C-Reactive Protein Makes Human Endothelium Stiff and Tight

Kristina Kusche-Vihrog, Katarina Urbanova, Anja Blanqué, Marianne Wilhelmi, Hermann Schillers, Katrin Kliche, Hermann Pavenstädt, Eva Brand, Hans Oberleithner

Abstract—Elevation of C-reactive protein (CRP) in human blood accompanies inflammatory processes, including cardiovascular diseases. There is increasing evidence that the acute-phase reactant CRP is not only a passive marker protein for systemic inflammation but also affects the vascular system. Further, CRP is an independent risk factor for atherosclerosis and the development of hypertension. Another crucial player in atherosclerotic processes is the mineralocorticoid hormone aldosterone. Even in low physiological concentrations, it stimulates the expression and membrane insertion of the epithelial sodium channel, thereby increasing the mechanical stiffness of endothelial cells. This contributes to the progression of endothelial dysfunction. In the present study, the hypothesis was tested that the acute application of CRP (25 mg/L), in presence of aldosterone (0.5 nmol/L; 24 hour incubation), modifies the mechanical stiffness and permeability of the endothelium. We found that endothelial cells stiffen in response to CRP. In parallel, endothelial epithelial sodium channel is inserted into the plasma membrane, while, surprisingly, the endothelial permeability decreases. CRP actions are prevented either by the inhibition of the intracellular aldosterone receptors using spironolactone (5 nmol/L) or by the inactivation of epithelial sodium channel using specific blockers. In contrast, inhibition of the release of the vasodilating gas nitric oxide via blockade of the phosphoinositide 3-kinase/Akt pathway has no effect on the CRP-induced stiffening of endothelial cells. The data indicate that CRP enhances the effects of aldosterone on the mechanical properties of the endothelium. Thus, CRP could counteract any decrease in arterial blood pressure that accompanies severe acute inflammatory processes. (Hypertension. 2011; 57:231-237.)

Key Words: aldosterone ■ CRP ■ ENaC ■ AFM ■ immunofluorescence ■ PI3K ■ NO
associated with vascular inflammation.\textsuperscript{19,20} The proinflammatory action of aldosterone has been documented further by the recent observation of Weibel–Palade Body exocytosis and the enhanced release of the prothrombotic von Willebrand factor.\textsuperscript{21}

Alerted by recent reports showing direct CRP actions on human endothelial cells,\textsuperscript{5,22,23} we tested the hypothesis that CRP could influence the biomechanical properties of endothelial cells. Some key parameters of endothelial cells were therefore studied by means of atomic force microscopy (AFM) and immunofluorescence techniques. By using the AFM as a nanomechanical sensor, the stiffness of individual endothelial cells was measured. Further, we tested the endothelial permeability in response to CRP. Because it is known that ENaC expression correlates positively with endothelial stiffening,\textsuperscript{17,24,25} the surface abundance of ENaC in response to CRP treatment was studied. By using fluorescent nanocrystals (Quantum Dots [QD]), covalently bound to secondary antibodies, immunofluorescence measurements were performed to estimate the abundance of ENaC, which appears on the endothelial cell surface.

The study shows that in the presence of aldosterone, CRP induces the insertion of ENaC into the plasma membrane, stiffens endothelial cells, and decreases endothelial permeability. This may be a compensatory mechanism to prevent a severe decrease in blood pressure during acute inflammatory processes.

\textbf{Methods}

\textbf{Endothelial Cell Culture}

Bovine aortic endothelial GM 7373 cells (DSMZ; Braunschweig, Germany) and human endothelial cells (EAhy.926, kindly donated by Cora-Jean S. Edgell, University of North Carolina, Chapel Hill, NC) were grown in culture as described previously.\textsuperscript{17,26} Briefly, endothelial cells\textsuperscript{27,28} were cultured in T\textsubscript{25} culture flasks using DMEM medium (InVitrogen Corp.; Karlsruhe, Germany) supplemented with NaHCO\textsubscript{3}, penicillin G, streptomycin (Biochrom AG; Berlin, Germany), and fetal bovine serum (PAA Clone; Coelbe, Germany). After reaching confluence, the cells were then cultured either on thin (15 mm) glass coverslips for AFM experiments or on 8-well diagnostic microscope slides (Menzel GmbH) for immunofluorescence studies. The coverslips and slides were placed in Petri dishes filled with culture medium. Both endothelial cell lines formed confluent monolayers within 48 hours (at 37°C; 5% CO\textsubscript{2}).

