

# Mechanisms of C-Reactive Protein-Induced Blood–Brain Barrier Disruption

Christoph R.W. Kuhlmann, MD; Laura Librizzi, PhD; Dorothea Closhen, MD; Thorsten Pflanzner; Volkmar Lessmann, PhD; Claus U. Pietrzik, PhD; Marco de Curtis, MD; Heiko J. Luhmann, PhD

**Background and Purpose**—Increased mortality after stroke is associated with brain edema formation and high plasma levels of the acute phase reactant C-reactive protein (CRP). The aim of this study was to examine whether CRP directly affects blood–brain barrier stability and to analyze the underlying signaling pathways.

**Methods**—We used a cell coculture model of the blood–brain barrier and the guinea pig isolated whole brain preparation.

**Results**—We could show that CRP at clinically relevant concentrations (10 to 20  $\mu\text{g}/\text{mL}$ ) causes a disruption of the blood–brain barrier in both approaches. The results of our study further demonstrate CRP-induced activation of surface Fc $\gamma$  receptors CD16/32 followed by p38-mitogen-activated protein kinase-dependent reactive oxygen species formation by the NAD(P)H-oxidase. The resulting oxidative stress increased myosin light chain kinase activity leading to an activation of the contractile machinery. Blocking myosin light chain phosphorylation prevented the CRP-induced blood–brain barrier breakdown and the disruption of tight junctions.

**Conclusions**—Our data identify a previously unrecognized mechanism linking CRP and brain edema formation and present a signaling pathway that offers new sites of therapeutic intervention. (*Stroke*. 2009;40:1458-1466.)

**Key Words:** blood–brain barrier ■ edema ■ myosin light chain ■ stroke

Clinical studies have identified inflammatory processes as risk factors for ischemic stroke.<sup>1,2</sup> After arterial occlusion, ischemic brain injury in patients is accompanied by acute local inflammation and a dramatic plasma level rise of inflammatory cytokines.<sup>3</sup> The elevated plasma level of the acute phase reactant C-reactive protein (CRP) is an outcome-predicting factor after stroke or myocardial infarction.<sup>4–6</sup> A recent study demonstrated that antagonizing CRP improves the outcome after experimental myocardial infarction.<sup>7</sup> CRP is an acute phase reactant produced by the liver in response to acute inflammatory stimuli and has been demonstrated to have direct effects (increased expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, lactate dehydrogenase release) on cerebral brain microvascular endothelial cells (ECs).<sup>8</sup> The blood–brain barrier (BBB), which is mainly formed by the ECs, plays an important role in maintaining a precisely controlled ion homeostasis in the brain.<sup>9</sup> Disruption of the BBB results in the formation of a vasogenic edema, which is the most common lethal complication of ischemic stroke.<sup>10</sup> Recent in vitro studies have demonstrated that an increased phosphorylation of myosin light chains (MLC) causes BBB breakdown by activating the cellular contractile machinery, which subsequently results in

enhanced intercellular gap formation between brain ECs.<sup>11,12</sup> However, it is currently unknown whether a causal relationship between CRP formation and BBB disruption exists. The aim of our study was to examine the direct effects of CRP on BBB function and to analyze the signaling pathways with a special focus on the endothelial contractile machinery.

## Methods

### Cell Culture

Cell culture was performed as described previously.<sup>12</sup> Primary postnatal day 0 to 2 neocortical rat astrocytes were isolated and cultured as described in more detail previously<sup>13</sup> (see Supplemental Methods for more details, available online at <http://stroke.ahajournals.org>).

### Measurement of Transendothelial Electric Resistance

Transendothelial electric resistance (TEER) measurements were performed as described before after 7 to 10 days of endothelial and glial cell coculture.<sup>12,14</sup> All inhibitors were preincubated for 60 minutes.

### Measurement of Sodium Fluorescein Permeability

The permeability of sodium fluorescein through bovine brain microvascular endothelial cells (BBMVEC) was measured as a second

Received August 29, 2008; final revision received October 8, 2008; accepted October 16, 2008.

From the Institute of Physiology and Pathophysiology (C.R.W.K., D.C., V.L., H.J.L.), Johannes Gutenberg University of Mainz, Mainz, Germany; the Department of Experimental Neurophysiology (L.L., M.d.C.), Fondazione I.R.C.C.S. Istituto Neurologico Carlo Besta via Celoria 11, Milan, Italy; the Institute for Physiological Chemistry and Pathobiochemistry (T.P., C.U.P.), Johannes Gutenberg University of Mainz, Mainz, Germany; and the Institute for Physiology (V.L.), Otto-von-Guericke University, Magdeburg, Germany.

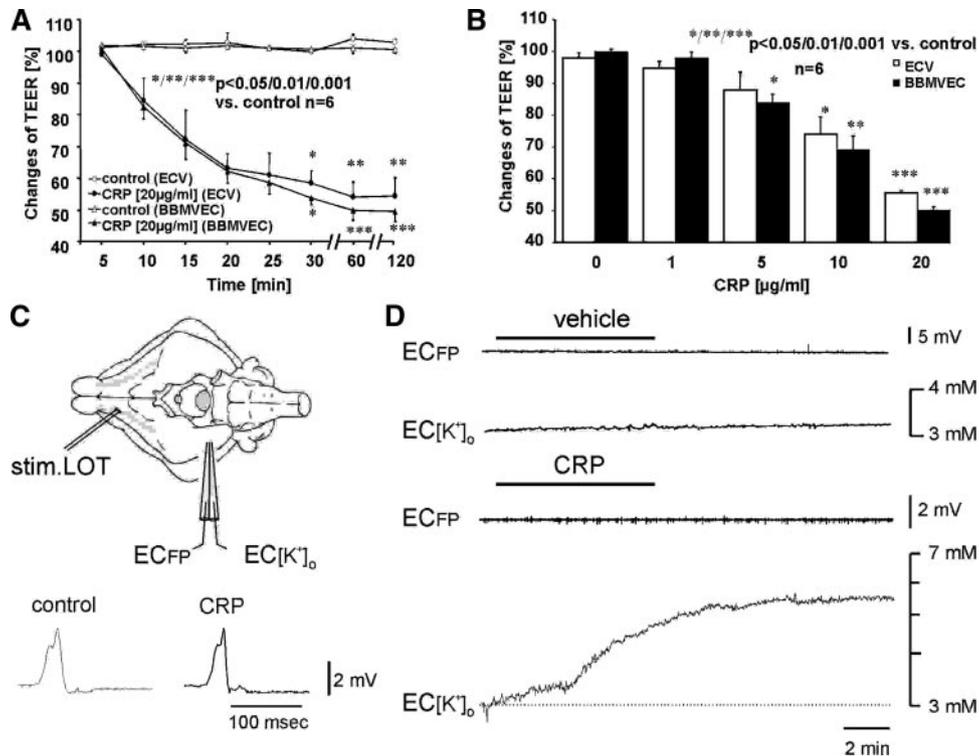
C.R.W.K. and L.L. contributed equally to this work.

Correspondence to Heiko J. Luhmann, PhD, Johannes Gutenberg University of Mainz, Institute of Physiology and Pathophysiology, Duesbergweg 6, 55128 Mainz, Germany. E-mail [luhmann@uni-mainz.de](mailto:luhmann@uni-mainz.de)

© 2009 American Heart Association, Inc.

