C1-Inhibitor Protects From Brain Ischemia-Reperfusion Injury by Combined Antiinflammatory and Antithrombotic Mechanisms

Nadine Heydenreich; Marc W. Nolte, PhD; Eva Göb, PhD; Friederike Langhauser, PhD; Marion Hofmeister; Peter Kraft, MD; Christiane Albert-Weissenberger, PhD; Marc Brede, MD; Csanad Varallyay, MD; Kerstin Göbel, MD; Sven G. Meuth, MD, PhD; Bernhard Niewendt, PhD; Gerhard Dickneite, PhD; Guido Stoll, MD; Christoph Kleinschnitz, MD

Background and Purpose—Inflammation and thrombosis are pathophysiological hallmarks of ischemic stroke still unamenable to therapeutic interventions. The contact-kinin system represents an interface between inflammatory and thrombotic circuits and is involved in stroke development. C1-inhibitor counteracts activation of the contact-kinin system at multiple levels. We investigated the therapeutic potential of C1-inhibitor in models of ischemic stroke.

Methods—Male and female C57Bl/6 mice and rats of different ages were subjected to middle cerebral artery occlusion and treated with C1-inhibitor after 1 hour or 6 hours. Infarct volumes and functional outcomes were assessed between day 1 and day 7, and findings were validated by magnetic resonance imaging. Blood–brain barrier damage, thrombus formation, and the local inflammatory response were determined poststroke.

Results—Treatment with 15.0 U C1-inhibitor, but not 7.5 U, 1 hour after stroke reduced infarct volumes by ≈60% and improved clinical scores in mice of either sex on day 1. This protective effect was preserved at later stages of infarction as well as in elderly mice and in another species, ie, rats. Delayed C1-inhibitor treatment still improved clinical outcome. Blood–brain barrier damage, edema formation, and inflammation were significantly lower compared with controls. Moreover, C1-inhibitor showed strong antithrombotic effects.

Conclusions—C1-inhibitor is a multifaceted antiinflammatory and antithrombotic compound that protects from ischemic neurodegeneration in clinically meaningful settings. (Stroke. 2012;43:00-00.)

Key Words: blood–brain barrier • C1-inhibitor • inflammation • kallikrein-kinin system • middle cerebral artery occlusion • thrombosis

The pathology of brain ischemia-reperfusion injury is complex and involves a myriad of distinct molecular and cellular pathways. Among these inflammation is one of the most relevant processes.1,2 Activation of the cerebral endothelium early after the ischemic event triggers upregulation of cellular adhesion molecules and successive trafficking of inflammatory cells (neutrophils, macrophages, T cells) from the circulation into the brain parenchyma. Those cells recruited from the periphery in concert with locally activated cell populations (endothelial cells, microglia, astrocytes) produce an array of highly active mediators such as cytokines and chemokines that permeate the inflammatory circuits, thereby causing direct or indirect tissue damage. Another characteristic of persisting ischemia is structural disintegration of the blood–brain barrier, which in consequence leads to the formation of brain edema.3,4 Excessive edema can harm otherwise healthy brain regions by mechanical compression and is a frequent cause of worsening of neurological symptoms in stroke patients. Until now, convincing pharmacological strategies to combat inflammation or edema formation in acute ischemic stroke are lacking.5

Current pathophysiological concepts also emphasize the importance of progressive thrombus formation in the cerebral microvasculature for secondary infarct growth.6 We could
show recently that blocking of either platelet adhesion receptors or coagulation factors reliably protects from ischemic neurodegeneration.7–9 Most interestingly, there is increasing evidence of a tightly regulated interplay between thrombotic and inflammatory mechanisms during the course of an ischemic insult, and this thromboinflammation might be accessible to specific therapeutic interventions.10

The contact-kinin system constitutes a framework of serially connected serine proteases, namely coagulation factor XII (FXII), kininogen, and plasma kallikrein, and takes a central position in the pathophysiology of acute ischemic stroke.11 This system fosters vascular permeability and stroke-related inflammation by the formation of short-lived kinins while at the same time is linked to thrombus formation via the FXII-driven intrinsic coagulation cascade.12,13 Therefore, the contact-kinin system and its different molecular constituents represent a promising multifunctional target for potential stroke therapies.

C1-inhibitor is a 478 amino acid glycoprotein belonging to the superfamily of serine protease inhibitors called serpins.14 Its designation originates from the initial description as the only known physiological inhibitor of the classical complement pathway in blood and tissue. However, C1-inhibitor is also a major regulator of the contact-kinin system by blocking of activated FXII (FXIIa) and plasma kallikrein.14 Moreover, C1-inhibitor is known to directly interfere with the attraction of circulating leukocytes during inflammatory reactions and application of C1-inhibitor has been proven to be beneficial in ischemia-reperfusion injury in a variety of organs.15,16

We show that plasma-derived C1-inhibitor protects from reversible brain ischemia in mice and rats in several clinically relevant scenarios by a combined antiinflammatory and antithrombotic mode of action.

Materials and Methods
A detailed description of the surgical procedures, the stroke study population, and the methods is provided in the Online Data Supplement (available online at http://stroke.ahajournals.org).

Ischemia Model
Three hundred sixty-nine C57Bl/6 mice (349 males, 20 females) and 33 male CD rats were included in the study, which was approved by institutional panels on animal care and governmental authorities (Regierung von Unterfranken, Würzburg, Germany; Regierungspräsidium Giessen, Germany). Focal cerebral ischemia was induced for 60 minutes (mice) or 90 minutes (rats) by transient middle cerebral artery occlusion (tMCAO) using the intraluminal filament technique.17,18 Animals were anesthetized with 2.5% isoflurane (Abbott) in a 70% N2O/30% O2 mixture. For permanent middle cerebral artery occlusion (pMCAO), the filament was left in situ. Mice were controlled for several physiological parameters that can critically affect stroke outcome (cerebral blood flow, blood pressure, heart rate, arterial blood gases; Supplemental Figures I, II, and Supplemental Table I, available online at http://stroke.ahajournals.org). We calculated edema-corrected brain volumes from computed tomography images and blood-sensitive T2-weighted gradient echo constructed in a 1.5-Tesla unit (Vision, Siemens) using T2-weighted imaging sequences and blood-sensitive T2-weighted gradient echo constructed interference in steady-state sequences as previously described.7,8

Magnetic Resonance Imaging
Serial stroke assessment by magnetic resonance imaging was performed in mice 24 hours and again 7 days after tMCAO on a 1.5-Tesla unit (Vision, Siemens) using T2-weighted imaging sequences and blood-sensitive T2-weighted gradient echo constructed interference in steady-state sequences as previously described.7,8