It is known that sodium azide is present in the commercially available preparations of CRP and therefore could cause significant intrinsic side effects such as changes in NO release when high CRP concentrations are applied.\textsuperscript{29} Such effects were observed when sodium azide concentrations were >10 mg/L, therefore, we did not exceed this concentration. Further, the concentration of sodium azide as in the various CRP experiments was the same as present in the respective control experiments.

\textbf{Endothelial Cell Stiffness Measurements}

The endothelial cell stiffness was measured with colloidal AFM probes following previously published protocols.\textsuperscript{24,30}

Measurements were performed on living cells at 37°C using a feedback-controlled heating device (Veeco). Cells were bathed in HEPES-buffered solution (standard composition in mmol/L: 140 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, and 10 HEPES [N-2-hydroxyethylpiperezine-N’-2-ethanesulfonic acid], pH 7.4). GM 7373 cells were grown for 24 hours in culture medium supplemented with aldosterone (0.5 nmol/L) or aldosterone (0.5 nmol/L) plus spironolactone (5 nmol/L). Stiffness was monitored continuously after adding CRP (25 mg/L; MP Biochemicals). The perfusate was a standard HEPES buffer that also contained either aldosterone (0.5 nmol/L) or aldosterone (0.5 nmol/L) and spironolactone (5 nmol/L).

\textbf{Endothelial Cell Stiffness of Ex Vivo Mouse Aorta}

Mouse aortas were isolated and freed from surrounding tissue. A small patch (~1 mm\textsuperscript{2}) of the whole aorta was removed and attached on Cell-Tak–coated glass, with the endothelial surface facing upward. Stiffness was determined with AFM techniques as described previously.\textsuperscript{26} AFM measurements were performed at 37°C using a Multimode AFM with a feedback-controlled heating device (Veeco). The artery preparation was bathed in HEPES–buffered solution (standard composition in mmol/L: 135 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, and 10 HEPES [N-2-hydroxyethylpiperezine-N’-2-ethanesulfonic acid], pH 7.4). Stiffness was monitored continuously after adding sodium azide (10 mg/L) as control, sodium azide (10 mg/L) plus 5% fetal calf serum (FCS), CRP (25 mg/L), CRP+FCS, CRP+FCS+benzamil, and CRP+FCS+benzamil+LY 294002.

\textbf{Immunofluorescence Measurements}

Cells were grown in culture medium supplemented with 0.5 nmol/L aldosterone. Another group was grown without aldosterone. Before fixation of the cells, 10 mg/L sodium azide or 25 mg/L CRP was added for 30 minutes, respectively.

Antibody staining was performed as described previously.\textsuperscript{31} Briefly, after fixation, EAhy.926 were gently washed 5× in phosphate-buffered saline (PBS; in mmol/L: 140 NaCl, 2 KCl, 4 Na\textsubscript{2}HPO\textsubscript{4}, and KH\textsubscript{2}PO\textsubscript{4}, pH 7.4) at room temperature and then incubated for 30 minutes in 100 mmol/L glycine/PBS solution. Antibody binding was then blocked with 10% normal goat serum at room temperature for 1 hour. The primary polyclonal rabbit anti–α-ENaC antibody (a kind gift from Dr M.S. Awayda, Department of Physiology and Biophysics, Buffalo University School of Medicine, Buffalo, NY) was diluted 1:100 in 10% normal goat serum and applied to the cells. For QD labeling, we incubated the cells with QD655 goat F(ab’\textsubscript{2}) anti-rabbit IgG conjugates (Invitrogen; Karlsruhe, Germany) 1:100 at room temperature in the dark for 1 hour. Cells were then fixed with 0.05% glutaraldehyde.

