inflammatory response, predominantly mediated by chemokines (MCP-1/CCL2, CX3CL1, CXCL7, CCL11, and CCL17), hepatokines (hepassocin, fetuin-A, FGF-21, and GDF-15), proteases (MMP-2 and MMP-3), and protease inhibitors (PAI-1). Our *in vitro* and *in vivo* data support a pronounced pro-inflammatory effect of HRP. A 1-hour time window was chosen for this study based on previous studies [13], taking into account the rapid excretion of excess calcium from the blood via its binding by acidic serum

proteins, removal of CPM by the kidneys, and recycling of HRP in the liver [5]. The concentration of ionized calcium is one of the most strictly regulated biochemical parameters of human blood (similar to pH); stable hypercalcemia is relatively rare, and even transient hypercalcemia can lead to arrhythmias [83-86]. The model of transient hypercalcemia, a condition that often occurs in patients with hyperparathyroidism or excessive vitamin D intake, is highly relevant [83-86]. The pathologic effects of calcium stress observed 1 h after intravenous administration of $CaCl_2$, CPM, or HRP to Wistar line rats were associated with chronic low-intensity sterile inflammation, defined as a moderate increase in cytokine levels in the bloodstream. Thus, calcium stress is capable of leading to a proinflammatory state that itself may support endothelial activation. If such a state remains uncontrolled (e.g., in elderly patients with more than one comorbid condition such as diabetes or chronic kidney disease), such temporary elevations in proinflammatory cytokines may contribute to the senile asthenia syndrome, in which biological age exceeds chronologic age [56-59].

Our results are in agreement with the data obtained by the group of W. Jahnen-Dechent, who showed the absence of toxicity or proinflammatory effects of CPMs after their internalization by hepatic sinusoidal ECs and epithelial cells of proximal tubules, in contrast to CHR, which showed significant cytotoxic effects and caused rapid activation of NLRP3 inflammasome already 2 h after the addition of CHR [18]. The combined analysis of *in vitro* and *in vivo* results showed that PAI-1 was the only molecule whose level was stably increased in ECs, monocytes and serum of rats after incubation with HRP-A. Reasons for this sustained increase in PAI-1 levels may include its relatively high expression (which allows us to capture the increase in its release by dot-blotting) and calcium-dependent regulation. Other cytokines whose concentrations were upregulated in culture medium from cells incubated with HRC-A/HRC-F or in the serum of rats intravenously injected with HRC-A/HRC-F (uPAR, CXCL1/GROα, MIP-3α/CCL20, MCP-1/CCL2, and MMP-9) showed less stable expression in different experimental models. For example, uPAR and CXCL1/GROα were characterized by a moderate signal in culture medium from ECs and monocytes, whereas MIP-3α/CCL20 and MMP-9 were highly expressed in culture medium from monocytes but not ECs. Similar to PAI-1, MCP-1/CCL2 showed relatively high expression in all models but was not upregulated in culture medium from monocytes. PAI-1 is produced [87, 88] and its activity is maintained [89] in a calcium-dependent manner, suggesting a mechanism that ensures activation of its release after incubation with CHR-A or CHR-F. Unfortunately, previous studies have not examined PAI-1 production by different cell populations after exposure to HRC.

Subsequent studies may focus on investigating the effects of CPM and CHR, given the high affinity of fetuin-A for calcium and the unique function of this protein as a mineral

chaperone regulating CHR formation, although the average serum fetuin-A level (1 g/L) is much lower than that of albumin (34 g/L) [90-93]. A promising direction is to investigate the combined effects of Ca^{2+} and CPM or HRP, since the calcium-sensitive receptor promotes the internalization of HRP [39], which may enhance their pathogenic effects. Another challenge is to determine the hierarchy of ability of acidic serum proteins (e.g., such as albumin, fetuin-A, osteonectin, osteoprotegerin, osteopontin, matrix Gla-protein, Gla-rich protein, alpha-1-acid glycoprotein, transferrin, haptoglobin, fibrinogen, ceruloplasmin, alpha-2-macroglobulin, immunoglobulin A, fibronectin, and antithrombin III) bind minerals. From a diagnostic point of view, differential detection of CPM and CHR can be performed using flow cytometry with a fluorescently labeled bisphosphonate (e.g., IVISense Osteo 680) and artificially synthesized CPM and CHR. Measurement of CPM and HRP concentrations in serum of healthy individuals and in various diseases may help to better understand the pathophysiologic significance of these parameters.

The results suggest that the negative effects of calcium stress are determined by the form of calcium delivery and not only by its amount. This may indicate the need to reconsider approaches to quantifying HRQ, although alternative methods (fluorescence labeling combined with flow cytometry, turbidimetry, nephelometry, dynamic light scattering, and scanning electron microscopy) are less standardized and their use for quantifying HRQ *in vitro* is widely debated. Dynamic light scattering and scanning electron microscopy are quite time-consuming, while turbidimetry results depend on the particle size distribution. Future research in this direction is likely to focus on the development of new methods for quantitative assessment of HRP.

CONCLUSION

We found that a physiologic increase in Ca^{2+} concentration (by 10%, equivalent to the population interquartile range) was achieved by the addition of 10 μg of calcium per 1 mL of serum-free culture medium or rat serum. Incubation of ECs and monocytes with this amount of HRP-A or HRP-F initiated their proinflammatory activation, manifested as transcriptional reprogramming and increased release of cytokines produced by endothelium (IL-6, IL-8, MCP-1/CCL2, uPAR, MIP-3α/CCL20, PAI-1) and monocytes (IL-6, IL-8, MIP-1α/1β, MIP-3α/CCL20, uPAR, PAI-1, CXCL1, CXCL5). Addition of free Ca^{2+} ions and CPM-A caused limited negative effects in ECs and monocytes, although CPMs were internalized by ECs under flow conditions similar to HRP. All forms of calcium delivery (free Ca^{2+} ions, colloidal CPM-A

and corpuscular CHR-A) induced a systemic inflammatory response in the Wistar rat line (Ca^{2+}: 22 cytokines, CPM-A: 30 cytokines, CHR-A: 24). PAI-1 was the only molecule whose level was stably increased in both ECs, monocytes, and serum of rats after incubation with KHR-A. The increased release of chemokines (CX3CL1, MCP-1/CCL2, CXCL7, CCL11, CCL17) and hepatokines (hepassocin, fetuin-A, FGF-21, GDF-15) after exposure to HRC indicates their role in the development of chronic low-intensity sterile inflammation.

Supplementary materials. The article supplement is published on the website of the Journal of Biochemistry (https://biochemistrymoscow.com).

AUTHOR CONTRIBUTIONS

D.S. and A.K. developed the study design and performed data validation; D.S., Victoria M., Y.M., M.S., A.S., Vera M., E.T., A.L., and A.S. developed the study methodology, performed the experiments, and analyzed the data; A.K. analyzed and validated the data, obtained funding, provided overall project management, prepared the figures, and wrote and edited the text of the article. All authors are familiarized and agree with the published version of the article.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

ETHICS DECLARATION

All animal experiment protocols were approved by the Local Ethical Committee of the Research Institute of Complex Problems of Cardiovascular Diseases (protocol code: 042/2023; approval date: April 42023). All animal experiments were conducted in accordance with the European Convention for the Protection of Vertebrate Animals (Strasbourg, 1986) and Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

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