Real-Time Polymerase Chain Reaction Studies
We determined relative gene expression levels of endothelin-1, interleukin-1β, and tumor necrosis factor-α in the ischemic cortices and basal ganglia by real-time polymerase chain reaction (StepOnePlus Real-Time PCR System; Applied Biosystems) as described.12

Determination of Blood–Brain Barrier Leakage and Brain Edema
Blood–brain barrier leakage after tMCAO was quantified using the vascular tracer Evan’s Blue (Sigma Aldrich) as described.12 The free water content of the brains (edema) was calculated from the brain wet/ dry weights.12
Immunohistology
Invading immune cells were detected by a rat antimouse Ly-6B.2 antibody (neutrophilic granulocytes; MCA771GA, 1:1000; AbD Serotec) and rat antimouse CD11b antibody (microglia/macrophages; MCA711, 1:100; AbD Serotec). For immunofluorescence staining against occludin, a rabbit antimouse occludin antibody (ab 31721, 1:100; Abcam) was applied. DNA stainings were performed using a fluorescent Hoechst dye (Hoechst 33342, 0.4 mg/mL; Sigma-Aldrich). Human C1-inhibitor was detected by a polyclonal sheep antibody against human C1-inhibitor (1:30 000; Abcam). For calculation of the thrombosis index, the whole brain was sliced 24 hours after tMCAO. Hematoxylin and eosin staining was performed according to standard procedures. The number of occluded blood vessels within the ischemic basal ganglia was counted in every tenth slice for control mice and mice treated with 7.5 U C1-inhibitor or 15.0 U C1-inhibitor, respectively, under 40-fold magnification (Axioptot2 microscope; Zeiss). Negative controls for all histological experiments included omission of primary or secondary antibody and produced no signals (not shown).

Western Blot
Immunoreactivity for fibrinogen (anti-Fibrinogen pAb 1:500; Acris Antibodies)24 and occludin (antioccludin pAB 1:1000; Abcam) in the ischemic cortices and basal ganglia was detected and quantified by Western blot.

Statistics
All results were expressed as mean±standard error of the mean except for ordinal functional outcome scales that were depicted as scatter plots including median with the 25% percentile and the 75% percentile given in brackets in the text. Numbers of animals (n=10) necessary to detect a standardized effect size on infarct volumes ≥0.2 (untreated mice vs mice treated with 15.0 U C1-inhibitor) were determined via a priori sample size calculation with the following assumptions: α=0.05, β=0.2, mean, and 20% standard deviation of the mean (GraphPad Stat Mate 2.0; GraphPad Software). For statistical analysis, the GraphPad Prism 5.0 software package was used. Data were tested for Gaussian distribution with the D’Agostino and Pearson omnibus normality test and then analyzed by 1-way analysis of variance or in case of measuring the effects of 2 factors simultaneously 2-way analysis of variance with post hoc Bonferroni adjustment for probability values. Nonparametric functional outcome scores were compared by Kruskal-Wallis test with post hoc Dunn multiple comparison test. For comparison of survival curves, the log rank test was used. Rat data were compared by unpaired 2-tailed Student t test (stroke size, brain edema) or nonparametric Mann-Whitney test (functional scores). P<0.05 was considered statistically significant.

Results
C1-Inhibitor Protects From Ischemic Brain Damage in Clinically Relevant Scenarios
To investigate the efficacy of plasma-derived C1-inhibitor in acute ischemic stroke, we chose a model of focal cerebral ischemia in which mice are subjected to tMCAO. This model induces a rapid and strong inflammatory response and massive edema formation within the brain and in addition depends on progressive microvascular thrombosis.25 First, 6-week-old male C57Bl/6 mice were subjected to tMCAO and treated with 7.5 U or 15.0 U C1-inhibitor 1 hour after stroke (Figure 1A). Infarct volumes on day 1 after tMCAO as assessed by staining of brain sections with 2,3,5-triphenyltetrazolium chloride were significantly smaller, by >60%, in male mice treated with 15.0 U C1-inhibitor than in vehicletreated controls (mean 110.5±8.0 mm3 [control] vs 41.9±8.4 mm3 [15.0 U], respectively; P<0.0001; Figure 1A). The smaller infarct volume was functionally relevant. Compared with control mice, mice receiving 15.0 U C1-inhibitor had significantly better overall neurological function 24 hours after tMCAO (Bederson score: median 3.0 [3.0–4.25] for control vs 1.0 [1.0–3.0] for 15.0 U, respectively; P<0.001) as well as improved motor function and coordination (grip test score: median 3.0 [3.0–4.0] for control vs 4.5 [4.0–5.0] for 15.0 U, respectively; P<0.001; Figure 1B). The 7.5-U C1-inhibitor failed to significantly lower infarct volumes (mean 110.5±8.0 mm3 [control] vs 81.3±10.4 mm3 [7.5 U], respectively; P>0.05) or improve functional outcomes (P>0.05; Figure 1A, B), indicating a dose-dependent effect of C1-inhibitor in brain ischemia.

Ischemic stroke usually is a disease of the elderly and, consequently, it is recommended to verify any stroke-protective effects observed in young laboratory animals in an older cohort, also.26,27 Adult middle-aged mice (age 6 months) also had development of significantly smaller brain infarctions (mean 95.9±4.9 mm3 [control] vs 44.5±5.9 mm3 [15.0 U], respectively; P<0.05) and less neurological deficits (Bederson score median: 3.5 [3.0–4.0] for control vs 3.0 [2.0–3.0] for 15.0 U, respectively; P<0.05; grip test score median 2.0 [0.0–2.25] for control vs 4.0 [3.5–4.0] for 15.0 U, respectively; P<0.0001) when treated with 15.0 U C1-inhibitor 1 hour after the onset of tMCAO, confirming our results in young animals (Figure 1A, B). We also tested old mice with an age of 12 months. Again, infarct volumes were significantly smaller (mean 152.7±10.0 mm3 [control] vs 72.4±21.6 mm3 [15.0 U], respectively; P<0.0001) and neurological deficits were less pronounced (grip test score median 1.0 [0.0–2.0] for control vs 4.0 [3.0–4.0] for 15.0 U, respectively; P<0.001) in the group treated with 15.0 U C1-inhibitor compared with vehicle-treated controls (Figure 1A, B).

