Original Research *Highlight article*

Proteinase 3 contributes to endothelial dysfunction in an experimental model of sepsis

Eric K Patterson[1], Carolina Gillio-Meina[2], Claudio M Martin[1,3], Douglas D Fraser[1,2,4], Logan R Van Nynatten[5], Marat Slessarev[1,5] and Gediminas Cepinskas[1,6]

[1]Centre for Critical Illness Research, Lawson Health Research Institute, Lawson Health Research Institute, London, N6A 5W9, Canada; [2]Children's Health Research Institute and Translational Research Centre, Lawson Health Research Institute, London, N6A 5W9, Canada; [3]Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, Western University, London, N6A 5C1, Canada; [4]Department of Paediatrics, Schulich School of Medicine and Dentistry, Western University, London, N6A 5C1, Canada; [5]Department of Medicine, Schulich School of Medicine and Dentistry, Western University, London, N6A 5C1, Canada; [6]Department of Medical Biophysics, Western University, London, N6A 5C1, Canada
Corresponding author: Gediminas Cepinskas. Email: gcepinsk@uwo.ca

Impact statement

Proteinase 3's contribution to sepsis-induced endothelial barrier dysfunction is under-recognized. In the current study, we demonstrate through functional assays that proteinase 3 is of significant consequence to sepsis-induced endothelial barrier damage and dysfunction. Furthermore, we provide the first evidence that carbon monoxide-releasing molecules (e.g. CORM-3), which are an emerging class of anti-inflammatory compounds, ameliorate this damage. This work advances the field of leukocyte protease-induced endothelial dysfunction in sepsis and expands therapeutic potential of carbon monoxide-releasing molecules.

Abstract

In sepsis-induced inflammation, polymorphonuclear neutrophils (PMNs) contribute to vascular dysfunction. The serine proteases proteinase 3 (PR3) and human leukocyte elastase (HLE) are abundant in PMNs and are released upon degranulation. While HLE's role in inflammation-induced endothelial dysfunction is well studied, PR3's role is largely uninvestigated. We hypothesized that PR3, similarly to HLE, contributes to vascular barrier dysfunction in sepsis. Plasma PR3 and HLE concentrations and their leukocyte mRNA levels were measured by ELISA and qPCR, respectively, in sepsis patients and controls. Exogenous PR3 or HLE was applied to human umbilical vein endothelial cells (HUVECs) and HUVEC dysfunction was assessed by FITC-dextran permeability and electrical resistance. Both PR3 and HLE protein and mRNA levels were significantly increased in sepsis patients ($P < 0.0001$ and $P < 0.05$, respectively). Additionally, each enzyme independently increased HUVEC monolayer FITC-dextran permeability ($P < 0.01$), and decreased electrical resistance in a time- and dose-dependent manner ($P < 0.001$), an effect that could be ameliorated by novel treatment with carbon monoxide-releasing molecule 3 (CORM-3). The serine protease PR3, in addition to HLE, lead to vascular dysfunction and increased endothelial permeability, a hallmark pathological consequence of sepsis-induced inflammation. CORMs may offer a new strategy to reduce serine protease-induced vascular dysfunction.

Keywords: Neutrophils, inflammation, carbon monoxide releasing molecule, proteinase 3, sepsis, endothelium

***Experimental Biology and Medicine* 2021; 246: 2338–2345. DOI: 10.1177/15353702211029284**

Introduction

Sepsis, a syndrome of physiologic, pathologic, and biochemical abnormalities induced by infection is a major public health concern worldwide.[1] Despite modern practices in critical care medicine, sepsis remains a leading cause of morbidity and mortality in hospital settings.[2] In 2017, the World Health Assembly declared sepsis a global emergency.[3]

The pathophysiology of sepsis is complex and not well defined. One of the key features of sepsis is increased microvascular permeability, contributing to the formation of interstitial edema and organ dysfunction.[4] During sepsis, systemic inflammation subjects the vascular endothelium to circulating pro-inflammatory mediators (e.g. lipopolysaccharide and cytokines) and to the damaging paracellular effects of polymorphonuclear neutrophils (PMNs).

PMNs contain azurophilic granules that encapsulate large amounts of proteolytic and oxidative enzymes, including the serine proteases, proteinase 3 (PR3) and human leukocyte elastase (HLE).[5,6] Azurophilic enzymes are mobilized to the plasma membrane upon neutrophil stimulation by inflammatory mediators,[6,7] while PR3 is also constitutively present on resting neutrophil plasma membranes.[8] These enzymes contribute to endothelial dysfunction directly or through the formation of neutrophil extracellular traps.[9–12] PR3 also can activate pro-IL-1β,[13] while both HLE and PR3 enhance neutrophil extravasation.[14–16] Our previous work demonstrated that PR3 can degrade some junctional proteins (e.g. VE-cadherin and occludin) in brain microvascular endothelial cells.[10] As junctional proteins are an integral part of creating the microvascular endothelial barrier, these enzymes may play a role in the progression to endothelial barrier dysfunction. Further, these enzymes degrade extracellular matrix,[13] thus accelerating PMN recruitment and exacerbating end-organ damage. Plasma from patients with septic shock shows increased proteolysis products, including those from elastase-like enzymes.[17]

As such, strategies to limit proteolytic injury to the vascular endothelium and/or inflamed tissue may offer therapeutic benefits.

Carbon monoxide releasing molecules (CORMs) are an emerging class of powerful anti-inflammatory drugs which have shown benefit in providing protective effects in several *in vivo* animal models of severe inflammation[18–21] including sepsis.[22] While CORMs protective mechanisms are not fully understood, we demonstrated previously that CORM-3 inhibits the damaging oxidant enzyme, myeloperoxidase.[9] In the current study we measured PR3 and HLE concentrations in plasma samples from patients with sepsis and investigated PR3 and HLE's proteolytic actions on endothelial barrier integrity in the presence or absence of CORM-3 treatment.