Immunofluorescence images were obtained with an inverted fluorescence microscope (Axiovert 200; Zeiss) equipped with a 100×1.45 oil immersion objective. We used the following filter: QD655, 420 nm excitation, 655 nm emission (XF302-1 filter; Omega Optical). Data acquisition and analysis were performed with the MetaVue Software (Visitron). Numbers of QD-labeled ENaC molecules at the apical surface of the cells were counted and corrected for QD background levels in cell-free areas.

\textbf{Determination of Endothelial Fluid Permeability}

The passage of fluorescein isothiocyanate (FITC)–labeled dextran (40 kDa) across a confluent layer of human endothelial cells was determined as described previously.\textsuperscript{32} To dilute unbound fluorophores, the FITC-labeled dextran was dialyzed for 24 hours against PBS. For flux measurements, cells (~2×10\textsuperscript{5} cells/cm\textsuperscript{2}) were seeded onto filter membrane inserts (ThinCert; Greiner; pore diameter 0.4 μm) and cultured with or without 0.5 mmol/L aldosterone. Only confluent cells were used for measurements. At time 0, FITC–dextran (100 μmol/L final concentration) was added together with the respective compounds (10 mg/L sodium azide, 25 mg/L of CRP, or 1 μmol/L ionomycin as a positive control) to the upper compartment (filter insert), and aliquots were taken as a 100% reference value. At time 0, 15, 30, 45, and 60 minutes, 10-μL aliquots were collected from the lower compartment, and the amounts of FITC–dextran transported across cell monolayers determined in a fluorescence 96-well microplate reader (Labsystem Fluoroskan II; GMI, Inc.) at excitation 485 nm and emission 538 nm.

\textbf{Statistics}

Data are shown as mean values±SEM. Significance of differences was evaluated by the paired or unpaired Student t test if applicable. Overall significance level is P<0.05.
**Results**

**Dose–Response Curve of CRP Action**
In health and disease, the plasma concentration of CRP covers a wide dynamic range of up to 10,000-fold (0.05 to 500 mg/L). In Figure 1, changes of endothelial stiffness in response to increasing CRP concentrations are displayed. Endothelial cells significantly stiffen in response to a 30-minute exposure of 25 mg/L CRP. For the present experiments, a concentration of 25 mg/L was chosen, which is a concentration observed in many inflammatory diseases. Further, we used an aldosterone concentration of 0.5 nmol/L, which is in the high physiological range.

**CRP Induces Sustained Stiffening of Endothelial Cells**
Endothelial cell stiffness was studied, a mechanical parameter that is altered in response to aldosterone. Within the first minutes after exposure to CRP, a transient softening of the cells is observed. However, this response is elusive, variable in magnitude, and only observable in aldosterone-exposed cells. After a lag-phase of ~15 minutes of CRP exposure, cells clearly stiffen. Spironolactone prevents this response (Figure 2A). Further, the functional ENaC blocker amiloride (1 μmol/L), a diuretic that acts on vascular endothelia, prevents the CRP-induced cell stiffening. This indicates that the membrane insertion of ENaC is a prerequisite for CRP-induced cell stiffening. The data are summarized in Figure 2B.

Stiffness measurements were also performed on endothelial cells in ex vivo preparations of mouse aortas (Figure 3A and B). Addition of CRP significantly increases cell stiffness, independent of the presence or absence of FCS. Inhibition of ENaC with benzamil (1 μmol/L) prevents the CRP-induced stiffening of the endothelial cells. The specific blocker of the PI3K pathway LY294002 does not abolish the effects of ENaC inhibition, indicating that the activity of endothelial NO synthase does not determine the endothelial stiffening. Data are summarized in Figure 3C.