Gender can significantly influence stroke outcome in rodents.27 Therefore, we also subjected 6-week-old female mice to 60 minutes tMCAO. In line with the results in male mice, C1-inhibitor-treated (15.0 U) female mice likewise had development of significantly smaller infarctions (mean 139.9±8.1 mm3 [control] vs 87.3±9.6 mm3 [15.0 U], respectively; P<0.0001) and less severe neurological deficits (Bederson score median 3.5 [3.0–4.0] for control vs 2.0 [1.75–3.0] for 15.0 U, respectively; P<0.001; grip test score median 3.0 [1.5–1.25] for control vs 4.0 [3.75–4.25] for 15.0 U, respectively; P<0.05) compared with female controls (Figure 1A, B).

Serial magnetic resonance imaging of living mice after tMCAO reaffirmed smaller infarctions on day 1 in animals receiving a single injection of C1-inhibitor (15.0 U) 1 hour after stroke (P<0.001; Figure 1C). Importantly, the infarct volumes did not increase between day 1 and day 7 (P>0.05), thus indicating that C1-inhibitor provides sustained protection against stroke. The alleged shrinkage in stroke size after 1 week in both groups was attributable to infarct maturation and subsequent fogging effects on magnetic resonance imaging rather than true infarct size reduction. Importantly, infarcts always appeared hyperintense on blood-sensitive constructed interference in steady-state sequences. Hypointense areas, which typically indicate intracerebral hemorrhage, were absent from C1-inhibitor–treated mice and
vehicle-treated controls (Figure 1C). This finding excludes the possibility of increased bleeding complications caused by an excess of plasma C1-inhibitor.

We also determined the functional outcome and mortality of C1-inhibitor–treated mice and controls over a longer time period after ischemic stroke (Figure 1D, E). Seven days after 60 minutes of tMCAO, 9 out of 10 control mice (90%) had died, which is in line with previous reports.29 In contrast, 7 out of 10 mice (70%) treated with 7.5 U C1-inhibitor and 9 out of 10 mice (90%) treated with the higher dose of 15.0 U C1-inhibitor...
survived until day 7 \((P<0.0087\) or \(P<0.0215\), respectively; Figure 1D). In line with these findings, mice receiving 15.0 U C1-inhibitor showed significantly better Bederson scores than controls at more advanced stages of infarct development, ie, on day 5 after tMCAO (Bederson score median 2.0 [2.0–3.0] for control vs 0.0 [0.0–1.0] for 15.0 U, respectively; \(P<0.001\); Figure 1E). This observation excludes that C1-inhibitor simply induces faster recovery from stroke but underlines its sustained effect on functional outcome.

In an attempt to extend the therapeutic time window of exogenously applied C1-inhibitor, wild-type mice also received 7.5 U or 15.0 U C1-inhibitor in a delayed setting that is 6 hours after the induction of tMCAO. The higher dose of 15.0 U C1-inhibitor showed a tendency toward smaller infarct volumes, but differences compared with controls or mice receiving 7.5 U C1-inhibitor were not statistically significant (mean stroke area: 23.8% ± 1.1% [control] vs 102.4 ± 9.9 mm\(^3\) [7.5 U] or 94.7 ± 15.3 mm\(^3\) [15.0 U], respectively; \(P>0.05\); Figure 2A). Notably, however, neurological dysfunction was still significantly less in the 15.0 U C1-inhibitor group compared with the 7.5 U C1-inhibitor group or control animals on day 1 (Bederson score median 3.0 [3.0–4.5] for control vs 3.0 [2.0–3.0] for 7.5 U or 1.0 [1.0–3.0] for 15.0 U, respectively; \(P<0.001\) [control vs 15.0 U]; grip test score median 3.0 [0.0–3.0] for control vs 4.0 [3.0–4.0] for 7.5 U or 5.0 [3.0–5.0] for 15.0 U, respectively; \(P<0.001\) [control vs 15.0 U]; Figure 2B).

According to the current experimental stroke guidelines, any protective effect requires evaluation in models of both transient and permanent ischemia. We therefore subjected C1-inhibitor–treated mice to filament-induced permanent MCAO, a procedure in which no tissue reperfusion occurs. In contrast to the striking effects observed after tMCAO, C1-inhibitor could not influence stroke size \((P>0.05)\) or neurological outcome \((P>0.05)\) 24 hours after permanent MCAO \((n=7–8/group)\). Injection of C1-INH 1 hour after stroke did not influence stroke volumes \((C)\) or functional deficits \((D)\) 24 hours after permanent MCAO \((n=7–8/group)\). Injection of C1-INH 1 hour after stroke did not influence stroke volumes \((C)\) or functional deficits \((D)\) 24 hours after permanent MCAO \((n=7–8/group)\). Injection of C1-INH 1 hour after stroke did not influence stroke volumes \((C)\) or functional deficits \((D)\) 24 hours after permanent MCAO \((n=7–8/group)\). Injection of C1-INH 1 hour after stroke did not influence stroke volumes \((C)\) or functional deficits \((D)\) 24 hours after permanent MCAO \((n=7–8/group)\). Injection of C1-INH 1 hour after stroke did not influence stroke volumes \((C)\) or functional deficits \((D)\) 24 hours after permanent MCAO \((n=7–8/group)\).
Protection From Ischemic Stroke in C1-Inhibitor–Treated Mice Is a Result of Reduced Edema Formation, Inflammation, and Thrombosis

Next, we sought to elucidate the underlying mechanisms of this C1-inhibitor–specific neuroprotection in transient stroke. C1-inhibitor plays an important role in the regulation of vascular permeability and suppression of inflammation by inactivating key proteases of the contact-kinin system such as factor XIIa (FXIIa) or plasma kallikrein.14 Consequently, the extent of blood–brain barrier damage and edema formation in the ischemic hemispheres was addressed. On day 1 after tMCAO, the integrity of the blood–brain barrier as reflected by the volume of the vascular tracer Evan’s Blue leaking into the brain parenchyma was preserved in mice treated with 15.0 U C1-inhibitor 1 hour after stroke and less pronounced after injection of 7.5 U C1-inhibitor in comparison with untreated controls (mean 51.6 ± 10.8 mm³ [control] vs 33.1 ± 7.9 mm³ [7.5 U] or 13.9 ± 4.3 mm³ [15.0 U], respectively; P<0.05 [control vs 15.0 U]; Figure 4A). This finding correlated with dramatically less brain edema formation (wet/dry weight method) after therapeutic C1-inhibitor application (mean 4.3% ± 0.5% [control] vs 2.9% ± 0.4% [7.5 U] or 0.2% ± 0.4% [15.0 U], respectively; P<0.0001 [control vs 15.0 U]; Figure 4B), a result that also could be confirmed in rats (Supplemental Figure III, available online at http://stroke.ahajournals.org). Importantly, almost no blood–brain barrier disruption was found in the brain regions (basal ganglia) where infarcts were regularly present in C1-inhibitor–treated mice (Figure 1A, 4A, red arrow). This indicates that the lesser edema seen in the C1-inhibitor group was a specific phenomenon and mechanistically relevant but not simply because of smaller infarct volumes in these animals.

