Innate Immunity OnlineFirst, published on August 26, 2009 as doi:10.1177/1753425909340420

Research article

Innate Immunity

0(0) (2009) 1–11

ISSN 1753-4259 (print)
10.1177/1753425909340420

Anti-inflammatory effects of C1-Inhibitor in porcine and human whole blood are independent of its protease inhibition activity

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C1-Inhibitor (C1-INH) is an important biological inhibitor, regulating several protein cascade systems. Recent research has shown that the molecule exhibits properties not dependent on its protease inhibition activity. Serum and whole blood from pigs and humans were pre-incubated with C1-INH, iC1-INH or the complement inhibitors SPICE or compstatin. Whole, live *Escherichia coli* were then added for further incubation. Complement activation, a range of cytokines, chemokines and growth factors, as well as the leukocyte activation markers wCD11R3 (pig) and CD11b (human) were measured. Both C1-INH and iC1-INH dose-dependently and significantly ($P < 0.05$) reduced a range of *E. coli*-induced pro-inflammatory cytokines and chemokines in porcine and human whole blood, as well as growth factors in human whole blood. Differences between the two forms of C1-INH and between the two species were modest. Most of these anti-inflammatory effects could not be explained by complement inhibition, as specific complement inhibitors had minor effect on several of the mediators. C1-Inhibitor had no inhibitory effect on *E. coli*-induced complement activation, while iC1-INH enhanced complement activation. The presented data indicate that C1-INH has broad anti-inflammatory effects in *E. coli*-induced inflammation in pig and human whole blood. These effects are largely independent of the protease inhibition activity.

Keywords: C1-Inhibitor, complement, cytokines, *E. coli*, iC1-Inhibitor, inflammation

Introduction

C1-Inhibitor (C1-INH) is an acute-phase protein with an average plasma level of 0.24 g/l corresponding to 1 U/ml, which is a much used functional unit. The protein belongs to the family of serine protease inhibitors and regulates both the complement and plasma kallikrein–kinin systems.[1] Because of these properties, C1-INH has been tested as a potential inhibitory agent in several diseases with known activation of these cascades.[2] C1-Inhibitor reduced the infarct size and improved myocardial contractility in myocardial ischemia-reperfusion injury (IRI) in pigs,[3] and reduced IRI in skeletal muscles in mice.[4] In experimental brain IRI in mice, C1-INH showed neuroprotection which was independent of C1q,[5] and C1-INH has also been shown to attenuate damage after brain contusion.[6] C1-Inhibitor has shown beneficial effects in various xenotransplantation models, like pig-to-human *ex vivo* kidney xenotransplantation.[7] C1-Inhibitor has also been studied in sepsis where both

Received 17 April 2009; Revised 22 May 2009; Accepted 25 May 2009

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the complement and plasma kallikrein–kinin systems are known to be activated.[8,9] In baboons infused with *Escherichia coli* bacteria, Jansen and co-workers[10] showed that C1-INH blocked the classical complement pathway, reduced the levels of factor XII and prekallikrein, and significantly reduced formation of pro-inflammatory cytokines.

In recent years, there has been focus on anti-inflammatory effects of C1-INH that are not related to its serine protease inhibitory activity. Liu and co-workers[11] showed that reactive center-cleaved, inactive C1-INH (iC1-INH) protected mice from lethal Gram-negative endotoxemia through direct interaction with LPS. Later, this interaction was shown to be dependent on glycosylation and positively charged residues within the amino-terminal non-serpin domain of the molecule.[12,13] It was also shown that C1-INH through its sialyl Lewis^x-saccharides on the non-serpin domain, can bind to E- and P-selectins on endothelial cells and inhibit rolling and transmigration of leukocytes.[14,15] In a study where mice underwent cecal ligation and puncture (CLP), both C1-INH and iC1-INH were shown to decrease mortality in the mice, possibly through enhanced phagocytosis or killing by neutrophils and macrophages, suggesting that effects other than complement and plasma kallikrein–kinin system inhibition was also important.[16]

To study the anti-inflammatory effects of C1-INH on Gram-negative induced inflammation further, and compare them to effects of iC1-INH, we used a whole blood model where blood was anticoagulated with lepirudin, a specific thrombin inhibitor leaving the remaining inflammatory network intact.[17] As pigs are often used to study human diseases, whole blood from both pigs and humans were used to study the effect of C1-INH and iC1-INH on a broad range of inflammatory mediators. These included cytokines, chemokines, growth factors and leukocyte activation markers.

Materials and Methods

Reagents

Lepirudin (Refludan) was purchased from Hoechst Marion Roussel (Frankfurt am Main, Germany). C1-Inhibitor (Cetor®) was purchased from Sanquin (Amsterdam, The Netherlands). Cleaved C1-INH (iC1-INH inhibitor) was made by trypsin treatment of C1-INH (Cetor®) as described below. Smallpox inhibitor of complement enzymes (SPICE) described previously,[18] was produced by cloning and site-directed mutagenesis of VCP.[19] The Compstatin analog Ac-I[CV(1MeW)QDWGAHRC]T was produced as described previously.[20] *Escherichia coli* was from the American Type Culture Collection (ATCC; Manassas, VA, USA). Human serum albumin (HSA), 200 mg/ml, was purchased from Octapharma AG (Lachen, Switzerland). Sterile phosphate-buffered saline (PBS) was purchased from Gibco, Invitrogen Corporation (Paisley, UK).

Production of cleaved C1-INH

C1-Inhibitor was cleaved by incubation with trypsin Sepharose (30 mg/ml) for 6 h. The Sepharose was removed by centrifugation for 3×2 min at 1000 *g*. Residual trypsin activity in the C1-INH containing supernatant was checked in a chromogenic assay with the chromogenic substrate S2222 (1 mM). Residual trypsin activity was <0.1%.