*Stroke* is available at <http://stroke.ahajournals.org>

DOI: 10.1161/STROKEAHA.108.535930



**Figure 1.** CRP disrupts the BBB in a time- and concentration-dependent manner. The effect of CRP on BBB integrity was examined using a coculture model of the BBB composed of either BBMVEC/rat astrocyte or ECV-304/C6. CRP induced a (A) time and (B) concentration (1 to 20  $\mu\text{g}/\text{mL}$ )-dependent decrease of TEER values (initial value:  $215.7 \pm 11.3 \Omega \times \text{cm}^2$ ), indicating a loss of barrier function. The arterial perfused isolated guinea pig brain (illustrated in a schematic drawing; C) was used to study the effects of CRP (10  $\mu\text{g}/\text{mL}$ ) in a complex model closer to the in vivo situation. Two-barrel micropipette in the entorhinal cortex (EC) used to record extracellular field potentials ( $\text{EC}_{\text{FP}}$ ) and extracellular potassium concentration ( $\text{EC}[\text{K}^+]_o$ ). Traces in the lower panel show evoked potentials recorded in the EC of the in vitro-isolated guinea pig brain after stimulation of lateral olfactory tract (LOT) in control condition and after perfusion with CRP. D, Changes in  $[\text{K}^+]_o$  induced by systemic perfusion of CRP (lower panel) or control vehicle (upper panel) are shown in the trace of a representative recording.

indicator of BBB integrity as described in more detail previously<sup>12</sup> (see Supplemental Methods for more details).

### Fluorescence Imaging of Intracellular Nitric Oxide and Reactive Oxygen Species

The ECs were pretreated for 60 minutes with CRP (1 to 20  $\mu\text{g}/\text{mL}$ ) and/or inhibitors. The generation of reactive oxygen species (ROS) and nitric oxide (NO) was analyzed using the fluorescent dyes 2',7'-dichlorodihydrofluorescein (DCF), dihydroethidium (DHE), and 4,5-diaminofluorescein (DAF) as described previously.<sup>14,15</sup> Pictures made with an upright microscope (BX51WI; Olympus, Hamburg, Germany), equipped with a Nipkow spinning disk confocal system (QLC10; Visitech, Sunderland, UK) and a krypton/argon laser (Laser Physics, Cheshire, UK) were analyzed using the Metamorph imaging software (Version 6.1; Molecular Devices Corporation, Downingtown, Pa).

### Immunofluorescent Confocal Microscopy

Immunostainings of ZO-1, occludin, p-MLC, CD16, CD32, p-p38-mitogen-activated protein kinase (MAPK) were performed as described previously.<sup>12</sup> For confocal microscopy, images were acquired using the previously mentioned laser confocal microscopy setup and processed using either Metamorph imaging or Adobe PhotoShop software (Adobe Systems Inc, San Jose, Calif). The phosphorylation of MLC was quantified by analyzing the average fluorescence intensity in each single cell, a quantification method that we previously showed to be equal to densitometric Western blot analysis.<sup>14</sup>

### Western Blot

Activation of the p38-MAPK was analyzed by detecting phosphorylated and total p38 in BBMVEC using Western blot analysis as described previously<sup>14</sup> (see Supplemental Methods for more details).

### Isolated Brain Experiments

Brains were isolated from young adult Hartley guinea pigs (150 to 200 g; Charles River Laboratories, Comerio, Italy) according to the standard technique previously described in detail.<sup>16–18</sup> (see Supplemental Methods for more details). The experimental protocol was reviewed and approved by the Committee on Animal Care and Use and by the Ethics Committee of the Fondazione Istituto Neurologico in accordance with the international guidelines on care and use of laboratory animals.

### Data Analysis

Results were expressed as mean values  $\pm$  SEM and a value of  $P < 0.05$  was considered significant. Statistically significant effects of CRP or inhibitory effects on TEER, MLC phosphorylation, and ROS production were assessed by 2-way analyses of variance followed by post hoc Tukey test for multiple comparisons. Statistical analysis for the isolated brain experiments was assessed by the  $U$  test and a value of  $P < 0.05$  was considered significant.

## Results

### Blood–Brain Barrier-Disrupting Effect of C-Reactive Protein

To address the question whether CRP contributes to the BBB disruption, we first analyzed the effect of CRP in a cell coculture model of the BBB and in the isolated guinea pig brain preparation. In the coculture model composed of either ECV304/C6 or BBMVEC/rat astrocytes, CRP induced a time- and concentration-dependent disruption of the barrier (Figure 1A–B). Interestingly, the

barrier-disrupting effect reached significance at a concentration of 5 to 10  $\mu\text{g/mL}$ , which has been demonstrated to correlate with worse poststroke prognosis in clinical studies.<sup>5</sup> To exclude unspecific effects, TEER measurements were repeated using heat-inactivated CRP, which showed no effect on TEER values. For further analysis, sodium fluorescein permeability was measured in BBMVEC. Sixty minutes of CRP (20  $\mu\text{g/mL}$ ) treatment significantly increased fluorescein permeability ( $P_e$  in  $\text{cm}/\text{min} \times 10^{-4}$ ): control  $1.17 \pm 0.03$ ; CRP  $2.93 \pm 0.13$  ( $n=3$ ,  $P<0.05$ ). We also examined the effect of CRP (10  $\mu\text{g/mL}$ ) in the more complex model of the arterial perfused isolated guinea pig brain, in which the BBB as neurovascular unit is completely preserved (Figure 1C). Arterial perfusion of the in vitro isolated guinea pig brain with a solution containing 10  $\mu\text{g/mL}$  CRP for 8 minutes induced a large and fast  $[\text{K}^+]_o$  increase, whereas vehicle perfusion had no effect (Figure 1D). To exclude unspecific effects, TEER measurements were repeated using heat-inactivated CRP, which showed no effect on TEER values.

### C-Reactive Protein-Induced Blood–Brain Barrier Disruption Involves Activation of the Contractile Machinery

Because we reported recently that activation of the contractile machinery is involved in stroke-associated brain edema formation,<sup>14</sup> we were interested whether the endothelial contractile machinery may contribute to the effects of CRP. The activation of the endothelial contractile machinery is controlled by the phosphorylation state of MLC. Because the phosphorylation state of MLC is regulated by the MLC-kinase (MLCK) and -phosphatase,<sup>19</sup> the cocultures or the isolated brains were treated with the MLCK inhibitor ML-7 before CRP application. ML-7 (10  $\mu\text{mol/L}$ ) itself increased the TEER values of the cocultures and completely prevented the barrier-disrupting effect of 10 or 20  $\mu\text{g/mL}$  CRP (Figure 2A). In addition, ML-7 treatment significantly reduced sodium fluorescein permeability in BBMVEC (CRP  $2.93 \pm 0.13$ ; CRP+ML-7  $1.28 \pm 0.01$ ; ML-7  $1.02 \pm 0.03$ ;  $n=3$ ;  $P<0.05$ ). This observation could be confirmed by immunostaining of MLC phosphorylation in brain ECs and the human cell line ECV304. CRP significantly increased MLC phosphorylation (Figure 2B–C) and induced the formation of cell crossing actin stress fibers, which was completely abolished in the presence of ML-7 (Figure 2D). In addition, the barrier-disrupting effect of CRP was also completely abolished by the MLCK-inhibitor ML-7 (10  $\mu\text{mol/L}$ ) in the isolated guinea pig brain (Figure 2E). Under these conditions,  $[\text{K}^+]_o$  did not differ from the control potassium concentration of 3.3  $\text{mmol/L}$  (Figure 2F).