We also analyzed the expression of endothelin-1 in the ischemic brains of C1-inhibitor–treated mice and controls. Endothelin-1 has been shown to be critically involved in the regulation of vascular integrity and edema formation under ischemic conditions.
various pathophysiological conditions, including ischemic stroke.30,31 Twenty-four hours after tMCAO, endothelin-1 mRNA levels were significantly elevated in the cortices and basal ganglia of vehicle-treated mice and mice receiving 7.5 U C1-inhibitor compared with sham-operated mice (relative gene expression cortex: 1.0 ± 0.1 [sham] vs 16.0 ± 2.2 [control] or 15.6 ± 2.3 [7.5 U], respectively; *P < 0.0001; relative gene expression basal ganglia: 1.0 ± 0.1 [sham] vs 4.3 ± 0.4 [control] or 4.2 ± 0.6 [7.5 U], respectively; **P < 0.0001; Figure 4C). In contrast, no significant induction of endothelin-1 transcripts was observed in either brain region after treatment with 15.0 U C1-inhibitor (P > 0.05). Again, endothelin-1 expression remained low in the basal ganglia after high-dose (15.0 U) C1-inhibitor treatment (Figure 4C), although significant parts of the basal ganglia were uniformly included into the infarcted areas in all animals (Figure 1A).

In line with a blood–brain barrier stabilizing effect of C1-inhibitor in stroke, immunoreactivity against the tight junction protein occludin was preserved in vessels of the ischemic basal ganglia from mice treated with 15.0 U C1-INH on day 1 after tMCAO (n = 5/group). ***P < 0.0001, 1-way ANOVA, Bonferroni post hoc test compared with untreated control mice. C, Relative gene expression of endothelin-1 (Edn-1) in the cortices and basal ganglia of sham-operated mice, untreated controls (Ctrl), and mice treated with 7.5 U C1-INH or 15.0 U C1-INH (n = 6–8/group) 24 hours after tMCAO. Note that 15.0 U C1-INH prevented the induction of endothelin-1 in both brain regions. ***P < 0.0001, ###P < 0.0001, +++P < 0.0001, 2-way ANOVA, Bonferroni post hoc test compared with sham-operated mice (cortex* or basal ganglia#, respectively) or control mice (cortex*). D, Upper panel shows occludin expression in the ischemic basal ganglia on day 1 after tMCAO in control mice (Ctrl) or mice receiving 7.5 U C1-INH or 15.0 U C1-INH as determined by immunoblot. Four representative blots of each group are shown. Lower panel shows densitometric quantification of occludin immunoreactivity in the mouse groups indicated (n = 4/group). *P < 0.05, 1-way ANOVA, Bonferroni post hoc test compared with untreated mice.
C1-inhibitor but was downregulated in control mice or mice receiving 7.5 U C1-inhibitor as demonstrated by immunohistochemistry (Supplemental Figure IV, available online at http://stroke.ahajournals.org). To quantify occludin protein expression in more detail, we also performed Western blot analysis (Figure 4D). Again, the amount of occludin on day 1 after tMCAO in the ischemic basal ganglia from untreated mice was low (optical density: 0.08 ± 0.10). In contrast, significantly more occludin protein was detectable after treatment with 7.5 U (optical density: 0.5 ± 0.2; P < 0.05) or 15.0 U (optical density 0.5 ± 0.1; P < 0.05) C1-inhibitor, respectively.

The C1-inhibitor has been shown to inhibit cell migration from the vasculature to inflammation sites by binding of cell adhesion molecules.32 We therefore quantified the numbers of immune cells invading the ischemic brain by immunohistochemistry (Figure 5A). Twenty-four hours after the induction of tMCAO, significantly more neutrophilic granulocytes (mean 299.1 ± 52.2 [control] vs 107.2 ± 44.7 [15.0 U]; P < 0.05) as well as macrophages/microglia cells (mean 676.3 ± 75.2 [control] vs 117.1 ± 32.5 [15.0 U]; P < 0.0001) had entered the ischemic basal ganglia of untreated control mice than of mice that had been treated with 15.0 U C1-inhibitor 1 hour after stroke. In contrast, the lower dose of 7.5 U of C1-inhibitor was unable to reduce cell trafficking after focal cerebral ischemia (P > 0.05; Figure 5A).

As a next step, we analyzed the gene expression profiles of the prototypic proinflammatory cytokines interleukin-1β and tumor necrosis factor-α in the brains of C1-inhibitor–treated mice and controls 24 hours after tMCAO (Figure 5B). Both cytokines are able to promote ischemic brain damage.1 The amount of interleukin-1β mRNA in the infarcted cortices was strongly elevated both in the untreated group as well as in the group receiving 7.5 U C1-inhibitor compared with sham-operated mice (P < 0.0001). In contrast, no significant increase of interleukin-1β could be observed in the cortex after high-dose (15.0 U) C1-inhibitor treatment (P > 0.05), and this dose also led to significantly lower interleukin-1β levels compared with untreated mice (P < 0.001; Figure 5B). Similar results were obtained for tumor necrosis factor-α. Again, application of 15.0 U C1-inhibitor attenuated the increase of tumor necrosis factor-α expression observed in control mice or after treatment with 7.5 U C1-inhibitor (P < 0.001, control vs 15.0 U).

The C1-inhibitor also acts on FXIIa, the prime activator of the intrinsic pathway of blood coagulation.14 Therefore, we analyzed the impact of C1-inhibitor on thrombotic activity after brain ischemia-reperfusion injury. The amount of fibrin(o-gen) detected by immunoblot in the ischemic cortex (mean optical density: 2.8 ± 0.5 [control] vs 1.7 ± 0.4 [7.5 U] or 0.03 ± 0.01 [15.0 U], respectively; P < 0.0001 [control vs 15.0 U]) was significantly reduced after treatment with 7.5 U C1-inhibitor but was downregulated in control mice or mice receiving 7.5 U C1-inhibitor as demonstrated by immunohistochemistry (Supplemental Figure IV, available online at http://stroke.ahajournals.org). To quantify occludin protein expression in more detail, we also performed Western blot analysis (Figure 4D). Again, the amount of occludin on day 1 after tMCAO in the ischemic basal ganglia from untreated mice was low (optical density: 0.08 ± 0.10). In contrast, significantly more occludin protein was detectable after treatment with 7.5 U (optical density: 0.5 ± 0.2; P < 0.05) or 15.0 U (optical density 0.5 ± 0.1; P < 0.05) C1-inhibitor, respectively.