Serum experiments

Sera from three different pigs and a serum pool of 20 humans were pre-incubated for 5 min with C1-INH, iC1-INH, SPICE (pig experiments), compstatin (human experiments) or HSA. *Escherichia coli* was added to a final concentration of 10^8 bacteria/ml serum and incubated for 30 min at 37°C. Complement activation was stopped by adding ethylenediaminetetraacetic acid (EDTA) to a final concentration of 20 mM. The samples were kept on ice and then centrifuged for 10 min at 1400 *g* (4°C), for removal of *E. coli* from the samples. The baseline sample (T0) was processed immediately. The sera were stored at –70°C until analysed for complement activation.

Whole-blood experiments

Blood was collected from seven different pigs (*Sus scrofa domesticus*, Landrace, out-bred stock) and six different humans. Both porcine and human blood was anticoagulated with lepirudin (50 μg/ml) as described previously,[17] and immediately split in 1.8-ml or 4.5 ml-Nunc tubes (Roskilde, Denmark) for incubation. The blood was used for complement, cytokine or flow cytometric analyses.

For complement and cytokine analyses, blood was pre-incubated at 37°C for 5 min with C1-INH, iC1-INH, a complement inhibitor (SPICE in porcine experiments and compstatin in human experiments), or HSA. *Escherichia coli* was then added to a final concentration of 10^8 bacteria/ml whole blood in the porcine experiments and 10^7 bacteria/ml whole blood in the human experiments. The samples were incubated for 2 h and 4 h. The baseline sample (T0) was processed immediately. Complement activation was stopped by adding EDTA to

a final concentration of 20 mM immediately after incubation. The blood samples were centrifuged for 10 min at 1400 *g* (4°C) and the plasma collected and stored at −70°C until analysed.

For the flow cytometry experiments, blood was pre-incubated at 37°C for 5 min with C1-INH, iC1-INH, a complement inhibitor (SPICE or compstatin), or HSA. *Escherichia coli* was then added to a final concentration of 10^8 bacteria/ml whole blood in both the porcine and human experiments and the samples were incubated for 10 min at 37°C before analysis.

Enzyme immunoassays

The soluble terminal C5b-9 complement complex (TCC) was measured in an enzyme immunoassay (EIA), as described previously,[21] and later modified.[22] Briefly, the monoclonal antibody (mAb) aE11 reacting with a neo-epitope exposed in C9 after incorporation in the C5b-9 complex was used as capture antibody at a final concentration of 3 μg/ml. A biotinylated anti-C6 mAb (Quidel Corporation, San Diego, CA, USA) was used as detection antibody at a final concentration of 4 μg/ml. Both antibodies cross-react with pig and the assay can be used to detect porcine TCC.[22] The standard was normal human serum activated with zymosan and defined to contain 1000 arbitrary units (AU)/ml. Zymosan-activated porcine serum was used as a positive control. The buffer used as a diluent for the standards and samples and as a negative control was PBS containing EDTA and a detergent (Tween).

Immunoassays detecting the porcine cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-8 (Quantikine) were purchased from R&D Systems (Minneapolis, MN, USA). They were used according to the instructions from the manufacturer. There are fewer porcine than human biomarker kits available. Fewer cytokine analyses were, therefore, conducted in the porcine part compared to the human part of the study.

Multiplex analysis

The human inflammatory mediators were measured in EDTA plasma. The samples were analysed using a multiplex cytokine assay (Bio-Plex Human Cytokine 27-Plex Panel; Bio-Rad Laboratories Inc., Hercules, CA, USA) containing the following cytokines, chemokines and growth factors: IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12 p70, IL-13, IL-15, IL-17, eotaxin (CCL11), basic fibroblast growth factor (bFGF), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage (GM) CSF, interferon-γ (IFN-γ), IFN-γ inducible protein (IP-10 or CXCL10), monocyte chemo-attractant protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), platelet-derived growth factor-BB, regulated upon activation normally T-cell expressed and secreted, TNF-α and vascular endothelial growth factor (VEGF). The multiplex 27-plex assay is based on 27 coloured beads, each with a unique cytokine detection antibody. The beads are incubated with the samples, a secondary biotin-conjugated antibody is added, and then a reporter molecule, streptavidin-PE, which binds to biotin. The samples are run in a modified flow cytometer, with two lasers, one which excites the beads and one which excites the reporter molecule. In this way, the beads are 'separated' and the amount of each of the 27 cytokines can be quantified. The analyses were performed according to the instructions from the manufacturer.

Flow cytometry

In the pig experiments, the whole blood was fixated after incubation using a 0.5% paraformaldehyde solution, and incubated for 4 min at 37°C. The cells were stained with a mouse anti-porcine wCD11R3 IgG1-FITC clone 2F4/11 or isotype-matched IgG1-FITC control antibody clone W3/25 (both from AbD Serotec, Oxford, UK) and incubated for 15 min at 20–22°C in the dark. The red cells where lysed, the samples centrifuged at 300 *g* for 5 min and the pellets were resuspended with PBS. The samples were centrifuged at 300 *g* for 5 min and the pellets were resuspended with PBS with 0.1% albumin. Cell samples were analysed on a flow cytometer (FACScan, BD Biosciences, Franklin Lakes, NJ, USA). Untreated cells, stained with the isotype-matched antibody, were used as negative control when adjusting the flow cytometer.

In the human experiments, the whole blood was stained after fixation with a mouse anti-human CD11b IgG2a-PE clone D12 or isotype-matched IgG2a-PE control antibody clone X39 (both from BD Biosciences, San Jose, CA, USA) and incubated for 15 min at 20–22°C in the dark. Then, PBS was added and the samples were analysed on a flow cytometer (BD LSR-II, BD Biosciences).

Data presentation and statistical analysis

C1-Inhibitor, iC1-INH and HSA were added in equimolar amounts, based on a 2-fold titration of C1-INH from 64 U/ml, corresponding to 152 μM. Data are presented as ratios between the measured value at each concentration of inhibitor or control and the baseline uninhibited value. Mean and 95% confidence interval (CI) of these values are presented in all the figures. Results were analysed by two-way ANOVA with repeated measurements with Fisher Least Significant Difference *post-hoc* test using

SPSS for Windows v.15.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $P < 0.05$.

Ethics

The study was approved by the Norwegian Regional Ethical Committee and the Norwegian Animal Experimental Board. Animals were treated according to Norwegian Laboratory Animal Regulations.