### CD16 and CD32 Receptors Mediate the Barrier-Disrupting C-Reactive Protein Effect

After demonstrating the contribution of MLC phosphorylation to CRP-induced BBB disruption in the coculture model and the intact brain preparation, we studied the underlying signaling cascades. Fc $\gamma$  receptors are known receptors of CRP on ECs.<sup>20</sup> For this reason, we analyzed whether pretreatment with neutralizing anti-CD16 or anti-CD32 antibodies affects CRP-induced BBB disruption in the coculture model. Interestingly, the CRP-induced decrease of TEER values was prevented in the presence of each one of these antibodies (Supplemental Table I, available

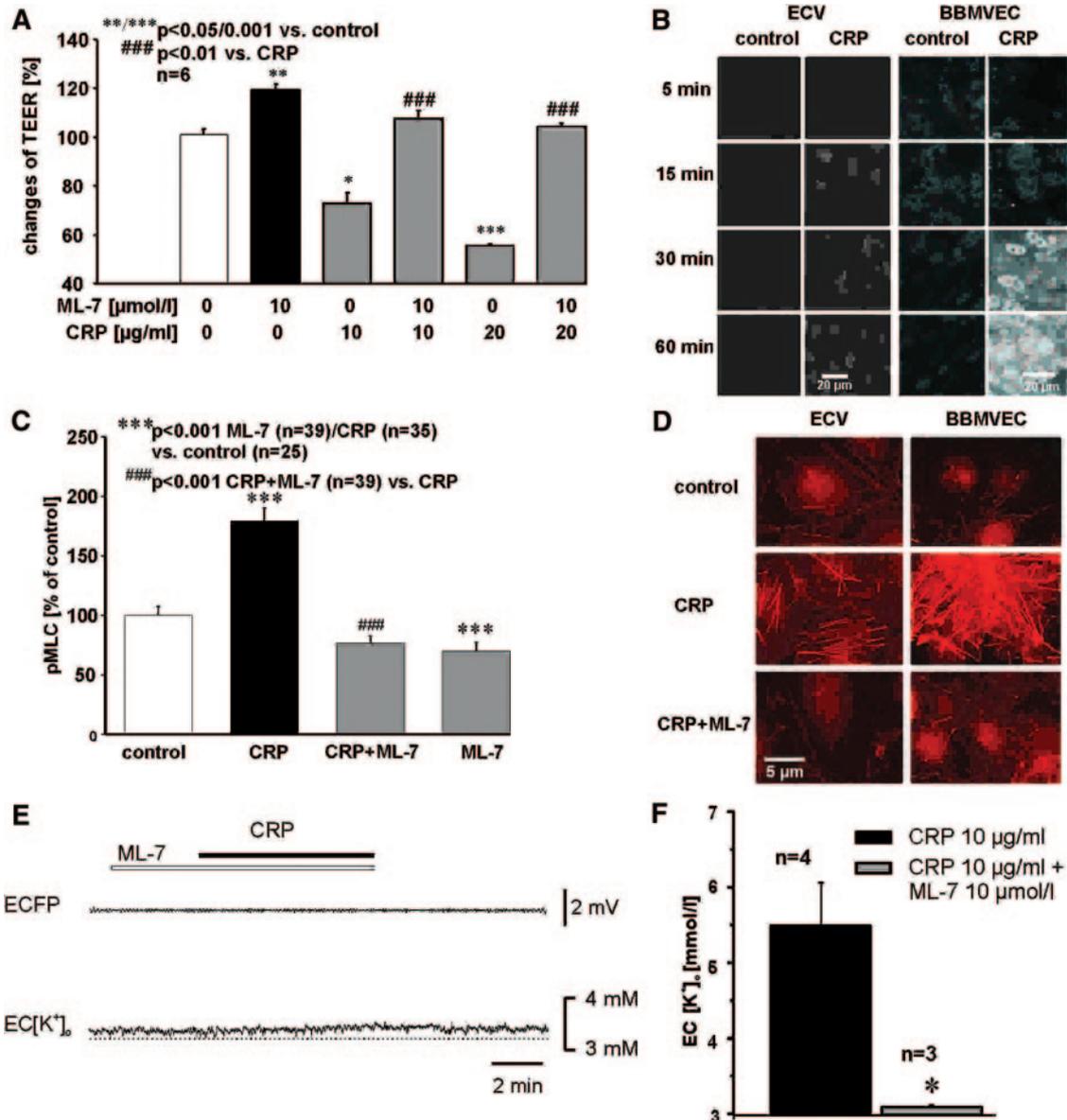
online at <http://stroke.ahajournals.org>). To further strengthen this finding, we performed immunohistochemical stainings of CD16 and CD32 to verify the expression of both receptors on protein level. As demonstrated in Supplemental Figure I, available online at <http://stroke.ahajournals.org>, both receptors are expressed on the cultured ECs.

### Contribution of Mitogen-Activated Protein Kinases to the Effects of C-Reactive Protein

As a next target, we examined the MAPK family. Inhibition of the p38-MAPK with SB203580 completely blocked the barrier disrupting effect of CRP (Supplemental Table I). In contrast, inhibition of JNK with SP600125 and ERK with PD98059 did not prevent the CRP-induced breakdown of the BBB (Supplemental Table I). CRP caused a time-dependent increase of p38-MAPK phosphorylation (Figure 3A) that was completely blocked by the p38-MAPK inhibitor SB203580 (Figure 3B). Identical results were obtained using quantitative Western blot analysis (Figure 3C).

### C-Reactive Protein-Induced Oxidative Stress Contributes to the Barrier-Disrupting Effect

Because ROS are involved in BBB disruption,<sup>14,21</sup> we also examined the role of oxidative stress in our model. Treatment of brain ECs or ECV304 with CRP resulted in a concentration- and time-dependent increase of ROS (Figure 4A–B). CRP-induced ROS formation was completely abolished in the presence of CD16- or CD32-neutralizing antibodies (data not shown). The NAD(P)H-oxidase is the major source of ROS in ECs and has recently been demonstrated to be highly expressed in cerebral blood vessels.<sup>22,23</sup> Application of the NAD(P)H-oxidase inhibitor apocynin resulted in a complete blockade of CRP-induced oxidative stress (Figure 4C) and reversed its barrier-disrupting effect (Supplemental Table I). Blocking the CRP-induced radical formation with apocynin also blocked the phosphorylation of MLC (Figure 4D) and the formation of actin stress fibers (Figure 4E). Furthermore, the barrier-disrupting effect of CRP was also completely abolished by apocynin (500  $\mu\text{mol/L}$ ) in the isolated guinea pig brain (Figure 4F). Under these conditions,  $[\text{K}^+]_o$  in the piriform cortex ( $\text{PC}[\text{K}^+]_o$ ) did not differ from the control potassium concentration (Figure 4G). To further strengthen this finding, we performed a 60-minute washout of the apocynin-treated brain. Thereafter, CRP perfusion resulted in BBB disruption as shown in a representative trace (Figure 4H). This barrier-disrupting effect of CRP in the piriform cortex was again blocked by the MLC-kinase inhibitor ML-7 (10  $\mu\text{mol/L}$ ; Figure 4G). Besides ROS, excessive NO release contributes to BBB disruption. Therefore, we analyzed whether CRP increases NO generation of cultured ECs. CRP treatment did not increase NO levels and blocking the NO-synthase had no effect on CRP-induced BBB disruption (data not shown). Because NO is constitutively produced by ECs, we further analyzed whether the CRP-induced increase of DCF fluorescence was caused by peroxynitrite ( $\text{ONOO}^-$ ) formation. Uric acid is a well-established  $\text{ONOO}^-$  scavenger.<sup>23–25</sup> However, uric acid (1  $\text{mmol/L}$ ) completely failed to block the CRP-dependent ROS production (Figure 4I).