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U]) and basal ganglia (mean optical density: 2.8±0.5 [control] vs 1.2±0.3 [7.5 U] or 0.3±0.1 [15.0 U], respectively; *P<0.001 [control vs 15.0 U]) was significantly reduced on day 1 after stroke after high-dose (15.0 U) C1-inhibitor application 1 hour after the induction of tMCAO (Figure 6A). The results from immunoblots also could be validated by immunohistochemistry showing less fibrin(ogen) deposits in the brain capillaries of C1-inhibitor–treated (15.0 U) mice (Supplemental Figure V, available online at http://stroke.ahajournals.org). Accordingly, the microvascular patency was significantly increased compared with naïve controls (thrombosis index: 15.8±1.4 [control] vs 12.2±1.2 [7.5 U] or 9.8±1.4 [15.0 U], respectively; *P<0.05 [control vs 15.0 U]; Figure 6B).

Discussion

We show that plasma-derived C1-inhibitor protects from brain ischemia-reperfusion injury in rodents in different “clinically” meaningful settings. C1-inhibitor halved the infarct size in male mice when applied 1 hour after the onset of stroke and was still effective up to 6 hours. Its beneficial activity was preserved in female mice, at later stages of infarction, as well as in older cohorts. Moreover, C1-inhibitor also was effective in another species, ie, rats. Combined antiinflammatory, antiedematous, and antithrombotic modes of C1-inhibitor action could be identified as underlying mechanisms.

We and others recently could demonstrate that the contact-kinin system is critically involved in the pathology of ischemic stroke at different levels. Genetic disruption7 or pharmacological blocking34 of FXIIa, the common origin of the contact-kinin system and activator of the intrinsic coagulation cascade, led to near-resistance against ischemic neurodegeneration by preventing microvascular thrombosis. However, inhibition of another component of the contact-
The C1-inhibitor formulation used in our experiments could positively influence neurological outcome, even when administered 6 hours after stroke, although beneficial effects on stroke size were no longer observable under these conditions. This mismatch is probably attributable to the relatively poor correlation between stroke volume and functional deficits in rodent models of ischemic stroke.

In contrast to our findings, another plasma-derived C1-inhibitor only showed a rather narrow therapeutic window after focal cerebral ischemia, ie, 30 minutes, but was ineffective already after 60 minutes. The exact reasons for this discrepancy are unclear at present but differences in the duration of brain ischemia (30 minutes vs 60 minutes) or the C1-inhibitor preparations used might play a role. This is further underlined by the fact that in our study 7.5 U of C1-inhibitor had only small effects on stroke outcome, whereas the minimal effective dose in mice was 5.0 U in the study by De Simoni et al.

In summary, C1-inhibitor improves stroke outcome by interfering with key mechanisms of ischemic brain damage, namely thrombosis, edema formation, and inflammation. The fact that stroke protection could be achieved in different scenarios mimicking the clinical situation together with the multifaceted modes of C1-inhibitor action is promising and should be taken as a basis for further translational studies in relevant disease models.

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**Disclosures**

Dr Nolte, Dr Hofmeister, and Dr Dickneite are employees of CSL Behring GmbH Marburg, Germany, and Dr Nolte and Dr Dickneite hold stocks in CSL Limited, Parkville, Australia. Dr Kleinschnitz received financial support for conducting this research project from CSL Behring GmbH Marburg, Germany. The other authors have nothing to disclose.

**References**


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Supplementary Methods

Induction of cerebral ischemia in mice. Focal cerebral ischemia was induced in 6 weeks old male and female mice, 6 months old male mice, or 12 months old male mice by 60 min transient middle cerebral artery occlusion (tMCAO) as described.\(^1\) Mice were anesthetized with 2.5% isoflurane (Abbott) in a 70% \(\text{N}_2\text{O}\)/30% \(\text{O}_2\) mixture. Core body temperature was maintained at 37°C throughout surgery by using a feedback-controlled heating device. Following a midline skin incision in the neck, the proximal common carotid artery and the external carotid artery were ligated and a standardized silicon rubber-coated 6.0 nylon monofilament (6021; Doccol) was inserted and advanced via the right internal carotid artery to occlude the origin of the right MCA. The intraluminal suture was left in situ for 60 minutes. Then animals were re-anesthetized and the occluding monofilament was withdrawn to allow for reperfusion. For permanent MCAO (pMCAO) the occluding filament was left in situ until sacrificing the animals. Operation time per animal did not exceed 15 minutes.

Assessment of functional outcome in mice. On day 1 and day 5 after MCAO, neurological deficits were scored and quantified according to Bederson.\(^3\) 0, no deficit; 1, forelimb flexion; 2, as for 1, plus decreased resistance to lateral push; 3, unidirectional circling; 4, longitudinal spinning; 5, no movement. For the grip test,\(^4\) the mouse was placed midway on a string between two supports and rated as follows: 0, falls off; 1, hangs on to string by one or both fore paws; 2, as for 1, and attempts to climb on to string; 3, hangs on to string by one or both fore paws plus one or both hind paws; 4, hangs on to string by fore and hind paws plus tail wrapped around string; 5, escape (to the supports).

Induction of cerebral ischemia in rats. 7-9 weeks old male CD rats were subjected to 90 min of tMCAO using the intraluminal filament technique.\(^5\) Anesthesia was induced in spontaneously breathing animals in an isoflurane chamber with 5% isoflurane (CP Pharma) and was subsequently maintained with 2.5% isoflurane via a face mask. During surgery, animals were placed on a heating device to ensure normothermia (37°C). After a midline skin incision in the neck, the left common carotid artery and external carotid artery were isolated and ligated. Following arteriotomy a 4.0 nylon monofilament (Ethilon®, Johnson & Johnson) with its tip blunted by heat was inserted into the internal carotid artery and advanced cranially to the origin of the middle cerebral artery until a gentle resistance was felt. The occluding filament was left in situ for 90 minutes. Then animals were re-anesthetized and the occluding monofilament was withdrawn to allow for reperfusion.