RESULTS

Effect of C1-INH and iC1-INH on complement activation in porcine and human serum and whole blood

In porcine serum, C1-INH non-significantly inhibited and iC1-INH non-significantly enhanced *E. coli*-induced complement activation, whereas HSA had no effect ($P = 0.065$; Fig. 1). The porcine complement inhibitor SPICE inhibited TCC to baseline values.

In porcine whole blood, C1-INH like HSA had no effect on TCC formation whereas iC1-INH significantly ($P < 0.0001$) enhanced complement activation (Fig. 1). SPICE inhibited TCC to baseline values.

In human serum and whole blood, C1-INH like HSA had no effect on TCC formation whereas iC1-INH significantly ($P < 0.0001$) enhanced complement activation compared to C1-INH and HSA (Fig. 1). The human complement inhibitor compstatin inhibited TCC to baseline values.

Effect of C1-INH and iC1-INH on production of cytokines in porcine whole blood

Tumor necrosis factor-α

C1-Inhibitor and iC1-INH dose-dependently and significantly ($P < 0.0001$ and $P = 0.001$, respectively)

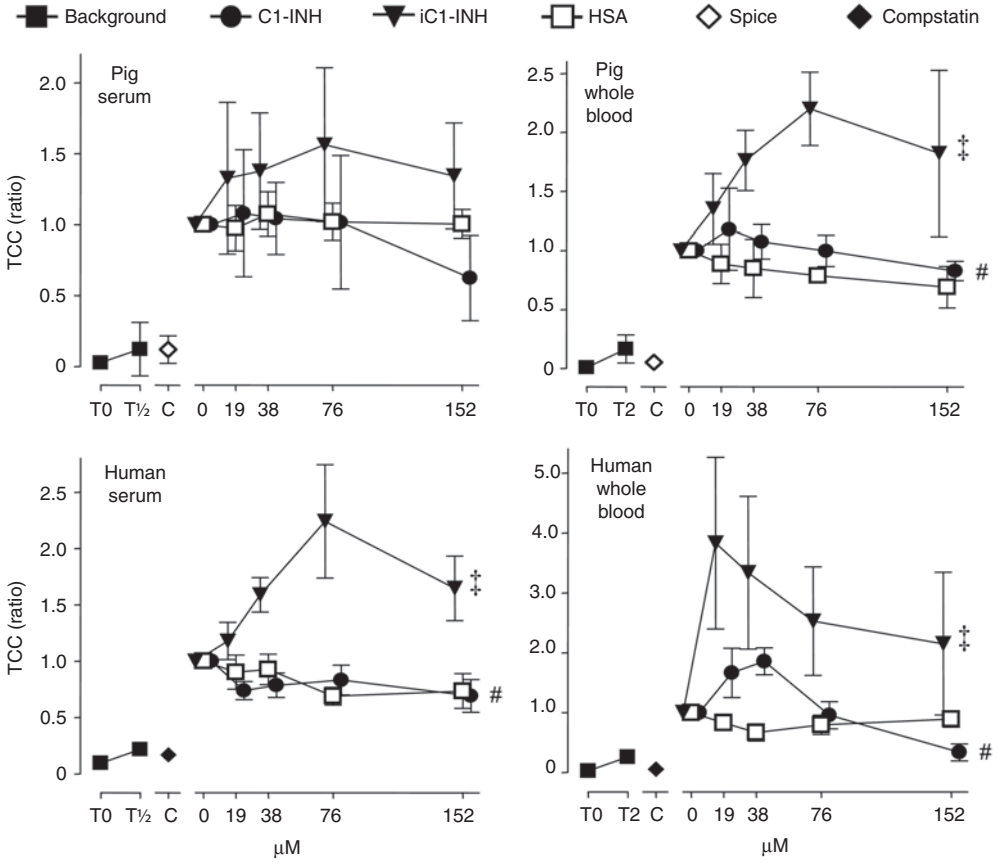

Fig. 1. Effect of C1-INH and iC1-INH on complement activation in porcine and human serum and whole blood. Sera from four different pigs and human serum from a pool of 20 donors were pre-incubated for 5 min at 37°C with C1-INH, iC1-INH, complement inhibitors (SPICE in porcine experiments and compstatin in human experiments) or HSA. A fixed dose of 10^8 *E. coli*/ml serum was then added and incubated for 30 min at 37°C. Whole blood from five different pigs and six different human donors were pre-incubated with C1-INH, iC1-INH, complement inhibitors or HSA, for 5 min at 37°C. A fixed dose of 10^8 *E. coli* bacteria/ml porcine whole blood and 10^7 *E. coli* bacteria/ml human whole blood was then added and incubated for 2 h at 37°C. Data are presented as mean and 95% CI. A two-way analysis of variance (ANOVA) with repeated measurements with Least Significant Difference (LSD) between groups *post-hoc* test was used to determine statistical significance. ‡iC1-INH compared to HSA; #C1-INH compared to iC1-INH, all $P < 0.0001$. T0, baseline sample. T½ and T2, negative control samples after 30 min and 2 h, respectively. C = complement inhibitor.

reduced *E. coli*-induced TNF-α production compared to HSA (Fig. 2). SPICE had no inhibitory effect on TNF-α production.

Interleukin-1β

C1-Inhibitor dose-dependently and significantly ($P = 0.003$) reduced *E. coli*-induced IL-1β production compared to HSA (Fig. 2), while the reduction observed with iC1-INH did not reach significance ($P = 0.080$). SPICE had no inhibitory effect on IL-1β production.

Interleukin-8

C1-Inhibitor and iC1-INH dose-dependently reduced *E. coli*-induced IL-8 production, but the reduction did not reach significance compared to HSA ($P = 0.084$; Fig. 2). SPICE had no inhibitory effect on IL-8 production.

Effect of c1-INH and IC1-INH on production of pro-inflammatory cytokines in human whole blood

Tumor necrosis factor-α

C1-Inhibitor dose-dependently and significantly ($P = 0.023$) reduced *E. coli*-induced TNF-α production compared to HSA (Fig. 3). At the highest dose, iC1-INH non-significantly ($P = 0.759$) reduced *E. coli*-induced TNF-α production. There was a significant difference between C1-INH and iC1-INH ($P = 0.042$). Compstatin reduced TNF-α production by 40%.