**CRP Increases ENaC Surface Expression**
As mentioned above, aldosterone leads to the expression of ENaC, which apparently controls cell deformability. Therefore, we labeled the extracellular domains of the apically expressed ENaC with specific anti-α-ENaC antibodies and performed single molecule detection experiments using secondary antibodies coupled to fluorescent nanocrystals. We found that incubation with aldosterone leads to a significant increase in ENaC, confirming a previous report. The results presented here show that an additional significant increase in the number of sodium channels by ~160% (compared with control) is observed when CRP is added to aldosterone-treated cells (Figure 4). In contrast, in the absence of aldosterone, CRP does not change the number of ENaC molecules in the membrane. This clearly indicates that aldosterone is a prerequisite for CRP action and CRP-driven sodium channel expression.
CRP Decreases Endothelial Fluid Permeability Only in Presence of Aldosterone

EAhy.926 cells form confluent monolayers, which limit paracellular transport. FITC–dextran was added to the apical compartment, and the flux of the tracer across the cell layer was calculated from the net increase of the fluorescence over time in the basolateral compartment. The background filter permeability of blank inserts (without cells) is significantly higher than the permeability of inserts with cell monolayers. Addition of the Ca\(^{2+}\)/H\(_{11001}\) ionophore ionomycin as a positive control\(^{35}\) significantly increases the paracellular movement of the marker (data not shown). Under control conditions and after addition of aldosterone or CRP alone, EAhy.926 monolayers exhibit no significant changes in permeability. Interestingly, only addition of a combination of both aldosterone and CRP significantly decreases endothelial permeability within 60 minutes by ~20% (Figure 5). This shows that the synergistic action of CRP and aldosterone affects endothelial permeability, a mechanism facilitating the retention of fluid in the vascular system.

Discussion

The concentration of CRP in the blood of hypertensive patients is increased and known to predict the development of cardiovascular diseases.\(^{2}\) The results described here show that CRP, in addition to being a clinical marker, directly affects human endothelium and open some new perspectives on the possible acute action of CRP on the vascular system. CRP does not change the biomechanical properties of an endothelial cell as long as aldosterone is absent. However, absence of aldosterone is a “nonphysiological” condition and may not occur in real life. In particular, during inflammatory processes, aldosterone may be in the rather high physiological range because edema, exudations, and elevated body temperature lead to a loss of fluid and electrolytes, which stimulate its release.\(^{19,20}\) In the initial phase of the hormone response (within 20 minutes), cells transiently soften followed by sustained stiffening.\(^{18,36}\) ENaC, the expression of which parallels endothelial cell stiffening,\(^{24,25,31}\) is one of the so-called aldosterone-induced proteins. Its surface abundance is regulated by aldosterone by 2 different pathways: via activating gene transcription using the genomic mineralocorticoid

Figure 3. Endothelial cell stiffness of ex vivo mouse aortas. A, Isolated mouse aorta ex vivo. B, A small patch of the arterial wall (~1 mm\(^2\)) with the endothelial surface facing upward is attached to the glass surface. C, Mean stiffness values of mouse endothelial cells ex vivo. Cells were incubated 24 hours before the experiments in (1) sodium azide (10 mg/L) as control, (2) sodium azide (10 mg/L) plus 5% FCS, (3) CRP (25 mg/L), (4) CRP + FCS, (5) CRP + FCS + benzamil, and (6) CRP + FCS + benzamil + LY 294002. Each mean value represents 20 individual experiments (* indicates significant differences between single bars marked with brackets; \(P<0.01\)).

Figure 4. Abundance of apically located sodium channels estimated by immunofluorescence measurements. Each mean value is based on single surface ENaC molecules of 10 to 12 cells. Cells were preincubated for 25 hours in 0.5 nmol/L aldosterone; CRP (25 mg/L) was added subsequently for 30 minutes. Amount of mean surface ENaC incubated in CRP, aldosterone (Aldo), and CRP + Aldo is shown relative to the control group (Control; * indicates statistical significance compared with Control and CRP; § statistical significance compared with all other groups; \(P<0.05\)).