**Figure 2.** Activation of the contractile machinery is involved in CRP-induced BBB disruption. The MLCK inhibitor ML-7 (10  $\mu\text{mol/L}$ ) was used in TEER (initial value:  $681.9 \pm 13.8 \Omega \times \text{cm}^2$ ) experiments to examine the contribution of MLC phosphorylation to the observed barrier-disrupting effect of CRP (10 and 20  $\mu\text{g/ml}$ ) in the coculture model of bovine brain endothelial cells and primary rat astrocytes (A). Phosphorylation of MLC (pMLC) was examined in bovine brain ECs and ECV304 using immunohistochemistry. CRP (20  $\mu\text{g/ml}$ ) induced a time-dependent increase of pMLC as demonstrated in representative immunostainings of ECV304 and BBMVEC (B). The maximum effect of CRP on pMLC in bovine brain endothelial cells after 60 minutes was completely abolished in the presence of ML-7 (10  $\mu\text{mol/L}$ ; shown as single-cell fluorescence intensity; C). Activation of the contractile machinery was monitored by the staining of cellular actin using Texas red labeled phalloidin. In ECV304 and BBMVEC, the CRP (20  $\mu\text{g/ml}$ )-dependent formation of cell-crossing actin stress fibers was blocked by ML-7 (10  $\mu\text{mol/L}$ ; D). The effect of intra-arterial perfusion of CRP (n=5) and coproduction of CRP+ML-7 (n=3) on the extracellular potassium concentration [ $\text{K}^+$ ]<sub>o</sub> in the isolated guinea pig brain preparation is shown as a representative trace (E). Results are expressed as mean values  $\pm$  SEM. Statistically significant effect of ML-7 on CRP-induced [ $\text{K}^+$ ]<sub>o</sub> increase was assessed by the U test and a value of  $*P < 0.05$  was considered significant ( $P = 0.02$ ; F).

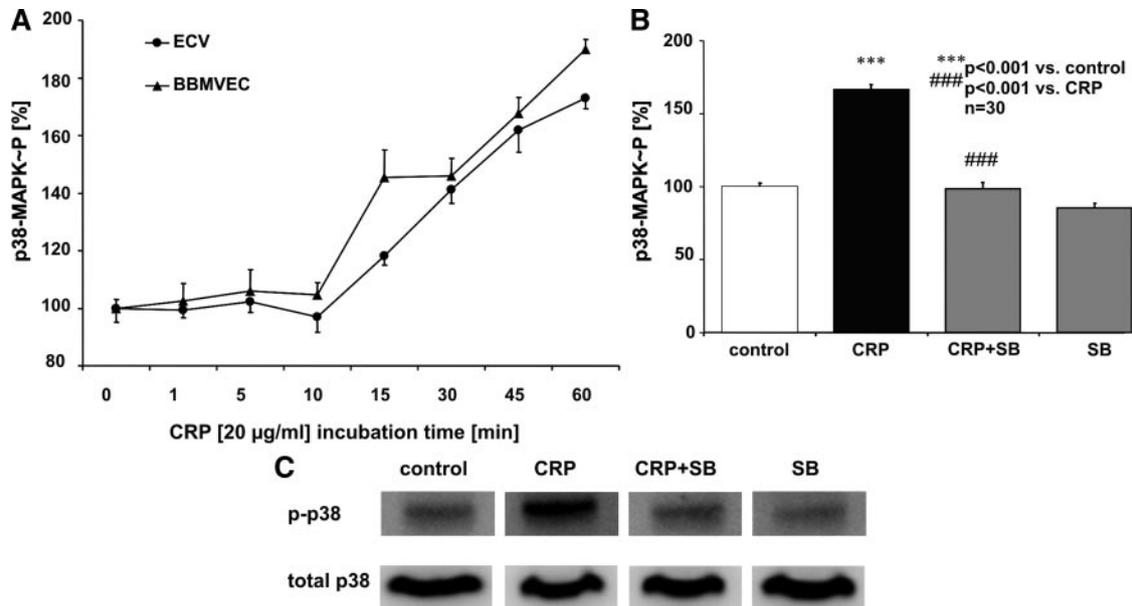
### Crosstalk of Reactive Oxygen Species and p38-Mitogen-Activated Protein Kinase

NAD(P)H-oxidase and p38-MAPK activation has been demonstrated to be closely related to each other and to interact.<sup>9,26</sup> To discriminate whether ROS activate p38-MAPK or vice versa, we examined the effect of SB203850 on ROS formation and of NAD(P)H-oxidase inhibition on p38-MAPK phosphorylation. Interestingly, inhibition of the NAD(P)H-oxidase had no effect on p38-MAPK phosphorylation (Figure 5A), whereas

SB203850 significantly reduced CRP-induced oxidative stress (Figure 5B) indicating that the NAD(P)H-oxidase is downstream of the p38-MAPK.

### C-Reactive Protein-Induced Tight Junction Rearrangement Depends on Reactive Oxygen Species and Myosin Light Chain Phosphorylation

Tight junctions (TJ) play a pivotal role for maintaining BBB integrity. For this reason, we were interested whether CRP



**Figure 3.** Signal transduction of CRP-induced barrier disruption involves p38-MAPK. Activation of the p38 MAPK was examined using phosphospecific immunostainings and qualitative Western blot analysis of the p38 MAPK. CRP (20 µg/mL) induced a time-dependent increase of p38 MAPK phosphorylation in ECV304 and BBMVEC (A) that was completely abolished in bovine brain ECs by the p38 MAPK inhibitor SB203580 (10 µmol/L; B). Representative Western blots of total and phosphorylated p38 MAPK in BBMVEC are shown (C).