Interleukin-1β

C1-Inhibitor and iC1-INH dose-dependently and significantly ($P < 0.0001$ for both) reduced *E. coli*-induced IL-1β production compared to HSA (Fig. 3). There was a significant difference between C1-INH and iC1-INH ($P = 0.030$). Compstatin had no effect on IL-1β production.

Interleukin-6

C1-Inhibitor and iC1-INH dose-dependently and significantly ($P = 0.007$ and $P = 0.040$, respectively) reduced *E. coli*-induced IL-6 production compared to HSA (Fig. 3). Compstatin had no effect on IL-6 production.

Interferon-γ

C1-Inhibitor and iC1-INH dose-dependently reduced *E. coli*-induced IFN-γ production, but the reduction did not reach significance compared to HSA ($P = 0.165$; Fig. 3). Compstatin reduced IFN-γ production by 15%.

Effect of C1-INH and iC1-INH on production of chemokines in human whole blood

Interleukin-8

C1-Inhibitor, like HSA, had no effect on *E. coli*-induced IL-8 production compared to HSA (Fig. 4). iC1-INH substantially enhanced IL-8 production compared to both C1-INH and HSA ($P = 0.005$ and $P = 0.001$, respectively). Compstatin reduced human IL-8 production by 45%.

Monocyte chemo-attractant chemokine 1

C1-Inhibitor and iC1-INH dose-dependently and significantly ($P < 0.0001$ for both) reduced *E. coli*-induced MCP-1 production compared to HSA (Fig. 4). Compstatin reduced MCP-1 production by 20%.

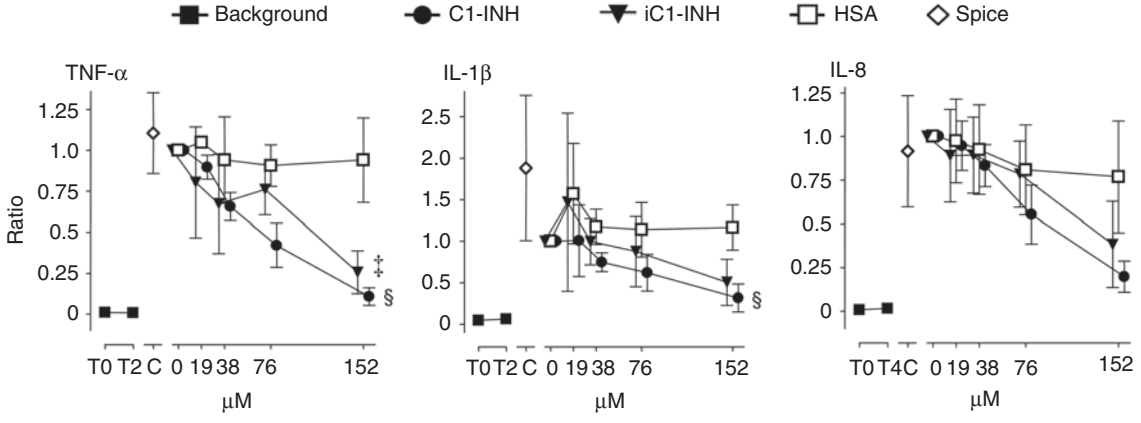

Fig. 2. Effect of C1-INH and iC1-INH on *E. coli*-induced cytokine production in porcine whole blood. Whole blood from five different pigs was pre-incubated for 5 min at 37°C with C1-INH, iC1-INH, complement inhibitor (SPICE) or HSA as control. A fixed dose of 10^8 *E. coli* bacteria/ml whole blood was then added and incubated for 2 h (TNF-α, IL-1β) or 4 h (IL-8) at 37°C. Data are presented as mean and 95% CI. ‡iC1-INH compared to HSA (TNF-α, $P = 0.001$); §C1-INH compared to HSA (TNF-α, $P < 0.0001$; IL-1β, $P = 0.003$). T0 = baseline sample. T2 and T4, negative control samples after 2 h and 4 h respectively. *C* = complement inhibitor.