Assessment of functional outcome in rats. On day 1 after tMCAO neurological deficits were quantified using a modified composite scoring system according to Reglodi.\(^6\) This functional assay is based on a battery of different neurological tests in which the sum of all sub-items constitutes the total neurological score (maximum score is 28 with a score of 0 indicating no neurological deficit). In detail, the test battery evaluates a) postural signs with ‘forelimb flexion’ (= degree of limb flexion when the animal is held by tail; 0–2 points) and ‘thorax twisting’ (= degree of body rotation when animal is held by tail, 0–2 points), b) gait disturbances with ‘circling’ (straight walking = 0 points, walking towards contralateral side = 1 point, alternate circling and walking straight = 2 points, alternate circling and walking towards paretic side = 3 points, circling and/or other gait disturbance (backing, crawling, walking on digits) = 4 points, and constant circling toward paretic side = 5 points) and ‘climbing’
(ability to climb up an inclined board (45°), 0–1 points), c) limb placing with ‘forelimb placing’ (normal, weak, or no placing; 0–2 points) and ‘hindlimb placing’ (normal, weak, or no placing; 0–2 points), d) balance on a cylindrical beam (10 cm above the floor, three times): 0 or 1 times falling off the beam with or without attempt to stay on the beam = 0 points; two times falling off the beam with or without attempt to stay on the beam = 1–2 points; three times falling off the beam with or without attempt to stay on the beam = 3–4 points, e) symmetry of muscle tone/strength with ‘lateral resistance’ (=degree of resistance against lateral push; 0–2 points) and ‘grasping strength’ (=symmetry of grasping strength onto wire cage; 0–1 points), f) sensory function with ‘grasping reflex of forepaw’ (=grasping onto tube when gently touched; 0–1 points) and ‘touching reflex’ (=withdrawal of forelimb when touched by needle; 0–1 points), g) motility/spontaneous activity (1 minute observation): Normal or slightly reduced exploratory behavior = 0–1 points, moving limbs without proceeding = 2 points, moving only to stimuli = 3 points, unresponsive to stimuli, with normal muscle tone = 4 points, severely decreased tone/premortal signs = 5 points.

C1-INH treatment. 1h or 6h after the induction of tMCAO or pMCAO, mice received a single intravenous injection of plasmatic human C1-INH (Berinert-P®, CSL Behring GmbH) at a dose of 7.5 units (U) or 15.0 U diluted in 150 µl carrier solution (isotonic saline). The respective doses were chosen based on previously published work in rodent models of cerebral ischemia and 15.0 U corresponds to the amount of C1-INH required to obtain 90% to 95% inhibition of complement haemolytic activity in mice.7,8 In rats, C1-INH (Berinert-P®, CSL Behring GmbH) was intravenously injected 90 min after the induction of tMCAO, i.e. immediately after induction of reperfusion, at a dose of 20 U/kg body weight. Control mice and rats received equal volumes of isotonic saline (vehicle).

Stroke study design. Vehicle-treated mice or rats or mice or rats receiving C1-INH were randomly assigned to the operators (N.H., E.G., F.L., M.H., C.K.) by an independent person not involved in data acquisition and analysis. We performed surgery and evaluation of all read-out parameters while blinded to the experimental groups. The following conditions excluded animals from end-point analyses (exclusion criteria):
1. Death within 24h after MCAO (except for analysis of survival)
2. Subarachnoidal hemorrhage (SAH) (as macroscopically assessed during brain sampling or by MRI)
3. Bederson score = 0 (immediately after reperfusion, mice only)

Of the 422 mice and 43 rats subjected to MCAO, 53 mice (12.6%) and 10 rats (23.3%) met at least one of the above exclusion criteria after randomization and were withdrawn from the study. Drop-out rates were evenly distributed between the groups, i.e. untreated controls or animals treated with C1-INH (P > 0.05). Statistical analysis was performed on a per-protocol basis not taking into account the excluded animals.

Determination of infarct size. Following MCAO the mouse and rat brains were quickly removed and cut in three (mice) or six (rats) 2-mm thick coronal sections using a mouse or rat brain slice matrix (Harvard Apparatus). The slices were stained for 20 min at 37°C with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) in PBS to visualize the infarctions.3
Indirect, i.e. corrected for brain edema, infarct volumes in mice were calculated by volumetry (ImageJ software, National Institutes of Health, USA) according to the following equation:

\[ V_{\text{indirect}} (\text{mm}^3) = V_{\text{infarct}} \times (1- (V_1 - VC)/VC), \]

whereas the term \((V_1 - VC)\) represents the volume difference between the ischemic hemisphere and the control hemisphere and \((V_1 - VC)/VC\) expresses this difference as a percentage of the control hemisphere.

In rats, edema-corrected infarct areas were calculated by planimetry as follows:

\[ \text{Infarct area} (\%) = 100 - \left[ \frac{(A_I + A_C) \times 100}{(A_C \times 2)} \right], \]

whereas \(A_I\) represents the total area of viable tissue of the ipsilateral (stroked) hemisphere and \(A_C\) represents the total area of the contralateral (healthy) hemisphere.

**Laser-Doppler flowmetry.** Laser-Doppler flowmetry (Moor Instruments) was used in vehicle-treated mice and C1-INH-treated mice to monitor regional cerebral blood flow (rCBF) in the MCA territory (6 mm lateral and 2 mm posterior from bregma).\(^9\)

**Invasive hemodynamics.** For the assessment of blood pressure and heart rate, C1-INH-treated mice (15.0 U or 7.5 U, respectively) and controls were anesthetized with 2.0% isoflurane and catheterized via the right carotid artery with a high-fidelity 1.4 F Millar microtip catheter (Milar Instruments) as described.\(^10\) Hemodynamic data were digitized via a MacLab system (AD Instruments) connected to an Apple G4 PowerPC computer and analyzed.

**Blood gas analysis.** 100 µl of arterial blood was drawn from the left cardiac ventricle of anesthetized mice by a heparinized syringe. We determined PaO\(_2\), PaCO\(_2\) and pH in C1-INH-treated mice (15.0 U or 7.5 U, respectively) and controls using an ABL 77 automated blood gas analyzer (Radiometer).

**Magnetic resonance imaging (MRI).** Magnetic resonance imaging of living C1-INH treated mice and controls was performed repeatedly at 24h and 7 days after tMCAO on a 1.5 Tesla unit (Vision, Siemens) under inhalation anesthesia as described previously.\(^1,11\) We used a custom-made dual channel surface coil designed for examining mice (A063HACG; Rapid Biomedical). The imaging protocol comprised a coronal T2-weighted sequence (slice thickness 2 mm) and a blood-sensitive coronal three-dimensional T2-weighted gradient echo constructed interference in steady state (CISS; slice thickness 1 mm) sequence. Images were transferred to an external workstation (Leonardo, Siemens) for data processing and were assessed with respect to infarct morphology and the occurrence of intracerebral bleeding. Infarct volumes were calculated by planimetry of the hyperintense area on high-resolution CISS images.