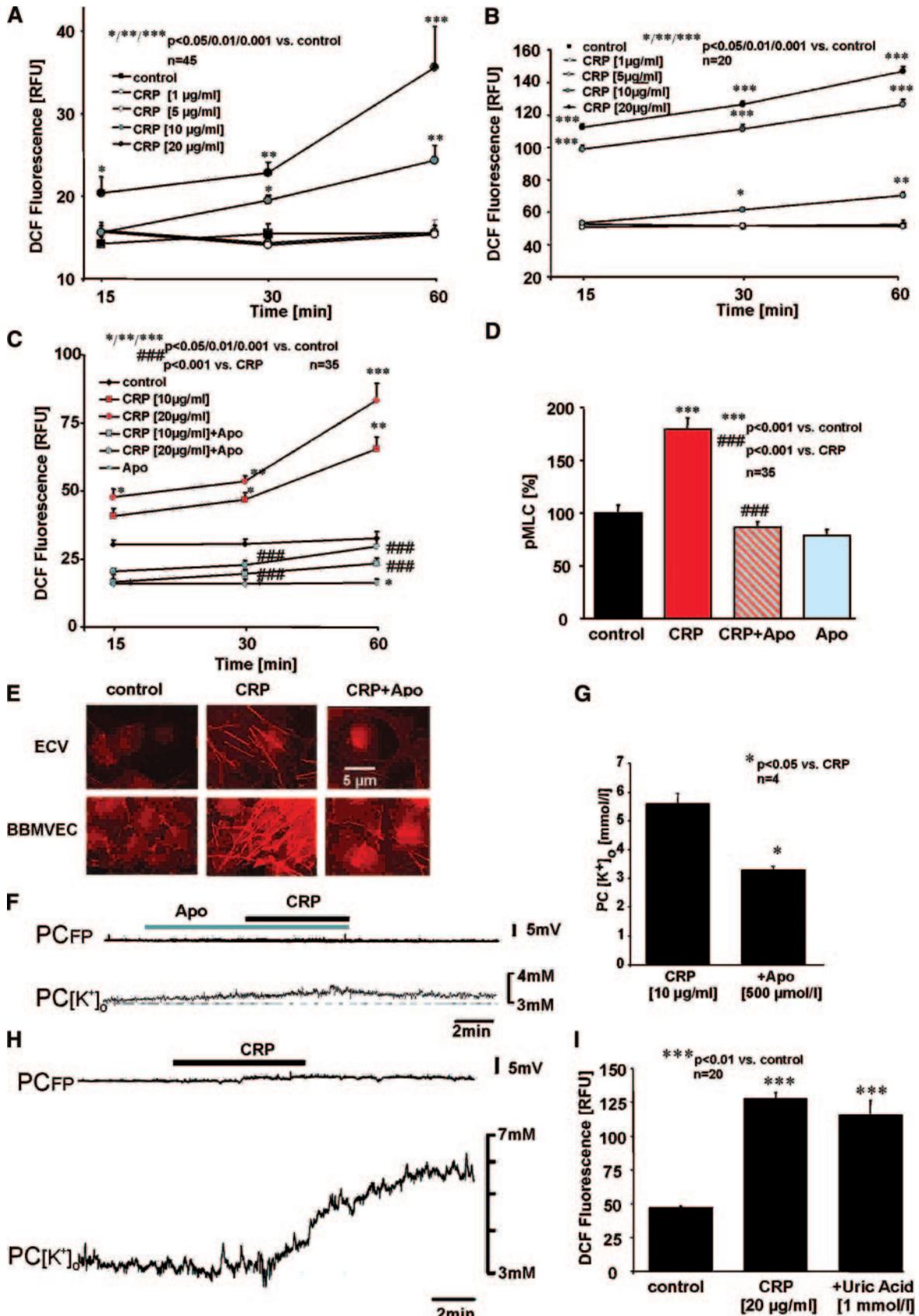
affects the TJ molecules occludin or zonula occludens protein 1 (ZO-1) in cultured ECs. ZO-1 is of special interest, because it is directly connected to the cellular contractile machinery through actin filaments. As demonstrated by representative immunostainings, CRP treatment resulted in disorganized ZO-1 staining at the cell borders that was reversed by the MLCK inhibitor ML-7 or the NAD(P)H-oxidase inhibitor apocynin in cultured bovine (Figure 6A) and human cells (Figure 6B). The CRP-induced rearrangement of occludin in cultured BBMVEC was also blocked by apocynin (Figure 6C).

### Discussion

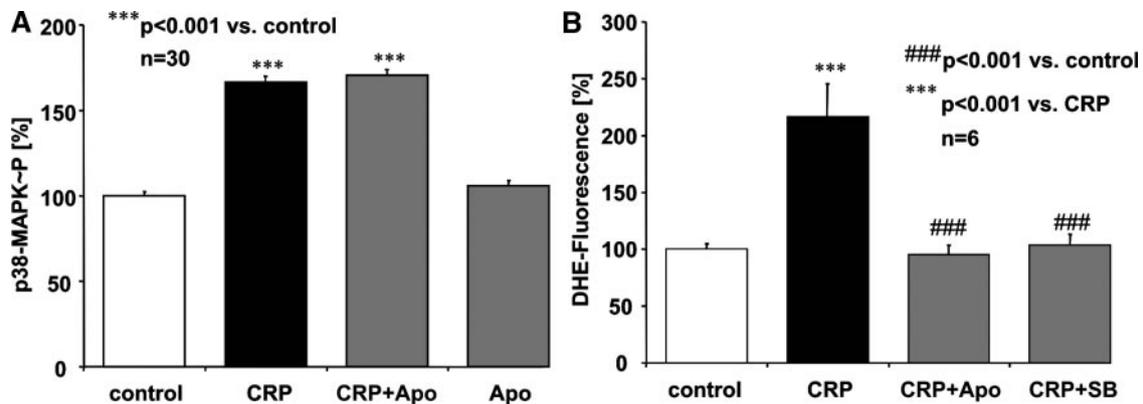
The aim of our present study was to examine whether CRP directly affects BBB integrity and to analyze the underlying mechanisms of CRP-induced barrier disruption. The major findings of our experiments are: (1) CRP exerts barrier-disrupting effects in the coculture model of the BBB and the more complex system of the arterial perfused guinea pig brain; (2) the barrier-disrupting effect of CRP involves the activation of the endothelial contractile machinery through MLC phosphorylation; and (3) p38-MAPK-induced ROS formation by the NAD(P)H-oxidase is a prerequisite for CRP-dependent MLC phosphorylation.

Elevated plasma levels of CRP have been demonstrated to be associated with a worse outcome of acute ischemic cardio- and cerebrovascular disease.<sup>4–6</sup> The clinical study of Di Napoli et al clearly demonstrates that patients with CRP plasma levels >15 µg/mL have a worse prognosis compared with those with lower CRP levels. Our study demonstrates for the first time a possible explanation for this clinical observation. The development of brain edema is the most common lethal complication after ischemic stroke.<sup>10</sup> Because our experimental data demonstrate a

barrier-disrupting effect of CRP near the clinically relevant level of 15 ng/mL, we hypothesize that direct barrier-disrupting effects of CRP are responsible for the worse prognosis of patients with elevated CRP plasma levels after stroke. The effect of CRP (10 to 20 µg/mL) on the BBB integrity was examined in a coculture model of the BBB-composed primary bovine brain ECs in coculture with primary rat astrocytes or in a barrier coculture model of human ECV304 and rat astrocytoma C6 cells. The coculture of bovine brain ECs with primary astrocytes is a well-accepted model for studying BBB integrity<sup>12,14</sup> but represents a rather artificial system. To reproduce our findings in a system closer to the in vivo situation, we further investigated the effect of CRP in the in vitro isolated guinea pig brain preparation, in which the complex interactions between the vascular and the neuronal compartments are preserved.<sup>17,27,28</sup> Using this model, functional alterations in BBB can be monitored by measuring changes in the extracellular potassium concentration ( $[K^+]_o$ ) in the brain parenchyma after arterial application of compounds acting on endothelial permeability.<sup>27</sup> Brain microvascular ECs are responsible for the transport of substances from the blood into the brain and are involved in the clearance of potassium ions from the brain. Brain potassium is transported to the blood by a specialized endothelial Na/K-ATPase.<sup>29,30</sup> The enhancement of intraparenchymal  $K^+$  determined by the arterial perfusion of the selective Na/K-ATPase blocker ouabain in the isolated guinea pig brain<sup>27</sup> confirms that alterations of  $K^+$  flux into and out of the brain can be used as a parameter for BBB impairment. In each system, the coculture model and the isolated guinea pig brain CRP (10 µg/mL) caused a permeabilization of the BBB that was antagonized by the MLCK inhibitor ML-7. This finding is in line with our