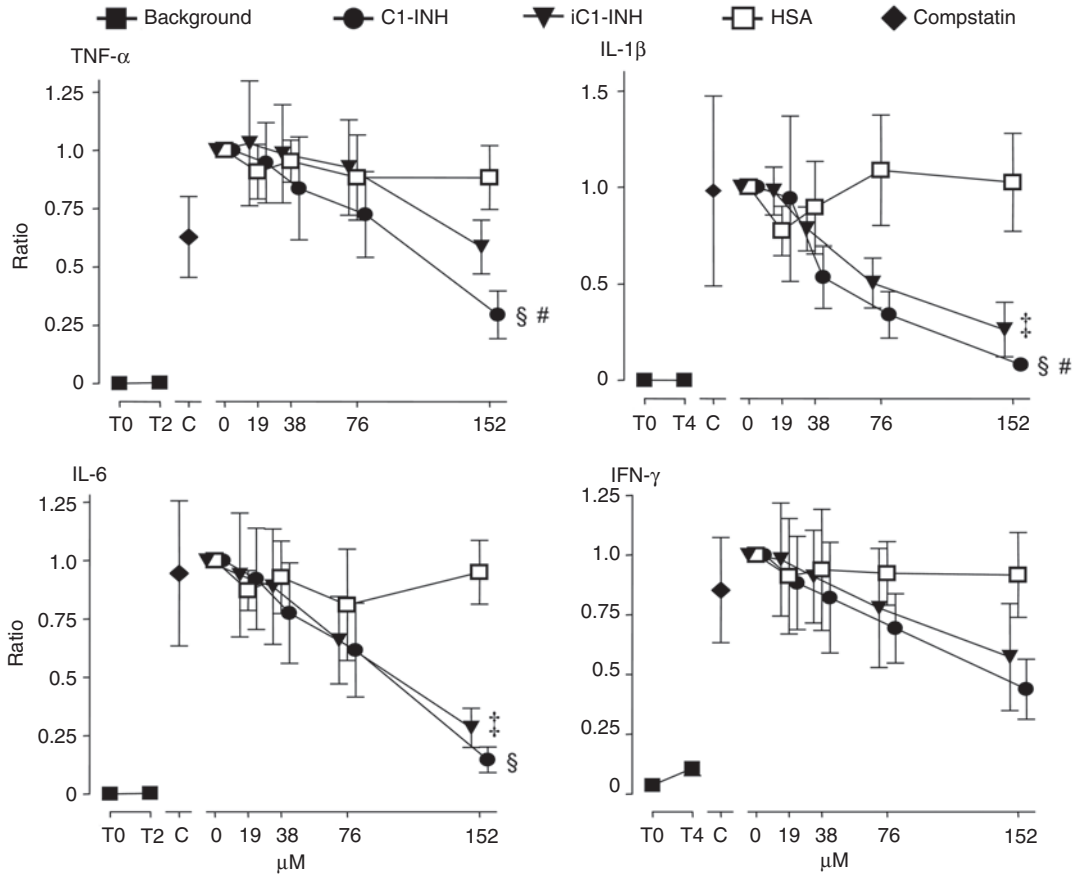

Fig. 3. Effect of C1-INH and iC1-INH on *E. coli*-induced cytokine production in human whole blood. Whole blood from six different donors was pre-incubated for 5 min at 37°C with C1-INH, iC1-INH, complement inhibitor (compstatin) or HSA. A fixed dose of 10^7 *E. coli*/ml whole blood was then added and incubated for 2 h (TNF-α and IL-6) or 4 h (IL-1β and IFN-γ) at 37°C. Data are presented as mean and 95% CI. ‡iC1-INH compared to HSA (IL-1β, $P<0.0001$; IL-6, $P=0.04$); §C1-INH compared to HSA (TNF-α, $P=0.023$; IL-1β, $P<0.0001$; IL-6, $P=0.007$); #C1-INH compared to iC1-INH (TNF-α, $P=0.042$; IL-1β, $P=0.03$). T0, baseline sample. T2 and T4, negative control samples after 2 h and 4 h, respectively. C = complement inhibitor.

Macrophage inflammatory protein-1α

C1-Inhibitor dose-dependently and significantly ($P<0.0001$) reduced *E. coli*-induced MIP-1α production compared to HSA (Fig. 4). iC1-INH reduced *E. coli*-induced MIP-1α production in human whole blood at the highest dose added, but the reduction did not reach significance ($P=0.149$). There was, however, a significant difference between C1-INH and iC1-INH ($P=0.002$). Compstatin reduced MIP-1α production by 10%.

Macrophage inflammatory protein-1β

C1-Inhibitor and iC1-INH had no effect on *E. coli*-induced MIP-1α production compared to HSA (Fig. 4). Compstatin had no effect on MIP-1α production.

Effect of C1-INH and iC1-INH on production of growth factors in human whole blood

Granulocyte colony stimulating factor

C1-Inhibitor and iC1-INH dose-dependently and significantly ($P<0.0001$ for both) reduced *E. coli*-induced G-CSF production compared to HSA (Fig. 5). Compstatin reduced human G-CSF production by 25%.

Granulocyte-macrophage colony stimulating factor

C1-Inhibitor and iC1-INH dose-dependently and significantly ($P<0.0001$ and $P=0.009$, respectively) reduced *E. coli*-induced GM-CSF production compared to HSA (Fig. 5). Compstatin reduced GM-CSF production by 15%.

Vascular endothelial growth factor

C1-Inhibitor and iC1-INH dose-dependently reduced *E. coli*-induced VEGF production, but the reduction did not reach significance compared to HSA ($P=0.167$; Fig. 5). Compstatin reduced VEGF production by 25%.

Fibroblast growth factor basic

C1-Inhibitor dose-dependently and significantly ($P=0.013$) reduced *E. coli*-induced FGF basic production compared to HSA (Fig. 5). iC1-INH reduced *E. coli*-induced FGF basic production at the highest dose added,