Materials and methods

Study subjects

The institutional review board of Western University (London, ON, Canada) approved this study. Consent was obtained from patients or a substitute decision-maker. Sepsis subjects were characterized as having a confirmed or suspected sepsis diagnosis using the Sepsis 3.0 criteria,[1] plasma was obtained within 24 h of admission to the intensive care unit. Healthy, medication-free volunteers without inflammatory conditions were also recruited and consented. Venous or arterial blood was drawn via indwelling catheters (sepsis patients) or venipuncture (controls) into tubes with 0.109 mol/L trisodium citrate. Whole blood was immediately centrifuged at 1500*g*, 15 min, 4°C. Plasma and buffy coat were separated and immediately stored at −80°C. Patient's characteristics were recorded and blood was drawn within 24 h of sepsis diagnosis. Sequential organ failure assessment (SOFA) score,[23] multiple organ dysfunction score (MODS),[24] and acute physiology and chronic health evaluation II (APACHE II)[25] were calculated from data collected within the first 24 h of admission to the intensive care unit.

PR3 and HLE blood plasma concentrations

Plasma concentrations were determined by ELISA. Samples were thawed on ice, then diluted to 1:50–1:200 for PR3 (Elabscience, E-EL-H1970) or 1:100 for HLE (Abcam, ab119553) in the respective kit's provided sample diluent and assayed according to the manufacturer's protocol.

RNA extraction and qPCR

RNA was extracted from buffy coat and qPCR preformed as described previously.[26] RNA integrity numbers (RINs) were determined for each sample to ensure sufficient quality for qPCR. Due to the archival nature of the samples, some subject's samples returned RIN < 6.3 and were excluded for qPCR and additional subject's samples obtained for qPCR only. Primer and probe sets were from Thermo Fisher; HLE (Hs00236952_m1) and PR3 (Hs01597751_g1) gene expression was normalized to GUSB (glucuronidase beta) (Hs00939627_m1) and UBC (ubiquitin C) (Hs00824723_m1) and scaled to the healthy controls.

Human umbilical vein endothelial cells (HUVECs)

HUVECs were isolated from normal deliveries as previously described.[27] Passages 1–5 were grown on fibronectin-coated vessels in M199 (Sigma, M5017) supplemented with 10% fetal bovine serum (Gibco, 12483-020), 50 μg/mL mitogen (Biomedical Technologies, BT-203), 10 U/mL heparin (Pharmaceutical Partners of Canada, C504801), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Wisent Inc, 450-201-EL).

Endothelial barrier integrity

Transendothelial movement of Texas Red-dextran (molecular weight 3000; Molecular Probes, D3329) was used to functionally assess HUVEC monolayer permeability. This was performed as described previously,[9] except HUVECs were treated with 200 μL of either 5 μg/mL PR3 (Athens Research & Technology, 16-14-161820) or 2 μg/mL HLE (Athens Research & Technology, 16-14-051200) in Hank's balanced salt solution (HBSS) for 60 min at 37°C. Texas Red-dextran was applied to the apical chamber for 4 min, and then the basal chamber was assayed for fluorescence. HBSS alone was used as a control.

In parallel experiments, monolayer integrity was assessed by monitoring real-time HUVEC monolayer electrical resistance. To this end, HUVECs were seeded at 1.5×10^5 cells per well in eight-well arrays (Applied Biophysics, 8W10E+ PET) in EBM-2 medium with EGM-2MV factors (Lonza, CC-3202) and grown for 2–3 days until confluent. Wells were washed three times with 400 μL HBSS and the cells allowed to acclimate in the incubator for 45–60 min before beginning enzyme treatments. Two hundred microliters were then replaced with PR3 or HLE to a final concentration of 1–5 μg/mL in duplicate wells while

resistance was continuously monitored at 4000 Hz for at least 90 min with an electric cell-substrate impedance sensing (ECIS) instrument (Model Zθ; Applied Biophysics). For experiments involving CORM-3 (Sigma, SML0496) the drug was mixed with the enzyme just before the solution was added to the cells. HBSS alone was added to control wells. Resistance was normalized to the average of the five readings just before adding the treatment to account for well-to-well variation.

Statistical analyses

Analyses were carried out with GraphPad Prism v 4.03 or 9.1. Differences in plasma enzyme concentrations or gene expression were analyzed with the Mann-Whitney U test. HUVEC permeability to dextran was analyzed by one-sample Wilcox (samples normalized to 1 to correct for day-to-day variation), and Bonferroni's correction, alpha adjusted to 0.025 for multiple comparisons. HUVEC resistance was analyzed with two-way ANOVA.

Results

The demographic and illness characteristics of sepsis patients and healthy controls are shown in Table 1. A total of 35 patients were recruited for each of the sepsis and control cohorts, the two cohorts were similar with regards to age and sex. For sepsis patients, the most common primary infection site was the lung, with gram positive bacteria as the most common pathogen. All but one sepsis patient received vasopressors for shock, and 66% of patients received intravenous corticosteroid treatment. The SOFA score within the first 24 h was 9.5 ± 3.2 (mean ± SD), indicating the patients had a high degree of organ dysfunction and a greater than 30% mortality risk. In keeping with the SOFA scores, our patient cohort had 37% mortality (Table 1).

Table 1. Patient demographics.

Characteristic	Sepsis patients	Healthy volunteers
Number of patients	35	35
Age (mean ± SD)	57 ± 13	55 ± 12
Male/total (%)	17/35 (49%)	16/35 (46%)
SOFA Score (mean ± SD)	9.5 ± 3.2	N/A
APACHE II (mean ± SD)	32.7 ± 6	N/A
MODS day 1 (mean ± SD)	8.5 ± 3.2	N/A
WBC count $\times 10^9$/L, ±SD (*n*)	19 ± 10.9 (35)	N/A
PMN count $\times 10^9$/L, ±SD (*n*)	14.1 ± 9.1 (33)[a]	N/A
Primary infection site, *n*[b] (%)		
Lung	19 (54%)	
Wound	14 (40%)	
Skin	1 (2.9%)	N/A
Urinary tract	14 (40%)	
Blood	13 (37%)	
Pathogen, *n*[b] (%)		
Gram −ve bacteria	15 (43%)	N/A
Gram +ve bacteria	16 (46%)	N/A
Fungal	2 (6%)	N/A
Culture negative	4 (11%)	N/A
Administered steroids, *n* (%)	23 (66%)	N/A
Administered vasopressors, *n* (%)	34 (97%)	N/A
Mortality, *n* (%)	13 (37%)	N/A

APACHE II: acute physiology and chronic health evaluation II; MODS: multiple organ dysfunction score; PMN: polymorphonuclear neutrophil; SOFA: sequential organ failure assessment score.
[a]Data not available for all patients; 27/33 were neutrophilic, 5 neutropenic.
[b]Some patients had multiple sites and/or pathogens, therefore the total is >35.

