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

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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 μg/mL) causes a disruption of the blood–brain barrier in both approaches. The results of our study further demonstrate CRP-induced activation of surface Fcγ 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.1,2 After arterial occlusion, ischemic brain injury in patients is accompanied by acute local inflammation and a dramatic plasma level rise of inflammatory cytokines.3 The elevated plasma level of the acute phase reactant C-reactive protein (CRP) is an outcome-predicting factor after stroke or myocardial infarction.4–6 A recent study demonstrated that antagonizing CRP improves the outcome after experimental myocardial infarction.7 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).8 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.9 Disruption of the BBB results in the formation of a vasogenic edema, which is the most common lethal complication of ischemic stroke.10 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.11,12 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.12 Primary postnatal day 0 to 2 neocortical rat astrocytes were isolated and cultured as described in more detail previously13 (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.12,14 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
Fluorescence Imaging of Intracellular Nitric Oxide and Reactive Oxygen Species

The ECs were pretreated for 60 minutes with CRP (1 to 20 μg/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. 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. 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.

Western Blot

Activation of the p38-MAPK was analyzed by detecting phosphorylated and total p38 in BBMVEC using Western blot analysis as described previously (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 (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±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 μg/mL, which has been demonstrated to correlate with worse poststroke prognosis in clinical studies.5 To exclude unspecified 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 μg/mL) treatment significantly increased fluorescein permeability (Pe in cm/min×10−6): control 1.17±0.03; CRP 2.93±0.13 (n=3, P<0.05). We also examined the effect of CRP (10 μg/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 CRP (10 μg/mL) for 8 minutes induced a large and fast [K+]o increase, whereas vehicle perfusion had no effect (Figure 1D). To exclude unspecified 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,14 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,19 the cocultures or the phosphorylation state of MLC is regulated by the MLC-kinase inhibitor ML-7 (10 μmol/L) 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).

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. Fcγ receptors are known receptors of CRP on ECs.20 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 immunohistochimical 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,14,21 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.22,23 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 μmol/L) in the isolated guinea pig brain (Figure 4F). Under these conditions, [K+]o in the piriform cortex (PC)[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 MLCK-inhibitor ML-7 (10 μmol/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 (ONOO−) formation. Uric acid is a well-established ONOO− scavenger.24,25 However, uric acid (1 mmol/L) completely failed to block the CRP-dependent ROS production (Figure 4I).
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.9,26 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...
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.4–6 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.10 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 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.17,27,28 Using this model, functional alterations in BBB can be monitored by measuring changes in the extracellular potassium concentration ([K⁺]₀) in the brain parenchyma after arterial application of compounds acting on endothelial permeability.27 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.29,30 The enhancement of intraparenchymal K⁺ determined by the arterial perfusion of the selective Na/K-ATPase blocker ouabain in the isolated guinea pig brain27 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 \( \mu \)g/mL) dependent manner as indicated by increasing relative DCF fluorescence values in ECV304 (A) and BBMVEC (B). In bovine
previous study and the studies of Haorah and workers.\textsuperscript{11,21} We were able to demonstrate that hypoxia-induced BBB disruption in vitro and in vivo involves the activation of the contractile machinery.\textsuperscript{14} Haorah et al could show similar results by treating an in vitro cell culture model with alcohol.\textsuperscript{11,21}

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 receptors.\textsuperscript{20} 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.\textsuperscript{31,32} 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.\textsuperscript{33} In contrast to previous observations demonstrating that blocking the p42/44 MAPK antagonizes hydrogen peroxide-induced BBB disruption,\textsuperscript{33} 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 observation is well in line with studies from the cardiovascular circulation demonstrating a CRP-dependent increase of cytokines and cell adhesion molecules in human coronary artery endothelial cells.\textsuperscript{34} and a p38-MAPK-dependent loss of serotonin-induced dilation of porcine coronary arterioles.\textsuperscript{26}

We and others demonstrated a pivotal role of oxidative stress in BBB disruption.\textsuperscript{14,21,33,35} Therefore, we were interested whether oxidative stress contributes to the CRP-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 neutrophiles and coronary arterioles.\textsuperscript{26,36} Importantly, Quamirani et al demonstrated that CRP-induced activation of the NAD(P)H-oxidase is significantly reduced by the p38-MAPK inhibitor SB203580.\textsuperscript{26} 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.\textsuperscript{14,21,33} Recently, we were able to demonstrate that hypoxia-induced ROS formation by the NAD(P)H-oxidase is followed by an increase of MLC.
phosphorylation.\textsuperscript{14} 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.\textsuperscript{11,21} 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.\textsuperscript{21}

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.\textsuperscript{33,37} Recently, NAD(P)H-oxidase-derived superoxide has been shown to play an important role in postischemic BBB disruption in vitro and in vivo.\textsuperscript{39} Exogenous peroxynitrite has been shown to mediate BBB permeabilization in an ECV304/C6 coculture model and in vivo.\textsuperscript{39,40} Because NO release as well has been demonstrated to be involved in BBB disruption (for review, see Thiel and Audus\textsuperscript{41}), 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\textsuperscript{-} in our BBB coculture models likely. Because the ROS indicator DCF also detects ONOO\textsuperscript{-}, we decided to analyze whether this signal is due to CRP-induced superoxide generation together with constantly synthesized NO ONOO\textsuperscript{-}. The ONOO\textsuperscript{-} 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.\textsuperscript{5} 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,\textsuperscript{14,42,43} 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.\textsuperscript{44} 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 (e.g., 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.

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**Disclosures**

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