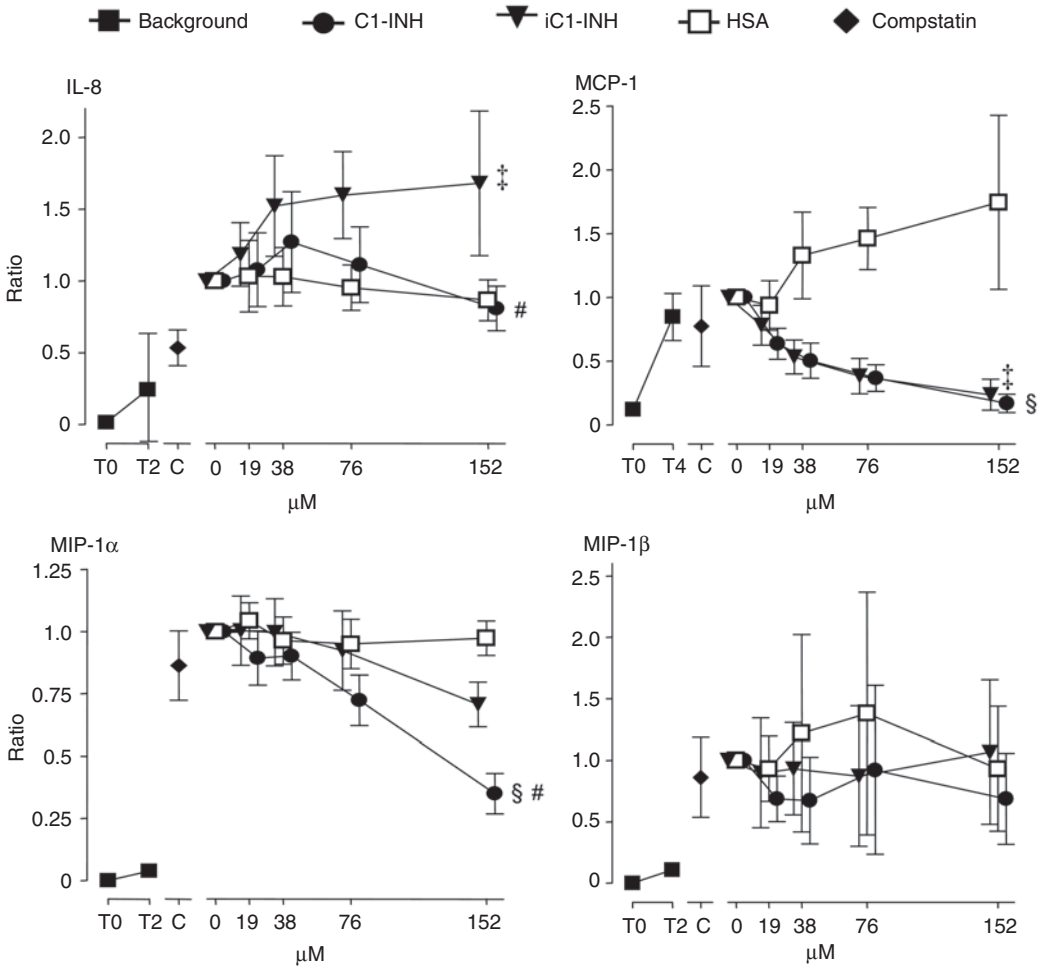

Fig. 4. Effect of C1-INH and iC1-INH on *E. coli*-induced chemokine production in human whole blood. Whole blood from six different donors was pre-incubated for 5 min at 37°C with C1-INH, iC1-INH, complement inhibitor (compstatin) or HSA. A fixed dose of 10^7 *E. coli*/ml whole blood was then added and incubated for 2 h (IL-8, MIP-1α and MIP-1β) or 4 h (MCP-1) at 37°C. Data are presented as mean and 95% CI. [‡]iC1-INH compared to HSA (IL-8, $P = 0.001$; MCP-1, $P < 0.0001$); [§]C1-INH compared to HSA (MCP-1, $P < 0.0001$; MIP-1α, $P < 0.0001$); [#]C1-INH compared to iC1-INH (IL-8, $P = 0.005$; MIP-1α, $P = 0.002$). T0, baseline sample. T2 and T4, negative control samples after 2 h and 4 h, respectively. C = complement inhibitor.

but the reduction did not reach significance compared to HSA ($P = 0.425$). Compstatin reduced FGF basic production by 25%.

Effect of C1-INH and iC1-INH on up-regulation of wCD11R3 in porcine whole blood and CD11b in human whole blood

Porcine wCD11R3

C1-Inhibitor reduced *E. coli*-induced wCD11R3 up-regulation on porcine granulocytes by 50% at the highest dose; however, the reduction did not reach significance compared to HSA ($P = 0.145$; Fig. 6, left panel). Neither iC1-INH nor HSA had any effect on the wCD11R3 up-regulation. SPICE reduced wCD11R3 up-regulation by 50%.

Human CD11b

Neither C1-INH nor HSA had any effect on CD11b up-regulation on human granulocytes (Fig. 6, middle panel), while iC1-INH substantially and significantly enhanced the CD11b up-regulation compared to C1-INH ($P = 0.006$) and HSA ($P = 0.001$). Compstatin reduced granulocyte CD11b up-regulation by 50%. C1-Inhibitor, iC1-INH and HSA had no significant effect on CD11b up-regulation on human monocytes (Fig. 6, right panel). Compstatin reduced monocyte CD11b up-regulation by 20%.

DISCUSSION

The present study shows that C1-INH efficiently reduced the production of a broad range of *E. coli*-induced