**figure 4.** CRP causes oxidative stress in endothelial cells through activation of the NAD(P)H-oxidase. ROS formation in ECV304 and BBMVEC was examined using the fluorescence dye DCF. Intracellular oxidative stress was augmented in a time- and concentration- (1 to 20 μg/mL) dependent manner as indicated by increasing relative DCF fluorescence values in ECV304 (A) and BBMVEC (B). In bovine



**Figure 5.** Crosstalk of p38-MAPK activation and NAD(P)H-oxidase-dependent radical formation induced by CRP. The crosstalk of NAD(P)H-oxidase and p38 MAPK was investigated in bovine brain ECs by examining the effect of apocynin (500  $\mu\text{mol/L}$ ) on CRP (20  $\mu\text{g/mL}$ )-induced p38-MAPK phosphorylation (A) or the effect of SB203580 (10  $\mu\text{mol/L}$ ) on CRP-dependent ROS formation (B). The superoxide-specific fluorescence dye DHE was used to detect CRP-induced radical generation and phosphospecific immunostainings of the p38-MAPK were performed to measure p38-MAPK activation.

previous study and the studies of Haorah and workers.<sup>11,21</sup> We were able to demonstrate that hypoxia-induced BBB disruption *in vitro* and *in vivo* involves the activation of the contractile machinery.<sup>14</sup> Haorah et al could show similar results by treating an *in vitro* cell culture model with alcohol.<sup>11,21</sup>

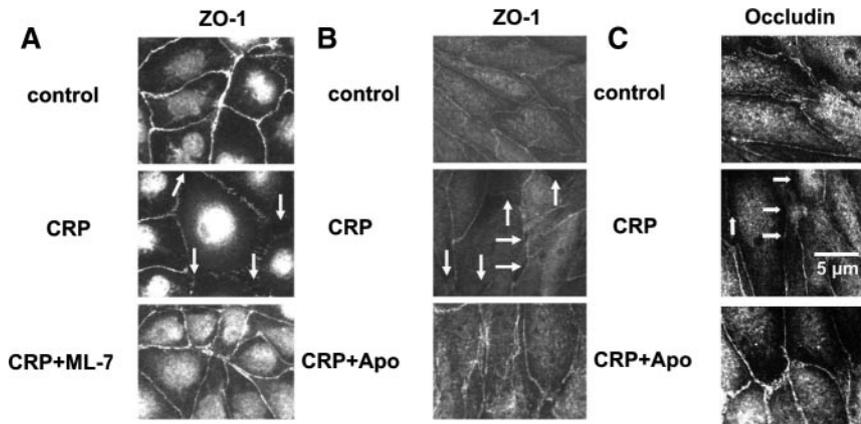
Next we were interested in exploring the signaling cascades underlying this process. First, we tried to identify a receptor for CRP that might be responsible for the effects on ECs. In endothelial cells, CRP induced activation of signaling pathways (eg, raised interleukin-8 production, increased expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) is commonly mediated by Fc $\gamma$  receptors.<sup>20</sup> Indeed, we were able to detect CD16 and CD32 expression by immunostainings, which is in line with other studies demonstrating CD16 expression at the human and rat BBB.<sup>31,32</sup> Pretreatment with neutralizing anti-CD16 or anti-CD32 antibodies significantly reduced the barrier-disrupting effect of CRP.

Because MAPK signaling has been demonstrated to be involved in BBB disruption, we analyzed whether inhibitors of different MAPK can antagonize the barrier-disrupting effect of CRP.<sup>33</sup> In contrast to previous observations demonstrating that blocking the p42/44 MAPK antagonizes hydrogen peroxide-induced BBB disruption,<sup>33</sup> we demonstrate that the p38-MAPK inhibitor SB203580 completely abolishes the effect of CRP. Inhibitors of the p42/44-MAPK and the JNK did not prevent the CRP-dependent loss of barrier integrity. This observations is well in line with studies from the cardiovascular circulation demonstrating a CRP-induced p38-MAPK-dependent increase of cytokines and cell adhesion molecules in human coronary artery endothelial cells<sup>34</sup> and a

p38-MAPK-dependent loss of serotonin-induced dilation of porcine coronary arterioles.<sup>26</sup>

We and others demonstrated a pivotal role of oxidative stress in BBB disruption.<sup>14,21,33,35</sup> Therefore, we were interested whether oxidative stress contributes to the CRP-induced effects. The barrier-disrupting effect of CRP in the coculture model as well as in the isolated guinea pig brain was completely abolished in the presence of the NAD(P)H-oxidase inhibitor apocynin. Furthermore, CRP caused a time- and concentration-dependent increase of ROS in human and bovine cells. In addition to the fluorescent indicator DCF, which detects a variety of ROS, we also used the more superoxide-specific dye DHE. Both indicators revealed a CRP-induced increase of fluorescence intensity that was blocked by DPI and apocynin, indicating that CRP-induced ROS are NAD(P)H-oxidase-derived superoxide. An interaction of p38-MAPK signaling and NAD(P)H-oxidase activity was observed in neutrophils and coronary arterioles.<sup>26,36</sup> Importantly, Quamirani et al demonstrated that CRP-induced activation of the NAD(P)H-oxidase is significantly reduced by the p38-MAPK inhibitor SB203580.<sup>26</sup> For this reason, we analyzed whether in our system the NAD(P)H-oxidase is a target of the p38-MAPK or vice versa. In good agreement with the Quamirani et al study, we observed that the NAD(P)H-oxidase is a downstream target of p38-MAPK signaling. As expected, blocking the NAD(P)H-oxidase prevented CRP-induced barrier disruption. Similar observations have been made for the BBB-disrupting effects of alcohol, glutamate, and hypoxia.<sup>14,21,33</sup> Recently, we were able to demonstrate that hypoxia-induced ROS formation by the NAD(P)H-oxidase is followed by an increase of MLC

**Figure 4. (continued)** brain, ECs blocking the NAD(P)H-oxidase with apocynin (500  $\mu\text{mol/L}$ ) completely blocked CRP (20  $\mu\text{g/mL}$ )-induced radical generation (C) and MLC phosphorylation (D). Furthermore, apocynin blocked stress fiber formation in ECV304 and BBMVEC (E). Effect of apocynin (500  $\mu\text{mol/L}$ ) in the isolated guinea pig model. Representative traces of the changes in extracellular potassium concentration  $[\text{K}^+]_o$  in the piriform cortex ( $\text{PC}[\text{K}^+]_o$ ) induced by systemic perfusion of apocynin+CRP (10  $\mu\text{g/mL}$ ) show no changes in  $[\text{K}^+]_o$  (F). In addition, ML-7 (10  $\mu\text{mol/L}$ ) suppressed the increase of  $[\text{K}^+]_o$  in the piriform cortex. After a washout period, arterial CRP (10  $\mu\text{g/mL}$ ) perfusion resulted in a significant increase of  $\text{PC}[\text{K}^+]_o$  (G), as also demonstrated by representative traces (H). The field responses recorded with the conventional extracellular barrel are also shown (upper traces). The dotted lines represent the baseline  $[\text{K}^+]_o$  during standard perfusion solution. Addition of the ONOO<sup>-</sup> scavenger uric acid (1 mmol/L) had no effect on the CRP (20  $\mu\text{g/mL}$ )-induced increase of oxidative stress (I).