**Determination of blood-brain-barrier leakage and brain edema.** To determine blood-brain-barrier leakage 100 µl of 2% Evan’s Blue tracer (Sigma Aldrich) diluted in 0.9% NaCl was i. v. injected 1h after the induction of tMCAO.\(^12\) After 24h C1-INH-treated mice and controls were sacrificed and brains were quickly removed and cut in 2 mm thick coronal sections using a mouse brain slice matrix (Harvard Apparatus). Planimetric measurements (ImageJ software, National Institutes of Health) of the brain parenchyma stained by Evan’s Blue were performed to estimate blood-brain-barrier damage.
To assess the extent of brain edema, C1-INH-treated mice or controls were sacrificed 24h after tMCAO. Brains were removed, hemispheres separated, and weighed to assess the wet weight (WW). Thereafter, the hemispheres were dried for 72h at 60°C and the dry weight (DW) was determined. Hemispheric water content (%) was calculated using the following formula: ((WW-DW)/WW) x 100. In rats, the extent of brain edema was calculated by planimetry from TTC-stained brain sections according to the following equation:

Brain edema area (%) = [(AL+Al+AC) x 100/(AC x 2)] – 100,

whereas AL represents the total area of TTC-negative (ischemic) brain tissue, Al represents the total area of viable tissue of the ipsilateral (stroked) hemisphere, and AC represents the total area of the contralateral (healthy) hemisphere.

**Histology and immunohistochemistry.** Cryo-embedded brains were cut into 10-µm thick slices and fixed in acetone for staining of neutrophilic granulocytes or in 4% PFA in PBS for staining of microglia/macrophages and occludin. Blocking of epitopes was achieved by pre-treatment with bovine serum albumin (BSA) in PBS for 45 min to prevent unspecific binding. For staining of invading immune cells rat anti-mouse Ly-6B.2 alloantigen (neutrophilic granulocytes; MCA771GA, AbD Serotec) at a dilution of 1:1000 and rat anti-mouse CD11b (microglia/macrophages; MCA711, AbD Serotec) at a dilution of 1:100 in PBS containing 1% BSA was added overnight at 4°C. Afterwards, slides were incubated with a biotinylated anti-rat IgG (BA-4001, Vector Laboratories) diluted 1:100 in PBS containing 1% BSA for 45 min at room temperature. Following treatment with Avidin/Biotin blocking solution (Avidin/Biotin Blocking Kit, Sp-2001, Vector Laboratories) to inhibit endogenous peroxidase activity, the secondary antibody was linked via streptavidin to a biotinylated peroxidase (POD) according to the manufacturer’s instructions (Vectorstain ABC Kit, Peroxidase Standard PK-4000, Vector Laboratories). Antigens were visualized via POD using the chromogen 3,3'- Diaminobenzid (DAB) (Kem-En-Tec Diagnostics). For quantification of immune cells identical brain sections (thickness 10-µm) at the level of the basal ganglia (0.5 mm anterior from bregma) from C1-INH-treated mice and controls were selected and cell counting was performed from 5 subsequent slices (distance 10 µm) from 4 different animals under a Nikon microscope Eclipse 50i (Nikon).

For immunofluorescence staining against occludin a rabbit anti-mouse occludin antibody (ab 31721, Abcam) was applied overnight (4°C) at a dilution of 1:100 in PBS containing 1% BSA. Proteins were detected with Cy3-labeled goat anti-rabbit secondary antibodies at a dilution of 1:300 in 1% BSA in PBS. For staining of DNA a fluorescent Hoechst dye (Hoechst 33342, Sigma-Aldrich) was added for 30 min at a concentration of 0.4 mg/ml. Sections were analyzed under an Axiophot 2 (Carl Zeiss AG).

For calculation of the thrombosis index the whole brain was sliced 24h after tMCAO. H&E staining was performed according to standard procedures. For quantification, stainings were examined in a blinded fashion under a microscope (Axiophot2, Carl Zeiss AG) equipped with a CCD camera (Visitron Systems). The number of occluded blood vessels within the ischemic basal ganglia was counted in every tenth slice for control mice, or mice treated with 7.5 U C1-INH or 15.0 U C1-INH, respectively, using a 40-fold magnification.

For staining of exogenously applied human C1-INH in the ischemic rat brain after tMCAO, animals were transcardially perfused with PBS followed by 4% formaldehyde. Brains were removed, postfixed in 4% formaldehyde at 4°C for 24h and transferred into 30% sucrose/PBS for 3 days. Then, 40-µm thick brain sections
were cut and rinsed three times with Tris-buffered saline Tween (TBST) buffer. To block endogenous peroxidase activity 0.3% H₂O₂/methanol solution was applied for 30 min. After that brain sections were washed with TBST and incubated with blocking solution (1.5% normal rabbit serum in TBST; Vectastain® Elite ABC Kit; Vector Laboratories) for 30 min at room temperature. Then, a polyclonal sheep antibody against human C1-INH (Abcam) diluted 1:30.000 in TBST was added for 20h at 4°C under free-floating conditions. After an additional washing step (TBST), a biotinylated secondary antibody (rabbit anti-sheep IgG in 1.5% normal rabbit serum/TBST; Vectastain® Elite ABC Kit; Vector Laboratories) was added for 40 min at room temperature. Sections were rinsed again and incubated with Vectastain® Elite ABC Reagent for 40 min (Vector Laboratories) at room temperature. The DAB technique was used for chromogenic reaction. Finally, brain sections were mounted on glass slides, air dried, dehydrated in graded ethanol, cleared in XTRA-Solve (J. T. Baker), and coverslipped with XTRA-Kit (J. T. Baker). Analysis of stained sections was performed using a Zeiss Z1-Imager (Carl Zeiss AG).

Negative controls for all histological experiments included omission of primary or secondary antibody and produced no signals (not shown).