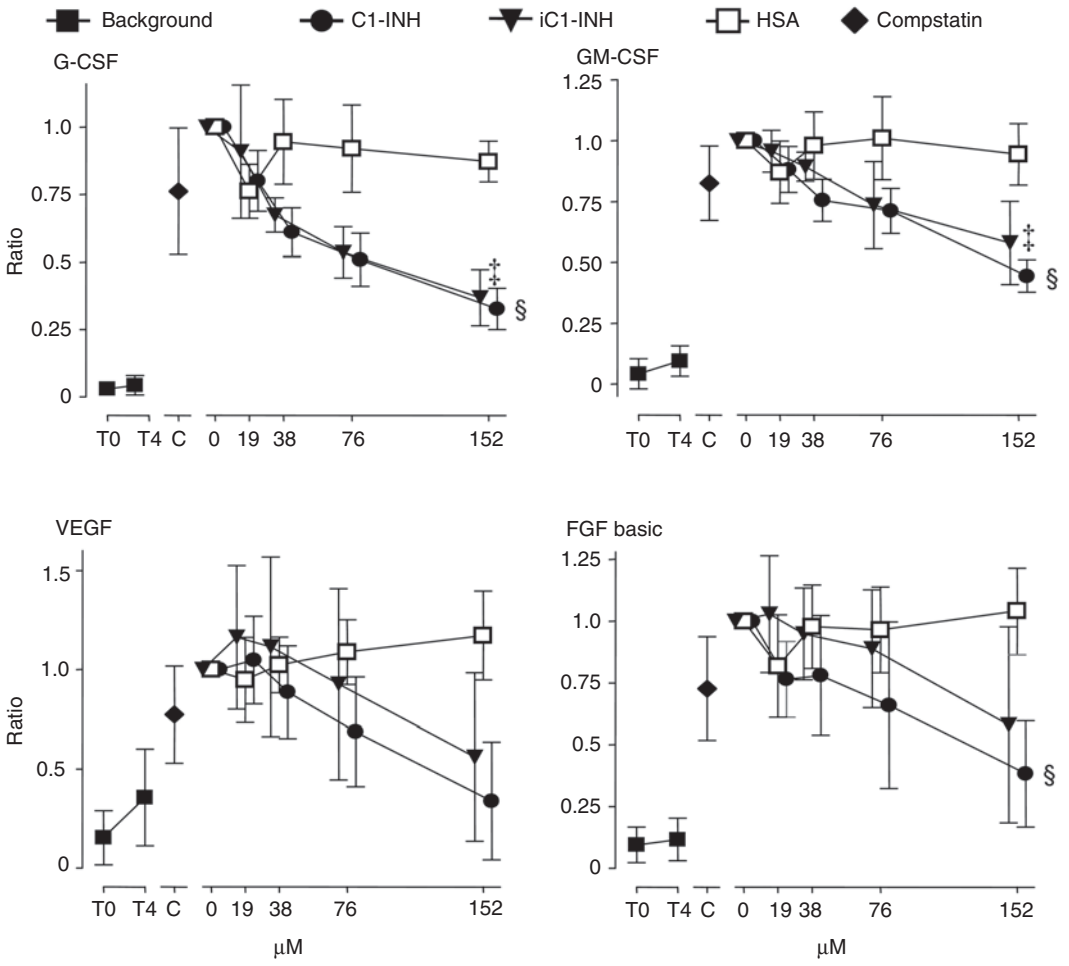

Fig. 5. Effect of C1-INH and iC1-INH on *E. coli*-induced growth factor production in human whole blood. Whole blood from six different donors was pre-incubated for 5 min at 37°C with C1-INH, iC1-INH, complement inhibitor (compstatin) or HSA. A fixed dose of 10^7 *E. coli* bacteria/ml whole blood was then added and incubated for 4 h at 37°C. Data are presented as mean and 95% CI. ‡iC1-INH compared to HSA (G-GSF, $P<0.0001$; GM-CSF, $P=0.009$); §C1-INH compared to HSA (G-CSF, $P<0.0001$; GM-CSF, $P<0.0001$; FGF basic, $P=0.013$). T0, baseline sample. T4, negative control samples after 4 h. C = complement inhibitor.

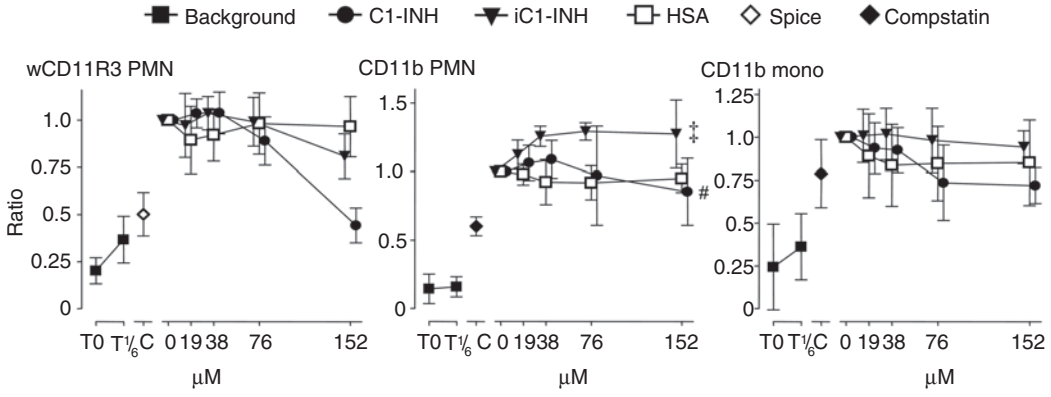

Fig. 6. Effect of C1-INH and iC1-INH on wCD11R3 expression on porcine granulocytes and CD11b expression on human granulocytes and monocytes. Whole blood from seven different pigs was pre-incubated for 5 min at 37°C with C1-INH, iC1-INH, complement inhibitor (SPICE) or HSA. A fixed dose of 10^8 *E. coli* bacteria/ml whole blood was then added and incubated for 10 min at 37°C (left panel). Whole blood from six different human donors was pre-incubated for 5 min at 37°C with C1INH, iC1INH, complement inhibitor (compstatin) or HSA. A fixed dose of 10^8 *E. coli* bacteria/ml whole blood was then added and incubated for 10 min at 37°C (middle and right panels). Data are presented as mean and 95% CI. ‡iC1-INH compared to HSA (PMN CD11b, $P=0.001$); #C1-INH compared to iC1-INH (PMN CD11b, $P=0.006$). T0, baseline sample. T1/6, negative control samples after 10 min. C = complement inhibitor.

cytokines in both human and porcine whole blood, and that these anti-inflammatory effects are largely independent of the protease inhibitory activity of C1-INH. These data are in accordance with recent findings documenting that C1-INH is a multifunctional molecule interacting with a number of non-complement related proteins participating in the inflammatory response, as recently reviewed.[23]

One of C1-INH's main functions is regulation of the classical pathway of complement. It is the only known inhibitor of the activated serine proteases C1r and C1s of this pathway.[1] Recent research has also revealed the inhibitory effect of C1-INH on the lectin pathway of complement[24] and, in particular, on this pathway's main protease MASP-2.[25] Jiang *et al*.[26] have also reported a mechanism where C1-INH can regulate the alternative complement pathway by non-covalent binding to C3b.

The reactive center (protease inhibitor site) on porcine and human C1-INH is shown to be highly homologous,[27] and we have previously found that human C1-INH in high doses inhibited complement activation in porcine serum to a certain extent.[28] In the present study, however, the inhibitory effect of C1-INH on both porcine and human complement was modest. It cannot be fully excluded that there are differences between batches of C1-INH that may explain the difference, in addition to potential differences in the experimental settings. It should, however, be emphasized that although C1-INH is an efficient inhibitor of the autocatalytic activity of C1,[29] recent data indicate that it is not similarly efficient in inhibiting exogenous activation of complement when induced on solid-phase,[24] and it is also needed in high doses in order to reduce the formation of fluid-phase TCC efficiently.[28,30]

It has previously been shown that several of the inhibitory functions of C1-INH are due to non-covalent interactions of C1-INH with target proteins in the complement cascade,[26,31] which means that these interactions are reversible. The possibility of iC1-INH influencing C1-INH's complement regulation thereby exists. Interestingly, in the present experiments, iC1-INH significantly enhanced complement activation in both species. This is a novel observation. It might be explained by competitive suppression of native C1-INH whereby iC1-INH represses C1-INH from controlling the complement autocatalytic activity, which could lead to increased spontaneous complement activation. This hypothesis would need to be further investigated in the future.