**Figure 6.** CRP-induced rearrangement of ZO-1/occludin involves ROS and MLCK activation. The effect of CRP (20 μg/mL) on the distribution of the TJ molecules ZO-1/occludin was examined using ZO-1-specific immunostainings in cultured (A) BBBMVEC, (B) ECV-304 and occludin-specific immunostainings in cultured (C) BBBMVEC. TJ rearrangement was inhibited by apocynin (500 μmol/L) or ML-7 (10 μmol/L). The formation of paracellular gaps is marked by white arrows.

phosphorylation.<sup>14</sup> Our present data, indicating a similar mechanism for CRP, are in line with our previous observations as well as with the studies of Haorah et al demonstrating the same mechanism for the effects of alcohol at the BBB.<sup>11,21</sup> In addition to our previously published study, we were further able to demonstrate the formation of cell-crossing stress fibers—which is a further indicator of the activation of the cellular contractile machinery—and a disorganization of ZO-1 and occludin at the cell borders. In line with previously published data, the effect of CRP on TJ was prevented by the inhibition of ROS formation and MLC phosphorylation.<sup>21</sup>

A contribution of ROS in BBB disruption has been previously shown in various reports. Hydrogen peroxide has been demonstrated to redistribute ZO-1, occludin and to induce actin stress fiber formation in cultured porcine and bovine brain endothelial cells.<sup>33,37</sup> Recently, NAD(P)H-oxidase-derived superoxide has been shown to play an important role in postischemic BBB disruption in vitro and in vivo.<sup>38</sup> Exogenous peroxynitrite has been shown to mediate BBB permeabilization in an ECV304/C6 coculture model and in vivo.<sup>39,40</sup> Because NO release as well has been demonstrated to be involved in BBB disruption (for review, see Thiel and Audus<sup>41</sup>), we examined whether CRP affects endothelial NO synthesis and whether this has an effect on the CRP-induced loss of barrier function. In the present experiments, CRP neither affected intracellular NO formation nor did blockade of the NO synthase prevent CRP-induced BBB disruption. However, NO is constitutively produced by ECs, which makes the formation of BBB disrupting ONOO<sup>-</sup> in our BBB coculture models likely. Because the ROS indicator DCF also detects ONOO<sup>-</sup>, we decided to analyze whether this signal is due to CRP-induced superoxide generation together with constantly synthesized NO ONOO<sup>-</sup>. The ONOO<sup>-</sup> scavenger uric acid did not affect the CRP-induced DCF signal. Therefore, we conclude that NO and NO-related radicals such as peroxynitrite are not involved in the CRP-induced effects on cerebral ECs.

### Summary

Our results demonstrate a causal role of CRP in BBB disruption. The signaling cascade involves the surface Fcγ receptors, p38-MAPK-dependent ROS formation by

the NAD(P)H-oxidase, and finally the phosphorylation of MLC.

In detail, CRP binds to its endothelial surface receptors CD16/CD32 mediating p38-MAPK activation, which is responsible for NAD(P)H-dependent ROS generation. The resulting oxidative stress activates the contractile machinery involving the MLCK. The EC contraction goes along with a disruption of ZO-1 TJ molecules, finally resulting in a loss of barrier function.

Importantly, the barrier-disrupting blood levels of CRP were identified as negative prognostic elements in patients with fatal stroke.<sup>5</sup> Although p38-MAPK, NAD(P)H-oxidase, and activation of the endothelial contractile machinery are independently known to be involved in stroke-associated brain edema formation,<sup>14,42,43</sup> there was a missing link between these individual factors and CRP. The experimental data of the isolated brain experiments also suggest that CRP may promote Na/K pump dysfunction that could contribute to generation of brain edema.<sup>44</sup> Our study highlights the causal role of CRP to brain edema formation and presents a signaling pathway that offers novel strategies for therapeutic intervention. A still open question results from the fact that the CRP concentrations examined in the present study are a feature of many other pathological conditions, which are not associated with brain edema formation. We hypothesize that the signaling pathway demonstrated in the present study is always induced by elevated CRP serum levels but causes clinically relevant brain edema only if the BBB integrity is impaired by additional pathophysiological factors (eg, ischemia in stroke). Further clinical work has to be done to confirm our findings in patients and to identify treatment strategies to prevent CRP-induced BBB disruption in stroke.

### Sources of Funding

This study was supported by DFG grant SFB 553/C12 to V.L. and H.J.L., a MAIFOR grant of the Medical Faculty of the University of Mainz to C.R.W.K., and in part by the Bundesministerium für Bildung und Forschung KNDD Grant 01GI0719 to C.U.P. and C.R.W.K., L.L. and M.d.C. were supported by the Italian Health Ministry and by Fondazione Cariplo grant 2005.1208/10.4963.

### Disclosures

H.J.L. and M.d.C. receive funding by the EC (LSH-CT-2006-037315, EPICURE).