Shown in Figure 1 are the measured concentrations of PR3 (Figure 1(a)) and HLE (Figure 1(b)) in plasma. There was a significantly greater PR3 concentration in sepsis patient plasma of 1163 ± 1502 ng/mL compared with 91.56 ± 53.94 ng/mL in healthy volunteers (mean ± SD, $P < 0.0001$ Mann-Whitney U test, $n = 33$ both groups). Similarly, there was a significantly greater HLE concentration in plasma of sepsis patients of 161.40 ± 164.70 ng/mL compared with approximately 17.53 ± 6.03 ng/mL in healthy volunteers (mean ± SD, $P < 0.0001$ Mann-Whitney U test, $n = 33$ both groups). There were no statistically significant differences between plasma PR3 and HLE in survivors versus non-survivors (P ~0.4–0.5) nor strong correlations between plasma enzyme concentrations and clinical scores (Spearman r was < 0.3).

As both PR3 and HLE were elevated in the plasma of sepsis patients, we used qPCR to determine if their mRNA expression was also increased in leukocytes. The qPCR results in Figure 2 show that both PR3 and HLE gene expressions are significantly up-regulated in the leukocytes of sepsis patients compared with healthy volunteers in a subset of patients from which we were able to extract high-quality RNA ($P < 0.05$ by Mann-Whitney U test, $n = 7$ and 8 for sepsis and control, respectively). High quality RNA could not be extracted from all samples and had to be excluded from analysis. The PR3 and HLE expression of one sepsis patient was too low to detect. There was a very strong correlation between neutrophil count (Table 1) and PR3 expression (Pearson $r = 0.801$, $P < 0.05$) and HLE expression (Pearson $r = 0.878$, $P < 0.01$). PMNs in healthy individuals express little PR3 or HLE mRNA[28] and their expression is restricted mostly to immature neutrophils.[29–31] This suggests that many of the sepsis patient's circulating neutrophils were very recently matured and/or with possibly more immature cells compared with healthy controls. Band cell and metamyelocyte information was unavailable for most patients. The correlations between neutrophil counts and the plasma concentrations of PR3 or HLE were very weak (Spearman $r < 0.2$).

To asses PR3's effect on endothelial barrier dysfunction we conducted functional assays on HUVECs grown on a permeable membrane. As seen in Figure 3, when HUVECs were treated with either PR3 (5 μg/mL) or HLE (2 μg/mL) for 45 min, the amount of Texas Red-dextran flow-through was significantly increased compared with HBSS alone suggesting the enzymes decreased endothelial barrier function ($P < 0.01$ by one-sample Wilcox test, α adjusted to 0.025, PR3 $n = 12$, HLE $n = 10$).

To further clarify time and/or dose-dependence, in parallel experiments we monitored real-time PR3- and HLE-induced damage to endothelial cells. As shown in Figure 4(a), PR3 caused both a dose- and time-dependent decrease in endothelial resistance over the course of 90 min. The decreased resistance reached significance earlier for increasing concentrations of PR3; with a significant

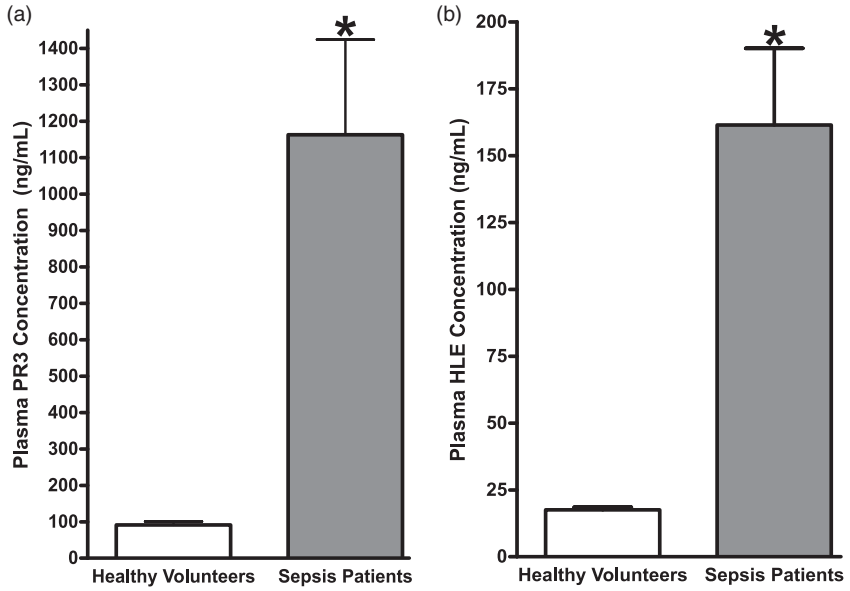

Figure 1. PR3 and HLE plasma concentrations. Plasma samples were stored at −80°C until use, then thawed on ice. (a) The PR3 concentration was determined using an Elabscience anti-human PR3 ELISA kit (E-EL-H1970) after plasma was diluted 1:50–1:200, $n = 33$ for both groups. (b) HLE plasma concentration was determined using an Abcam anti-human HLE ELISA kit (cat # ab119553) after plasma was diluted 1:100, $n = 33$. *$P < 0.0001$ vs. healthy volunteers using the Mann-Whitney U test. Data represent the mean ± SEM.