Figure 5. Changes in FITC–dextran fluid permeability of endothelial monolayers in the presence of aldosterone. Endothelial cells were preincubated for 25 hours in 0.5 nmol/L aldosterone; CRP (25 mg/L) was added at the beginning of the experiments. Permeability of endothelial cells incubated in CRP, aldosterone (Aldo), and CRP + Aldo is shown relative to the control group (Control; \(n=5\); mean±SEM). Asterisk indicates statistical significance compared with all other groups (\(P<0.05\)).
It is tempting to speculate that during inflammatory processes, when the levels of both aldosterone and CRP are in the high range, endothelial cells are supposed to be mechanically stiff. This most likely influences NO metabolism. Indeed, stiff endothelial cells release reduced amounts of NO, which leads to increased vascular resistance.17 Further, an active ENaC inhibits endothelial NO synthase phosphorylation via a blockade of the PI3K/Akt pathway, leading to reduced NO release.45 Here, we show that the inhibitor of the PI3K pathway, LY294002, has no effect on the decrease in stiffness attributable to ENaC inhibition. This indicates that inhibition of NO release does not influence the mechanical stiffness of the cells. Soft cells are more sensitive (ie, cells are more deformable) to shear stress and thus have the ability to release more NO. In contrast, stiff cells better resist shear stress (ie, cells are less deformable) and therefore release less NO.46 In other words, the nanomechanical properties of endothelial cells in vascular endothelium determine NO release and not vice versa.

During acute inflammatory processes and septic shock, a profound decrease in blood pressure caused by an increased NO production is described that could be life threatening.3,4 In such an acute situation, when the levels of CRP and aldosterone are high, the CRP-induced insertion of ENaC molecules into the plasma membrane and subsequent stiffening of the endothelial cells may therefore lead to reduced NO production and vasoconstriction.

During acute inflammation, endothelial permeability is increased because of enhanced NO production, which leads to an escape of exudates into the interstitial space.47,48 The data of the present study show that CRP acutely decreases endothelial permeability. This finding is supported by data showing that CRP inhibits the PI3K/Akt pathway, which is involved in the regulatory processes of inflammatory responses.6 The activation of the PI3K/Akt pathway stimulates NO release and therefore contributes to acute inflammatory processes including the increase in vascular permeability.48,49 The CRP-dependent reduction in endothelial permeability may help to retain fluid in the vascular system, thereby stabilizing the arterial blood pressure. In contrast, as expected, aldosterone alone did not increase endothelial permeability.50 From these results, we would suggest that CRP exerts its favorable effects during acute inflammatory processes by tightening the endothelial barrier. Indeed, it was shown that CRP inhibits protein leakage and vascular permeability in a manner consistent with its anti-inflammatory effect.51,52

Because CRP appears to affect endothelial cells only when aldosterone is present, it can be concluded that CRP action is mediated by aldosterone-induced proteins (eg, ENaC). It is tempting to speculate that the sequence of events is as follows: aldosterone induces the expression of ENaC, which, during inflammatory processes, is inserted into the plasma membrane facilitated by CRP. This leads to an increased mechanical stiffness of the endothelial cells and as thus limits NO release.

**Perspectives**

During acute inflammatory processes, when the levels of CRP and aldosterone are raised, the organism is threatened by a profound decrease in blood pressure resulting from enhanced production of NO. The results of the present study reveal that CRP potentiates aldosterone-mediated effects on endothelial cells. ENaC is inserted into the plasma mem-
brane, which increases the mechanical stiffness, whereas endothelial permeability is reduced. The stiff endothelial cells release reduced amounts of NO, which leads to increased vascular resistance. In parallel, the reduction of the endothelial permeability causes fluid to be retained in the vascular system, which stabilizes the arterial blood pressure. The results of the present study indicate that the synergistic action of CRP and aldosterone may help control blood pressure (Figure 6).

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Disclosures

None.

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