**PCR studies.** Tissue homogenization, RNA isolation and Real-time RT-PCR were performed as described.¹³ Total RNA was prepared with a Miccra D-8 power homogenizer (ART) using the TRizol reagent® (Invitrogen) and was quantified spectrophotometrically. Then, 1 µg of total RNA were reversely transcribed with the TaqMan® Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's protocol using random hexamers. Relative gene expression levels of endothelin-1 (assay ID: Mm 00438656_m1, Applied Biosystems), IL-1ß (assay ID: Mm 00434228_m1, Applied Biosystems), and TNFα (assay ID: Mm 00443258_m1, Applied Biosystems) were quantified with the fluorescent TaqMan® technology. GAPDH (TaqMan® Predeveloped Assay Reagents for gene expression, part number: 4352339E, Applied Biosystems) was used as an endogenous control to normalize the amount of sample RNA. The PCR was performed with equal amounts of cDNA in the StepOnePlus™ Real-Time PCR System (Applied Biosystems) using the TaqMan® Universal 2x PCR Master Mix (Applied Biosystems). Reactions (total volume 12.5 µl) were incubated at 50°C for 2 min, at 95°C for 10 min followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Water controls were included to ensure specificity. Each sample was measured in triplicate and data points were examined for integrity by analysis of the amplification plot. The comparative Ct method was used for relative quantification of gene expression as described.¹⁴

**Western blot.** Cortices or basal ganglia were dissected from native brains and homogenized in RIPA buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40) containing 0.1% SDS and 4% proteinase inhibitor (complete protease inhibitor cocktail, Roche). Samples were sonified for 10 sec. Afterwards tissue lysates were centrifuged at 15.000×g for 30 min at 4°C and supernatants were used for bicinchoninic acid (BCA) protein assay and subsequent Western blot analysis. The total lysates were treated with 4x SDS-PAGE loading buffer (final conc. 62.5 mM Tris pH 6.8, 3% beta-mercaptoethanol, 8% SDS, 15% glycerol) at 95°C for 5 min. 20 µg of total protein was electrophoresed and transferred to a PVDF membrane. After blocking for 30 min with blocking buffer (5% nonfat dry milk, 50 mM Tris-HCl pH 7.5, 0.05% Tween-20) membranes were incubated with the primary antibody at 4°C overnight at the following dilutions: anti-fibrinogen pAb 1:500 (Acris Antibodies), anti-occludin pAb 1:1000 (Abcam), and anti-actin mAb 1:75.000 (Dianova). After a
washing step with TBST (50 mM Tris-HCl pH 7.5, 0.05% Tween-20), membranes were incubated for 1h with HRP-conjugated donkey anti-rabbit IgG (for fibrinogen and occludin) (Dianova) or donkey anti-mouse IgG (for actin) (Dianova) at a dilution of 1:5000 and were finally developed using ECLplus (GE Healthcare).
Supplemental References


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**Table S1** Results of blood gas analysis in vehicle-treated C57Bl/6 mice (control) and mice receiving 7.5 U or 15.0 U C1-INH, respectively. No significant differences were observed between the groups, ns: not significant.
**Figure S1** Regional cerebral blood flow (rCBF) in the territory of the right middle cerebral artery (MCA) as measured by Laser Doppler flowmetry in vehicle-treated control mice (Ctrl) and mice treated with 7.5 U or 15.0 U C1-INH (n=3/group) at baseline levels, after insertion of the filament (Ischemia) and again ten minutes after removal of the filament (Reperfusion). No differences in rCBF were observed between the groups at any time point, 2-way ANOVA, Bonferroni’s post test, ns: not significant.
Figure S2 Treatment with 7.5 U or 15.0 U C1-INH does not alter mean arterial blood pressure or heart rate compared with vehicle-treated mice (Ctrl) as assessed by invasive hemodynamics (n=4/group), 1-way ANOVA, Bonferroni’s post test ns: not significant.
Figure S3 C1-INH treatment reduces brain edema formation upon stroke in rats. Rats were subjected to 90 min of tMCAO and treated with 20 U/kg C1-INH immediately after reperfusion. The extent of brain edema was calculated by planimetry from TTC-stained brain sections on day 1 (n=15/group); ***p<0.0001, two-tailed Student's t-test compared with vehicle-treated controls.
Figure S4 Expression of occludin on day 1 after tMCAO in the basal ganglia of control mice or mice receiving C1-INH. Immunohistochemistry revealed that occludin was predominately located within brain vessels (arrows) and expression was preserved after treatment with 15.0 U C1-INH but was markedly reduced in untreated mice or with 7.5 U C1-INH. Left panel: DAPI staining for cell nuclei, middle panel: occludin staining, right panel: merge. Bars represent 100 µm.
Figure S5 Immunohistochemical localization of fibrin(ogen) (white arrows) in the lumina of brain microvessels 24h after tMCAO in the infarcted hemispheres of mice treated with 15.0 U C1-INH, 7.5 U C1-INH or control mice. DAPI staining depicts cell nuclei. One representative panel per group is shown. Bars represents 100 μm.
C1-Inhibitor Protects From Brain Ischemia-Reperfusion Injury by Combined Antiinflammatory and Antithrombotic Mechanisms

Nadine Heydenreich1; Marc W. Nolte, PhD2; Eva Göb, PhD1; Friederike Langhauser, PhD1; Marion Hofmeister1; Peter Krafft, MD3; Christiane Albert-Weissenberger, PhD1; Marc Brede, MD3; Csanad Varallyay, MD4; Kerstin Göbel, MD5; Sven G. Meuth, MD, PhD5,6; Bernhard Nieswandt, PhD7,8; Gerhard Dickneite, PhD2; Guido Stoll, MD1; Christoph Kleinschnitz, MD7,8; Christiane Albert-Weissenberger, PhD1; Marc Brede, MD3; Csanad Varallyay, MD4; Kerstin Göbel, MD5; Sven G. Meuth, MD, PhD5,6; Bernhard Nieswandt, PhD7,8; Gerhard Dickneite, PhD2; Guido Stoll, MD1; Christoph Kleinschnitz, MD7,8;

1 Department of Neurology, University of Würzburg, Würzburg, Germany; 2 CSL Behring GmbH, Marburg, Germany; 3 Department of Anesthesiology and Critical Care, University of Würzburg, Würzburg, Germany; 4 Department of Neuroradiology, University of Würzburg, Würzburg, Germany; 5 Department of Neurology–Inflammatory Disorders of the Nervous System and Neurooncology, University of Münster, Münster, Germany; 6 Institute of Physiology-Neuropathophysiology, University of Münster, Münster, Germany; 7 Department of Vascular Medicine, University of Würzburg, Würzburg, Germany; 8 Rudolf Virchow Center, DFG Research Center for Experimental Biomedicine, University of Würzburg, Würzburg, Germany.

Abstract

C1-Inhibitor Protects From Brain Ischemia-Reperfusion Injury by Combined Antiinflammatory and Antithrombotic Mechanisms

C1 インヒビターは抗炎症と抗血栓の複合的機序により脳虚血-再灌流障害から脳を保護する

C1-Inhibitor Protects From Brain Ischemia-Reperfusion Injury by Combined Antiinflammatory and Antithrombotic Mechanisms

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