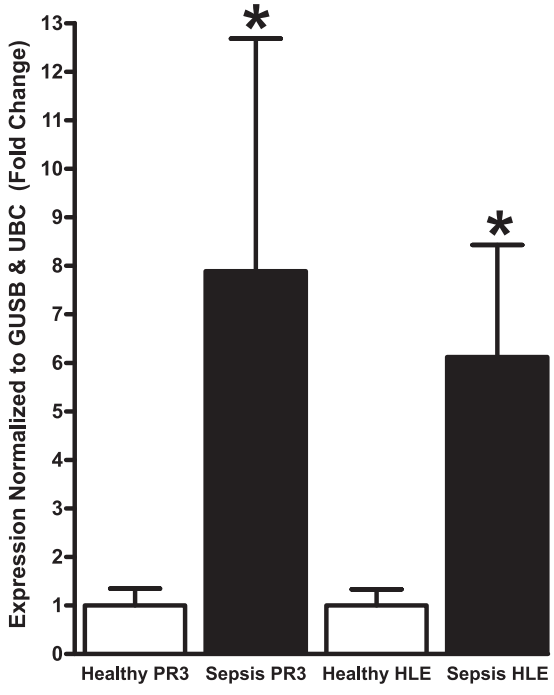

Figure 2. Leukocyte PR3 and HLE gene expression. Buffy coats were frozen at −80°C immediately after separation and RNA was extracted later using the Trizol method. Samples with a RIN ≥ 6.3 were used for qPCR; due to the archival nature of the samples, sufficient quality RNA could not be obtained from all subjects. PR3 (assay ID: Hs01597751_g1) or HLE (assay ID: Hs00236952_m1) gene expression was normalized to GUSB (assay id: Hs00939627_m1) and UBC (assay ID: Hs00824723_m1). *$P < 0.05$ vs. healthy controls, $n = 7$ and 8 for sepsis patients and controls, respectively. For graphing, expression was scaled to the corresponding healthy volunteer group ± SEM.

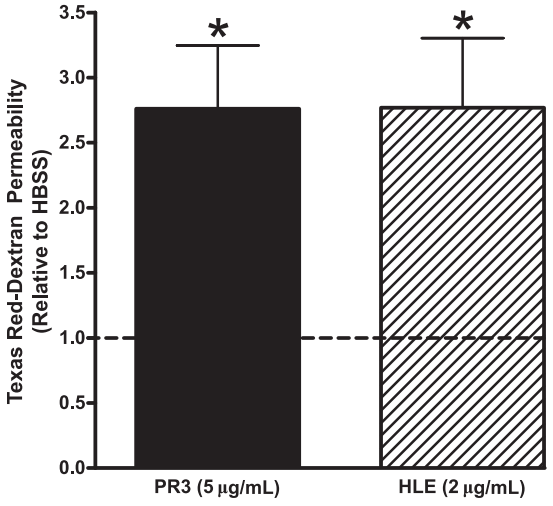

Figure 3. HUVEC permeability after PR3 or HLE treatment. HUVECs were grown in 24-well cell culture inserts (3.0 μm pore size). Confluent HUVEC monolayers were washed with HBSS and the apical chamber was treated with 200 μL of 5 μg/mL PR3 or 2 μg/mL HLE in HBSS for 60 min at 37°C, with 700 μL of HBSS in the basal chamber. Two hundred microliters of 50 μg/mL Texas Red-dextran (3000MW) were applied to the apical chamber for 4 min before the insert was removed and the liquid collected from the basal chamber was assayed for Texas Red fluorescence. HUVECs were treated with HBSS alone as a control. Values are normalized to flow-through of HBSS alone = 1 (dashed line), data represent the mean ± SEM. *$P < 0.01$ vs. HBSS alone by one-sample Wilcox test, α adjusted to 0.025, PR3 $n = 12$, HLE $n = 10$.

difference from HBSS only, seen after 23, 11, and 4 min for 1, 2 and 5 μg/mL PR3, respectively. A similar pattern emerged with HLE, shown in Figure 4(b), where the decreased resistance reached significance after 42, 15, and 4 min for 1, 2, and 5 μg/mL HLE, respectively, compared with HBSS only. It appears that at lower concentrations (<2 μg/mL), PR3 causes more severe damage to HUVEC than HLE; for instance, 1 μg/mL PR3-treated HUVEC monolayers resistance decreased to approximately 45% of untreated control after 90 min compared with approximately 65% for HLE.

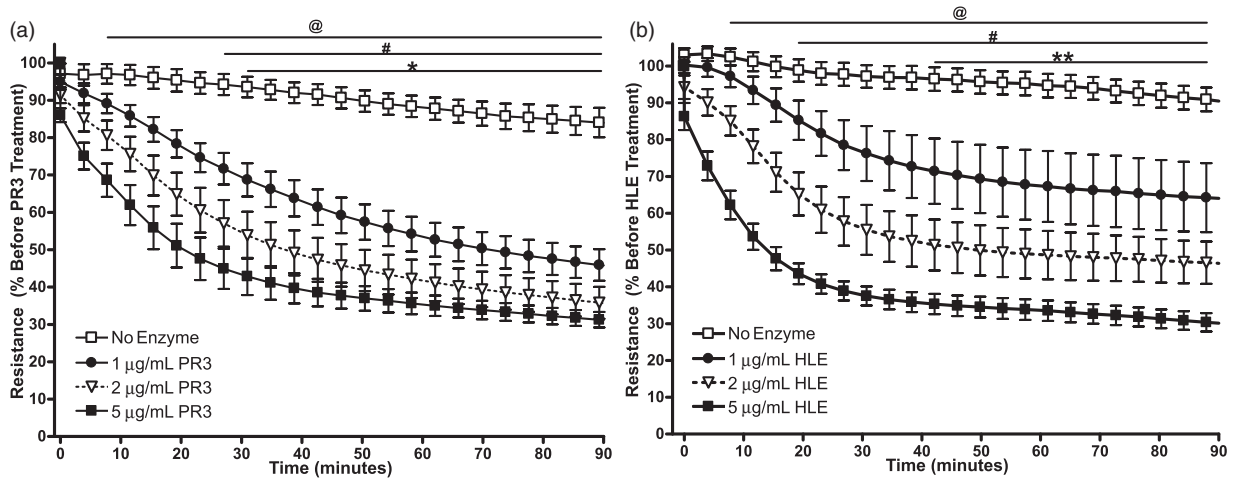

Figure 4. Real-time HUVEC resistance after PR3 or HLE treatment. HUVECs were grown to confluence in eight-well ECIS arrays (8W10E+). The indicated enzymes were added to duplicate wells and resistance was recorded for at least 90 min; HBSS alone was used as a control. Results are expressed as percent of baseline resistance (average of 5 readings before adding enzyme) ± SEM. (a) PR3 effect on HUVEC resistance, $n = 5$ in duplicate. (b) HLE effect on HUVEC resistance, $n = 7$ in duplicate. $^{*}P < 0.001$ no enzyme vs. 1 μg/mL, $^{\#}P < 0.001$ no enzyme vs. 2 μg/mL, $^{@}P < 0.001$ no enzyme vs. 5 μg/mL, $^{**}P < 0.05$ no enzyme vs. 1 μg/mL by two-way ANOVA. Data points represent the mean at that time ± SEM.