## References

- Bova IY, Bornstein NM, Korczyn AD. Acute infection as a risk factor for ischemic stroke. *Stroke*. 1996;27:2204–2206.
- Danton GH, Dietrich WD. Inflammatory mechanisms after ischemia and stroke. *J Neuropathol Exp Neurol*. 2003;62:127–136.
- Fassbender K, Rossol S, Kammer T, Daffertshofer M, Wirth S, Dollman M, Hennerici M. Proinflammatory cytokines in serum of patients with acute cerebral ischemia: kinetics of secretion and relation to the extent of brain damage and outcome of disease. *J Neurol Sci*. 1994;122:135–139.
- Brown DA, Breit SN, Buring J, Fairlie WD, Bauskin AR, Liu T, Ridker PM. Concentration in plasma of macrophage inhibitory cytokine-1 and risk of cardiovascular events in women: a nested case-control study. *Lancet*. 2002;359:2159–2163.
- Di Napoli M, Papa F, Bocola V. Prognostic influence of increased C-reactive protein and fibrinogen levels in ischemic stroke. *Stroke*. 2001;32:133–138.
- Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med*. 2002;347:1557–1565.
- Pepys MB, Hirschfield GM, Tennent GA, Gallimore JR, Kahan MC, Bellotti V, Hawkins PN, Myers RM, Smith MD, Polara A, Cobb AJ, Ley SV, Aquilina JA, Robinson CV, Sharif I, Gray GA, Sabin CA, Jenvey MC, Kolstoe SE, Thompson D, Wood SP. Targeting C-reactive protein for the treatment of cardiovascular disease. *Nature*. 2006;440:1217–1221.
- Zhang J, Rui YC, Yang PY, Lu L, Li TJ. C-reactive protein induced expression of adhesion molecules in cultured cerebral microvascular endothelial cells. *Life Sci*. 2006;78:2983–2988.
- Abbott NJ, Rönnbäck L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci*. 2006;7:41–53.
- Rosenberg GA. Ischemic brain edema. *Prog Cardiovasc Dis*. 1999;42:209–216.
- Haorah J, Heilman D, Knipe B, Chrastil J, Leibhart J, Ghorpade A, Miller DW, Persidsky Y. Ethanol-induced activation of myosin light chain kinase leads to dysfunction of tight junctions and blood-brain barrier compromise. *Alcohol Clin Exp Res*. 2005;29:999–1009.
- Kuhlmann CR, Lessmann V, Luhmann HJ. Fluvastatin stabilizes the blood-brain barrier in vitro by nitric oxide-dependent dephosphorylation of myosin light chains. *Neuropharmacology*. 2006;51:907–913.
- Lessmann V, Heumann R. Modulation of unitary glutamatergic synapses by neurotrophin-4/5 or brain-derived neurotrophic factor in hippocampal microcultures: presynaptic enhancement depends on pre-established paired-pulse facilitation. *Neuroscience*. 1998;86:399–413.
- Kuhlmann CR, Tamaki R, Gamberding M, Lessmann V, Behl C, Kempfski OS, Luhmann HJ. Inhibition of the myosin light chain kinase prevents hypoxia-induced blood-brain barrier disruption. *J Neurochem*. 2007;102:501–507.
- Erdogan A, Most AK, Wienecke B, Fehsecke A, Leckband C, Voss R, Grebe MT, Tillmanns H, Schaefer CA, Kuhlmann CR. Apigenin-induced nitric oxide production involves calcium-activated potassium channels and is responsible for antiangiogenic effects. *J Thromb Haemost*. 2007;5:1774–1781.
- de Curtis M, Pare D, Llinas RR. The electrophysiology of the olfactory-hippocampal circuit in the isolated and perfused adult mammalian brain in vitro. *Hippocampus*. 1991;1:341–354.
- de Curtis M, Biella G, Buccellati C, Folco G. Simultaneous investigation of the neuronal and vascular compartments in the guinea pig brain isolated in vitro. *Brain Res Brain Res Protoc*. 1998;3:221–228.
- Muhlethaler M, de Curtis M, Walton K, Llinas R. The isolated and perfused brain of the guinea-pig in vitro. *Eur J Neurosci*. 1993;5:915–926.
- Yuan Y, Huang Q, Wu HM. Myosin light chain phosphorylation: modulation of basal and agonist-stimulated venular permeability. *Am J Physiol*. 1997;272:H1437–H1443.
- Devaraj S, Du Clos TW, Jialal I. Binding and internalization of C-reactive protein by Fcγ receptors on human aortic endothelial cells mediates biological effects. *Arterioscler Thromb Vasc Biol*. 2005;25:1359–1363.
- Haorah J, Knipe B, Leibhart J, Ghorpade A, Persidsky Y. Alcohol-induced oxidative stress in brain endothelial cells causes blood-brain barrier dysfunction. *J Leukoc Biol*. 2005;78:1223–1232.
- Lander HM. An essential role for free radicals and derived species in signal transduction. *FASEB J*. 1997;11:118–124.
- Miller AA, Drummond GR, Schmidt HH, Sobey CG. NADPH oxidase activity and function are profoundly greater in cerebral versus systemic arteries. *Circ Res*. 2005;97:1055–1062.
- Doughan AK, Harrison DG, Dikalov SI. Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. *Circ Res*. 2008;102:488–496.
- Venugopal SK, Devaraj S, Jialal I. C-reactive protein decreases prostacyclin release from human aortic endothelial cells. *Circulation*. 2003;108:1676–1678.
- Dang PM, Stensballe A, Boussetta T, Raad H, Dewas C, Krovciarski Y, Hayem G, Jensen ON, Gougerot-Pocidal MA, El-Benna J. A specific p47phox-serine phosphorylated by convergent MAPKs mediates neutrophil NADPH oxidase priming at inflammatory sites. *J Clin Invest*. 2006;116:2033–2043.
- Qamirani E, Ren Y, Kuo L, Hein TW. C-reactive protein inhibits endothelium-dependent NO-mediated dilation in coronary arterioles by activating p38 kinase and NAD(P)H oxidase. *Arterioscler Thromb Vasc Biol*. 2005;25:995–1001.
- Librizzi L, Janigro D, De Biasi S, de Curtis M. Blood-brain barrier preservation in the in vitro isolated guinea pig brain preparation. *J Neurosci Res*. 2001;66:289–297.
- Mazzetti S, Librizzi L, Frigerio S, de Curtis M, Vitellaro-Zuccarello L. Molecular anatomy of the cerebral microvessels in the isolated guinea-pig brain. *Brain Res*. 2004;999:81–90.
- Eisenberg HM, Suddith RL. Cerebral vessels have the capacity to transport sodium and potassium. *Science*. 1979;206:1083–1085.
- Stummer W, Betz AL, Keep RF. Mechanisms of brain ion homeostasis during acute and chronic variations of plasma potassium. *J Cereb Blood Flow Metab*. 1995;15:336–344.
- Ulvestad E, Williams K, Vedeler C, Antel J, Nyland H, Mørk S, Matre R. Reactive microglia in multiple sclerosis lesions have an increased expression of receptors for the Fc part of IgG. *J Neurol Sci*. 1994;121:125–131.
- Schlachetzki F, Zhu C, Partridge WM. Expression of the neonatal Fc receptor (FcRn) at the blood-brain barrier. *J Neurochem*. 2002;81:203–206.
- Fischer S, Wiesnet M, Renz D, Schaper W. H2O2 induces paracellular permeability of porcine brain-derived microvascular endothelial cells by activation of the p44/42 MAP kinase pathway. *Eur J Cell Biol*. 2005;84:687–697.
- Khreis T, Jozsef L, Potempa LA, Filep JG. Conformational rearrangement in C-reactive protein is required for proinflammatory actions on human endothelial cells. *Circulation*. 2004;109:2016–2022.
- Sharp CD, Hines I, Houghton J, Warren A, Jackson TH, Jawahar A, Nanda A, Elrod JW, Long A, Chi A, Minagar A, Alexander JS. Glutamate causes a loss in human cerebral endothelial barrier integrity through activation of NMDA receptor. *Am J Physiol Heart Circ Physiol*. 2003;285:H2592–H2598.
- Lee HS, Namkoong K, Kim DH, Kim KJ, Cheong YH, Kim SS, Lee WB, Kim KY. Hydrogen peroxide-induced alterations of tight junction proteins in bovine brain microvascular endothelial cells. *Microvasc Res*. 2004;68:231–238.
- Kahles T, Luedike P, Endres M, Galla HJ, Steinmetz H, Busse R, Neumann-Haefelin T, Brandes RP. NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke. *Stroke*. 2007;38:3000–3006.
- Jiang J, Wang W, Sun YJ, Hu M, Li F, Zhu DY. Neuroprotective effect of curcumin on focal cerebral ischemic rats by preventing blood-brain barrier damage. *Eur J Pharmacol*. 2007;561:54–62.
- Tan KH, Harrington S, Purcell WM, Hurst RD. Peroxynitrite mediates nitric oxide-induced blood-brain barrier damage. *Neurochem Res*. 2004;29:579–587.
- Thiel VE, Audus KL. Nitric oxide and blood-brain barrier integrity. *Antioxid Redox Signal*. 2001;3:273–278.
- Kim-Mitsuyama S, Yamamoto E, Tanaka T, Zhan Y, Izumi Y, Izumiya Y, Ioroi T, Wanibuchi H, Iwao H. Critical role of angiotensin II in excess salt-induced brain oxidative stress of stroke-prone spontaneously hypertensive rats. *Stroke*. 2005;36:1083–1088.
- Lennmyr F, Ericsson A, Gerwins P, Ahlstrom H, Terent A. Increased brain injury and vascular leakage after pretreatment with p38-inhibitor SB203580 in transient ischemia. *Acta Neurol Scand*. 2003;108:339–345.
- Stanimirovic DB, Wong J, Ball R, Darkin JP. Free radical-induced endothelial membrane dysfunction at the site of blood-brain barrier: relationship between lipid peroxidation, Na,K-ATPase activity, and 51Cr release. *Neurochem Res*. 1995;20:1417–1427.