Pro-inflammatory cytokines are important mediators of inflammation. We found that *E. coli*-induced TNF-α and IL-1β were dose-dependently and significantly reduced by both C1-INH and iC1-INH in porcine whole blood. The TNF-α result is consistent with a previous finding that TNF-α mRNA from murine macrophages activated with LPS was reduced by both C1-INH and iC1-INH.[11] In human whole blood, iC1-INH seemed less potent in reducing TNF-α. This may be due to a more complement-dependent TNF-α production in humans than in pigs. A specific complement inhibitor reduced the human TNF-α level by approximately 40%, while porcine TNF-α levels was not reduced by complement inhibition, which is in accordance with previous findings.[32] Like porcine IL-1β, human IL-1β was highly significantly and dose-dependently reduced by both C1-INH and iC1-INH, while complement inhibition did not affect the production, consistent with non-protease inhibitory effects being quantitatively most important. Interleukin-6 showed a similar pattern to that of IL-1β. Our previous findings of IL-6 as one of the least complement-dependent cytokines in this whole blood model,[33] also indicate that the effect of C1-INH on IL-6 in the present study is largely independent of complement inhibition.

The central pro-inflammatory chemokine IL-8 was dose-dependently, but not significantly, reduced in porcine whole blood, while specific complement-inhibition did not influence the production. In human whole blood, however, IL-8 production was inhibited approximately 45% by specific complement inhibition, while C1-INH did not influence the production. Interleukin-8 was the only cytokine that clearly differed between the two species. This is in accordance with the fact that IL-8 production is more complement-dependent in human than in porcine whole blood. In human whole blood other important chemokines like MCP-1 and MIP-1α, were inhibited by both C1-INH and iC1-INH, while MIP-1β was not influenced by either C1-INH or iC1-INH. Thus, collectively our data indicate that the effect of C1-INH on cytokine production is mainly mediated via non-protease inhibition, and the contribution of complement inhibition is small.

The interest for growth factors in the pathogenesis of Gram-negative inflammation and sepsis is increasing. For instance, VEGF was shown to predict morbidity and mortality in human and animal sepsis.[34] Vascular endothelial growth factor was dose-dependently inhibited in the present study, but the inhibition was not statistically significant, reasonably explained by the large inter-individual variation in the experiments leading to a possible type II statistical error. Brekke and co-authors[33] showed that the combination of an anti-CD14 antibody and a complement inhibitor significantly reduced the *E. coli*-induced growth factors VEGF, FGF-basic, G-CSF and GM-CSF in human whole blood, while complement inhibition alone did not significantly reduce these growth factors. Both C1-INH and iC1-INH, however, had an impressive and highly significant inhibitory effect on G-CSF and GM-CSF in the present study. It may, therefore, be that C1-INH's

combined effect as both a complement inhibitor and an inhibitor of LPS also has a synergistic effect in these experiments. These two growth factors have attracted attention due to their role in proliferation and maturation of neutrophils and monocytes,[35,36] and may be important in the pathogenesis of sepsis. In sepsis, GM-CSF stimulate to differentiation of tissue macrophages,[37] and GM-CSF$^{-/-}$ mice show increased tolerance for LPS.[38]

A proposed mechanism for C1-INH's non-protease inhibition of the inflammatory response to Gram-negative bacteria is its interaction with lipopolysaccharide (LPS) as shown for *Salmonella enterica* sv. Typhimurium.[12] The glycosylated positively charged amino-terminal non-serpin domain of C1-INH binds to the lipid A part of the LPS molecule.[13] This binding interferes with LPS binding to LPS-binding protein and to the LPS-receptor complex on white blood cells.[11,23,39] The consequence may for instance be reduced production of TNF-α.[11] The binding between C1-INH and LPS from other Gram-negative organisms than *Salmonella* has also been demonstrated, as well as C1-INH's binding to whole Gram-negative bacteria.[23] Such binding with LPS or whole bacteria may well explain a substantial part of the anti-inflammatory effects by C1-INH shown in the present study.

C1-Inhibitor was, in general, a slightly (and for a few biomarkers significantly) more potent inhibitor of cytokines, chemokines and growth factors than iC1-INH, but the differences were, all-in-all, modest. The enhanced complement activation caused by iC1-INH might explain why there was a small inhibitory difference between the two molecules. In particular, human IL-8 was shown to be complement-dependent as compstatin inhibited the production substantially. According to this, IL-8 was the only cytokine where iC1-INH increased the production in the same manner as complement was activated. The same effect was seen for the complement-dependent biomarker CD11b on human PMNs. Neither C1-INH nor iC1-INH influenced the level of CD11b expression on human monocytes. In pigs, a substantial inhibition was obtained using C1-INH at the highest dose, but not iC1-INH, suggesting that there might have been a complement-dependent inhibition by C1-INH in these experiments. The data should, however, be interpreted with caution, since the overall change was not statistically significant. It should be noted that for both C1-INH and iC1-INH relatively high supraphysiological doses were needed to obtain the observed effects in both species.

Conclusions

We show, for the first time, that a range of *E. coli*-induced inflammatory biomarkers in whole blood from pigs and humans are reduced by protease inhibition independent effects of C1-INH. These effects dominate by far over complement inhibition. The data add novel information to the current knowledge of C1-INH's role as a multitask inhibitor of inflammatory responses, and emphasize the non-protease effects of the molecule.

Acknowledgements

The authors thank Anne Pharo for excellent laboratory technical assistance, Dorte Christiansen for growing and preparing the bacteria and Kristin Aasland and Harry Hjelmseth at The Norwegian Centre for Laboratory Animal and Alternatives, Norwegian School of Veterinary Science, Oslo, Norway for help with blood sampling of the pigs and for housing the animals. We also thank Dorina Roem and Ineke Wagenaar-Bos at Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, Amsterdam, The Netherlands for preparing the cleaved C1-INH preparation.

Financial support was kindly provided by The Research Council of Norway, The Norwegian Council on Cardiovascular Disease, NIH grant no R01-EB-003968-01, GM-62314, and AI-068730, The Working Environmental Fund, Confederation of Norwegian Enterprise, The Family Blix Foundation and The Odd Fellow Foundation.

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