Next, we used CORM-3 in conjunction with the proteases to determine if it could mitigate some of the barrier damage caused by the proteases. As seen in Figure 5(a), 100 μmol/L CORM-3 prevented the PR3-induced reduction in HUVEC monolayer resistance, maintaining the endothelial barrier function at levels similar to control (HBSS alone), the effect was significant after approximately 11 min ($P < 0.001$ by 2-way ANOVA). Similarly, Figure 5 (b) shows that CORM-3 prevented HLE-mediated endothelial barrier dysfunction with the effect becoming significant after approximately 4 min ($P < 0.0001$ by 2-way ANOVA).

Discussion

We have herein shown that PR3 and HLE concentrations are greater in the plasma of sepsis patients compared with healthy controls and that these enzymes are each able to compromise endothelial barrier function. Furthermore, this effect can be attenuated by the addition of CORM-3, one of an emerging class of anti-inflammatory compounds. While HLE's role in inflammation-induced endothelial dysfunction has received more attention, the role of PR3 is largely uninvestigated. This work shows PR3's important role in contributing to one of the hallmark pathological consequences of sepsis, endothelial barrier dysfunction. Furthermore, that this decrease in endothelial barrier function is attenuated by CORM-3 provides insight into the protective mechanisms of CORMs in severe inflammatory conditions.

Certain clinical manifestations of sepsis and septic shock, such as edema, hypovolemia, and hypotension result from inflammation-induced endothelial dysfunction.[32] The loss of endothelial barrier function allows fluids, proteins, and solutes to leak between endothelial cells into the intracellular space of organs, and vascular tone dysregulation contributes to hypotension.[12,32,33] Microvascular thrombi and neutrophil extracellular traps cause tissue hypoperfusion, resulting in localized hypoxia and tissue damage, contributing to organ damage. Together these failures of the endothelium contribute to organ dysfunction in sepsis.

Our findings of greater plasma concentrations of both PR3 and HLE in sepsis are consistent with previous studies.[34,35] In healthy individuals, PR3 and HLE are confined to leukocytes (primarily neutrophils),[13,36] with little to none of these enzymes in circulation, suggesting the greater concentrations in sepsis patient plasma come primarily from degranulated neutrophils and/or sloughing from neutrophil extracellular traps.[37] Indeed, the increased leukocytic expression of these two transcripts in sepsis patients confirms that neutrophils were released immediately upon or slightly before maturity in order to replace the large quantity of neutrophils degranulating.

The present data indicate that PR3 and HLE have similar effects on endothelial barrier integrity, possibly because both enzymes originated from the same ancestral gene[30] and have similar, though not identical, specificity.[13,38] However, important differences are evident as, particularly at lower concentrations, PR3 decreased endothelial resistance to a greater extent than HLE. These effects may be exacerbated *in vivo* because α1 antitrypsin (A1AT), which is a native inhibitor of PR3 and HLE, preferentially inhibits HLE over PR3[39] and PR3 activates the pro-inflammatory cytokine IL-1β.[40] Further, *in vivo*, A1AT is subject to oxidative inhibition from the neutrophil respiratory burst at the site of degranulation.[41] Moreover, PR3 and HLE bound to substrate are more resistant to inactivation by native inhibitors.[39,41,42] Interestingly, CORM-3 effectively ameliorates this decrease in endothelial barrier function caused by PR3 and HLE. While the potential mechanisms (e.g. CORM-3 directly inhibiting proteolytic activity or modulating endothelial cell responses) of this protective effect remain to be investigated in future studies, our previous studies demonstrate that CORM-3 inhibits myeloperoxidase activity.[9]

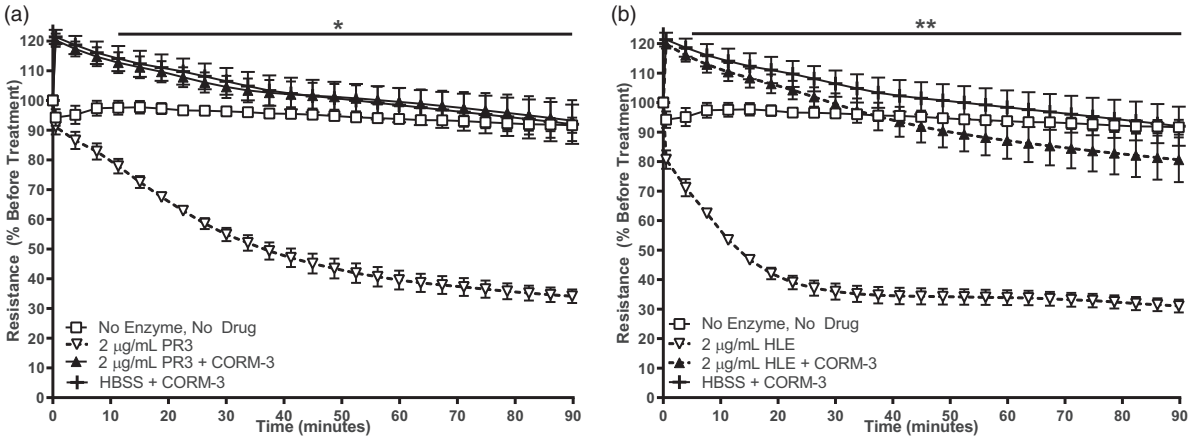

Figure 5. Real-time CORM-3 effect on PR3 or HLE-mediated HUVEC barrier function. HUVECs were grown to confluence in eight-well ECIS arrays (8W10E+). A concentration of 2 μg/mL of enzyme was mixed with 100 μmol/L CORM-3 then added to duplicate wells and resistance recorded for at least 90 min; HBSS alone was used as a control. Results are expressed as percent of baseline resistance (average of 5 readings before adding enzyme/CORM-3) ± SEM. (a) CORM-3 effect on PR3-mediated barrier function, $n = 5$ in duplicate. (b) CORM-3 effect on HLE-mediated damage, $n = 5$ in duplicate. *$P < 0.001$ vs. 2 μg/mL PR3, **$P < 0.0001$ vs. 2 μg/mL HLE by two-way ANOVA. Data points represent the mean ± SEM.

While enzymatically inhibited PR3 generates endothelial intracellular signals,[16] in this study we are modeling actions of the active enzyme that endothelial cells would encounter in inflammatory conditions, which may include both enzymatic and signaling effects. Previous work from our laboratory showed that the junctional proteins occludin and VE-cadherin were predominantly cleaved by PR3, rather than HLE in brain microvascular endothelial cells,[10] suggesting a critical role of PR3-induced proteolysis in endothelial barrier damage. Here we extend those findings to HUVEC as wells as demonstrating dose- and time-dependence of the barrier dysfunction. Although PR3 or HLE are individually sufficient to disrupt the endothelial barrier, this action is likely exacerbated *in vivo* by several other factors, e.g. oxidants and high mobility group box 1 protein.[12] These factors, along with neutrophil extravasation, likely provide PR3 increased access to endothelial junction proteins,[10,43] cellular adhesion molecules,[44] and the subendothelial space,[45] thus further impairing vascular barrier integrity leading to increased vascular permeability and PMN infiltration.

Regarding the above, it is important to note that protease concentrations experienced by endothelial cells at the site of PMN degranulation or extravasation are higher than what might be inferred from their plasma concentrations. While PR3 and HLE's plasma concentrations are in the ng/mL range (Figure 1), we should recognize that these enzymes are at far greater concentrations near the site of neutrophil degranulation.[41,42,46,47] These microenvironments (e.g. sites of neutrophil adhesion to the vascular endothelium, neutrophil migration or neutrophils in subendothelial spaces; discussed in Owen and Campbell[41]) exclude most native protease inhibitors. As such, PMN-derived proteases cause more pronounced local damage to the vascular endothelium[48] than would be inferred by their plasma concentrations.

Unfortunately, to date there are no definitive values for neutrophil enzyme concentrations at the endothelial barrier, and to our best knowledge, no studies were able to address this specific question; therefore, we chose a range of PR3 and HLE concentrations. In addition, it is well recognized that under acute inflammatory conditions, small blood vessels attract several PMN per endothelial cell,[49] creating a highly localized proteolytic microenvironment. This comports well with the common *in vitro* practice of applying 5:1 to 10:1 PMNs to endothelial cells in order to model more severe inflammation.[14,15,48,50–54] In the current study, applying the 5:1 or 10:1 ratio of PMN to HUVEC (Figure 4) would represent approximately 7.5×10^5 or 1.5×10^6 PMN per well containing approximately 2.4–4.8 μg of PR3 or 1.2–2.4 μg of HLE.[6,55] As such, while the concentrations of PR3 and HLE used in this study surpass the plasma levels of these enzymes, they are reasonably close to those we would expect to be experienced by the vascular endothelium due to degranulated PMNs in severe inflammatory conditions.

Despite these interesting findings, our study has several limitations. First, while the timing of blood draws was standardized across all patients, the timing of sepsis onset before patients arrived will differ, and we must therefore expect there to be heterogeneity in the patient samples. Second, as discussed above, there is no definitive value for the concentration of neutrophil enzymes at the endothelial barrier, so we have provided a range of concentrations for *in vitro* studies. In the current study, we are addressing only a very acute aspect of protease-mediated endothelial cell dysfunction, which is a multifactorial phenomenon and likely has longer-term consequences such as endothelial cells apoptosis, proliferation, and angiogenesis. Finally, the *in vitro* studies do not replicate the time-course of systemic PMN accumulation and enzyme build-up which occurs with *in vivo* inflammatory conditions. As a practical method of modeling this, we chose to add enzymes directly to the cultures rather than adding activated PMNs and causing degranulation though adding agents which could have their own effects on endothelial cells and would not have allowed us to study the effects of individual enzymes.

In summary, we demonstrate increased plasma concentrations of PR3 and HLE in sepsis patients relative to healthy controls. We also show that PR3 and HLE individually impair endothelial barrier function and that this is effectively prevented by CORM-3. We conclude that the PR3 released by PMNs, in addition to HLE, contributes to vascular dysfunction (a multifactorial phenomenon) and endothelial leak, a hallmark pathological consequence of sepsis, and that CORMs may offer protection against proteolytic damage to the vascular endothelium.

AUTHORS' CONTRIBUTIONS

DDF, CMM, and GC initiated the study. EKP, DDF, and GC conceived and designed experiments and analyzed data. EKP performed all experiments. CGM collected, prepared, banked, and catalogued all plasma samples and collected patient data. EKP, DDF, and GC wrote the initial manuscript. LRVN and MS collected and organized patient data and calculated clinical scores. All authors critically reviewed, revised, and approved the manuscript.

ACKNOWLEDGMENTS

The authors thank Inga Cepinskas for her assistance in isolating and preparing HUVECs as well as Eileen Campbell and Loretta Baratta for helping with clinical data collection.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The institutional review board of Western University (London, ON, Canada) approved this study.

FUNDING

This study was funded by the Heart and Stroke Foundation Ontario grant: G-17-0018622 (GC).

ORCID ID

Eric K Patterson https://orcid.org/0000-0003-3697-3042

REFERENCES

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche J-D, Coopersmith CM, Hotchkiss RS, Levy MM, Marshall JC, Martin GS, Opal SM, Rubenfeld GD, van der Poll T, Vincent J-L, Angus DC. The Third International Consensus definitions for sepsis and septic shock (Sepsis-3). *JAMA* 2016;**315**:801–10
2. Fleischmann C, Scherag A, Adhikari NKJ, Hartog CS, Tsaganos T, Schlattmann P, Angus DC, Reinhart K. Assessment of global incidence and mortality of hospital-treated sepsis. Current estimates and limitations. *Am J Respir Crit Care Med* 2015;**193**:259–72
3. Reinhart K, Daniels R, Kissoon N, Machado FR, Schachter RD, Finfer S. Recognizing sepsis as a global health priority – a WHO resolution. *N Engl J Med* 2017;**377**:414–7
4. Deutschman CS, Tracey KJ. Sepsis: current dogma and new perspectives. *Immunity* 2014;**40**:463–75
5. Wilde CG, Snable JL, Griffith JE, Scott RW. Characterization of two azurphil granule proteases with active-site homology to neutrophil elastase. *J Biol Chem* 1990;**265**:2038–41
6. Campbell EJ, Campbell MA, Owen CA. Bioactive proteinase 3 on the cell surface of human neutrophils: quantification, catalytic activity, and susceptibility to inhibition. *J Immunol* 2000;**165**:3366–74
7. Owen CA, Campbell MA, Boukedes SS, Campbell EJ. Cytokines regulate membrane-bound leukocyte elastase on neutrophils: a novel mechanism for effector activity. *Am J Physiol* 1997;**272**:L385–L393
8. Halbwachs-Mecarelli L, Bessou G, Lesavre P, Lopez S, Witko-Sarsat V. Bimodal distribution of proteinase 3 (PR3) surface expression reflects a constitutive heterogeneity in the polymorphonuclear neutrophil pool. *FEBS Lett* 1995;**374**:29–33
9. Patterson EK, Fraser DD, Capretta A, Potter RF, Cepinskas G. Carbon monoxide-releasing molecule 3 inhibits myeloperoxidase (MPO) and protects against MPO-induced vascular endothelial cell activation/dysfunction. *Free Radic Biol Med* 2014;**70**:167–73
10. Woo MMH, Patterson EK, Clarson C, Cepinskas G, Bani-Yaghoub M, Stanimirovic DB, Fraser DD. Elevated leukocyte azurophilic enzymes in human diabetic ketoacidosis plasma degrade cerebrovascular endothelial junctional proteins. *Crit Care Med* 2016;**44**:e846–53
11. Kaplan MJ, Radic M. Neutrophil extracellular traps: double-edged swords of innate immunity. *J Immunol* 2012;**189**:2689–95
12. Opal SM, van der Poll T. Endothelial barrier dysfunction in septic shock. *J Intern Med* 2015;**277**:277–93
13. Korkmaz B, Horwitz MS, Jenne DE, Gauthier F. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. *Pharmacol Rev* 2010;**62**:726–59
14. Cepinskas G, Noseworthy R, Kvietys PR. Transendothelial neutrophil migration. Role of neutrophil-derived proteases and relationship to transendothelial protein movement. *Circ Res* 1997;**81**:618–26
15. Cepinskas G, Sandig M, Kvietys PR. PAF-induced elastase-dependent neutrophil transendothelial migration is associated with the mobilization of elastase to the neutrophil surface and localization to the migrating front. *J Cell Sci* 1999;**112**:1937–45
16. Kuckleburg CJ, Tilkens SB, Santoso S, Newman PJ. Proteinase 3 contributes to transendothelial migration of NB1-positive neutrophils. *J Immunol* 2012;**188**:2419–26
17. Bauzá-Martinez J, Aletti F, Pinto BB, Ribas V, Odena MA, Díaz R, Romay E, Ferrer R, Kistler EB, Tedeschi G, Schmid-Schönbein GW, Herpain A, Bendjelid K, de Oliveira E. Proteolysis in septic shock patients: plasma peptidomic patterns are associated with mortality. *Br J Anaesth* 2018;**121**:1065–74
18. Katada K, Bihari A, Mizuguchi S, Yoshida N, Yoshikawa T, Fraser DD, Potter RF, Cepinskas G. Carbon monoxide liberated from CO-releasing molecule (CORM-2) attenuates ischemia/reperfusion (I/R)-induced inflammation in the small intestine. *Inflammation* 2010;**33**:92–100
19. Yabluchanskiy A, Sawle P, Homer-Vanniasinkam S, Green CJ, Foresti R, Motterlini R. CORM-3, a carbon monoxide-releasing molecule, alters the inflammatory response and reduces brain damage in a rat model of hemorrhagic stroke. *Crit Care Med* 2012;**40**:544–52
20. Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R, Motterlini R. Cardioprotective actions by a water-soluble carbon monoxide–releasing molecule. *Circ Res* 2003;**93**:e2–e8
21. Bihari A, Cepinskas G, Sanders D, Lawendy A-R. Systemic administration of carbon monoxide–releasing molecule-3 protects the skeletal muscle in porcine model of compartment syndrome. *Crit Care Med* 2018;**46**:e469
22. Cepinskas G, Katada K, Bihari A, Potter RF. Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice. *Am J Physiol Gastrointest Liver Physiol* 2008;**294**:G184–G191

23. Vincent JL, Moreno R, Takala J, Willatts S, De Mendonça A, Bruining H, Reinhart CK, Suter PM, Thijs LG. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the working group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. *Intensive Care Med* 1996;**22**:707–10
24. Marshall JC, Cook DJ, Christou NV, Bernard GR, Sprung CL, Sibbald WJ. Multiple Organ Dysfunction Score: a reliable descriptor of a complex clinical outcome. *Crit Care Med* 1995;**23**:1638
25. Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: a severity of disease classification system. *Crit Care Med* 1985;**13**:818–29
26. Woo M, Patterson EK, Cepinskas G, Clarson C, Omatsu T, Fraser DD. Dynamic regulation of plasma matrix metalloproteinases in human diabetic ketoacidosis. *Pediatr Res* 2016;**79**:295–300
27. Yoshida N, Granger DN, Anderson DC, Rothlein R, Lane C, Kvietys PR. Anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. *Am J Physiol* 1992;**262**:H1891–1898
28. Lakschevitz FS, Visser MB, Sun C, Glogauer M. Neutrophil transcriptional profile changes during transit from bone marrow to sites of inflammation. *Cell Mol Immunol* 2015;**12**:53–65
29. Cowland JB, Borregaard N. The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules. *J Leukoc Biol* 1999;**66**:989–95
30. Zimmer M, Medcalf RL, Fink TM, Mattmann C, Lichter P, Jenne DE. Three human elastase-like genes coordinately expressed in the myelomonocyte lineage are organized as a single genetic locus on 19pter. *Proc Natl Acad Sci U S A* 1992;**89**:8215–9
31. Garwicz D, Lennartsson A, Jacobsen SE, Gullberg U, Lindmark A. Biosynthetic profiles of neutrophil serine proteases in a human bone marrow-derived cellular myeloid differentiation model. *Haematologica* 2005;**90**:38–44
32. Trzeciak S, Rivers EP. Clinical manifestations of disordered microcirculatory perfusion in severe sepsis. *Crit Care* 2005;**9**:S20–S26
33. Abraham E, Singer M. Mechanisms of sepsis-induced organ dysfunction. *Crit Care Med* 2007;**35**:2408–16
34. Nuijens JH, Abbink JJ, Wachtfogel YT, Colman RW, Eerenberg AJ, Dors D, Kamp AJ, Strack van Schijndel RJ, Thijs LG, Hack CE. Plasma elastase alpha 1-antitrypsin and lactoferrin in sepsis: evidence for neutrophils as mediators in fatal sepsis. *J Lab Clin Med* 1992;**119**:159–68
35. Dolman KM, van de Wiel BA, Kam C-M, Abbink JJ, Hack CE, Sonnenberg A, Powers JC, von Dem Borne A, Goldschmeding R. Determination of proteinase 3–α1-antitrypsin complexes in inflammatory fluids. *FEBS Lett* 1992;**314**:117–21
36. Braun MG, Csernok E, Gross WL, Müller-Hermelink HK. Proteinase 3, the target antigen of anticytoplasmic antibodies circulating in Wegener's granulomatosis. Immunolocalization in normal and pathologic tissues. *Am J Pathol* 1991;**139**:831–8
37. Söderberg D, Segelmark M. Neutrophil Extracellular Traps in ANCA-Associated Vasculitis. *Front Immunol* 2016;**7**:256
38. Jerke U, Hernandez DP, Beaudette P, Korkmaz B, Dittmar G, Kettritz R. Neutrophil serine proteases exert proteolytic activity on endothelial cells. *Kidney Int* 2015;**88**:764–75
39. Korkmaz B, Poutrain P, Hazouard E, de Monte M, Attucci S, Gauthier FL. Competition between elastase and related proteases from human neutrophil for binding to α1-protease inhibitor. *Am J Respir Cell Mol Biol* 2005;**32**:553–9
40. Korkmaz B, Hajjar E, Kalupov T, Reuter N, Brillard-Bourdet M, Moreau T, Juliano L, Gauthier F. Influence of charge distribution at the active site surface on the substrate specificity of human neutrophil protease 3 and elastase a kinetic and molecular modeling analysis. *J Biol Chem* 2007;**282**:1989–97
41. Owen CA, Campbell EJ. Extracellular proteolysis: new paradigms for an old paradox. *J Lab Clin Med* 1999;**134**:341–51
42. Liou TG, Campbell EJ. Quantum proteolysis resulting from release of single granules by human neutrophils: a novel, nonoxidative mechanism of extracellular proteolytic activity. *J Immunol* 1996;**157**:2624–31
43. Carden D, Xiao F, Moak C, Willis BH, Robinson-Jackson S, Alexander S. Neutrophil elastase promotes lung microvascular injury and proteolysis of endothelial cadherins. *Am J Physiol* 1998;**275**:H385–H392
44. Champagne B, Tremblay P, Cantin A, Pierre YS. Proteolytic cleavage of ICAM-1 by human neutrophil elastase. *J Immunol* 1998;**161**:6398–405
45. Young RE, Voisin M-B, Wang S, Dangerfield J, Nourshargh S. Role of neutrophil elastase in LTB4-induced neutrophil transmigration in vivo assessed with a specific inhibitor and neutrophil elastase deficient mice. *Br J Pharmacol* 2007;**151**:628–37
46. LeRoy EC, Ager A, Gordon JL. Effects of neutrophil elastase and other proteases on porcine aortic endothelial prostaglandin I2 production, adenine nucleotide release, and responses to vasoactive agents. *J Clin Invest* 1984;**74**:1003–10
47. Liou TG, Campbell EJ. Nonisotropic enzyme-inhibitor interactions: a novel nonoxidative mechanism for quantum proteolysis by human neutrophils. *Biochemistry* 1995;**34**:16171–7
48. Ionescu CV, Cepinskas G, Savickiene J, Sandig M, Kvietys PR. Neutrophils induce sequential focal changes in endothelial adherens junction components: role of elastase. *Microcirculation* 2003;**10**:205–20
49. Thompson RD, Noble KE, Larbi KY, Dewar A, Duncan GS, Mak TW, Nourshargh S. Platelet-endothelial cell adhesion molecule-1 (PECAM-1)-deficient mice demonstrate a transient and cytokine-specific role for PECAM-1 in leukocyte migration through the perivascular basement membrane. *Blood* 2001;**97**:1854–60
50. Kaslovsky RA, Horgan MJ, Lum H, McCandless BK, Gilboa N, Wright SD, Malik AB. Pulmonary edema induced by phagocytosing neutrophils. Protective effect of monoclonal antibody against phagocyte CD18 integrin. *Circ Res* 1990;**67**:795–802
51. Ellerbroek PM, Hoepelman AIM, Wolbers F, Zwaginga JJ, Coenjaerts FEJ. Cryptococcal glucuronoxylomannan inhibits adhesion of neutrophils to stimulated endothelium in vitro by affecting both neutrophils and endothelial cells. *Infect Immun* 2002;**70**:4762–71
52. Hossain M, Qadri SM, Su Y, Liu L. ICAM-1-mediated leukocyte adhesion is critical for the activation of endothelial LSP1. *Am J Physiol Cell Physiol* 2013;**304**:C895–C904
53. Mittal M, Nepal S, Tiruppathi C, Malik AB. PMN mediated endothelial cell TRPM2 activation regulates neutrophil trafficking and lung vascular permeability. *FASEB J* 2017;**31**:995.2
54. Mittal M, Nepal S, Tsukasaki Y, Hecquet CM, Soni D, Rehman J, Tiruppathi C, Malik AB. Neutrophil activation of endothelial cell-expressed TRPM2 mediates transendothelial neutrophil migration and vascular injury. *Circ Res* 2017;**121**:1081–91
55. Damiano VV, Kucich U, Murer E, Laudenslager N, Weinbaum G. Ultrastructural quantitation of peroxidase- and elastase-containing granules in human neutrophils. *Am J Pathol* 1988;**131**:235–45

(Received April 20, 2021, Accepted June 13, 2021)