Endothelial Von Willebrand Factor Promotes Blood–Brain Barrier Flexibility and Provides Protection From Hypoxia and Seizures in Mice


Objective—Aberrant blood–brain barrier (BBB) permeability is a hallmark pathology of many central nervous system diseases. von Willebrand factor (VWF) is stored in endothelial Weibel–Palade bodies from where it is released on activation into plasma and basement membrane. The role of VWF in endothelial homeostasis is unclear. The goal of this study was to assess the role of VWF in disease models associated with increased BBB permeability.

Approach and Results—We did not find any differences in BBB permeability to Evans blue dye at baseline between wild-type and VWF−/− animals. We next used 2 models presenting with increased BBB permeability, hypoxia/reoxygenation and pilocarpine-induced status epilepticus, to assess the response of VWF−/− animals. In both models, VWF−/− mice maintained a tighter BBB than wild–type mice. VWF−/− mice fared worse in both conditions, with ≈100% of VWF−/− mice dying within 120 minutes after pilocarpine administration, whereas >80% of wild-type animals survived. Investigation into the status of tight junction proteins revealed that VWF−/− mice expressed more claudin-5 at baseline. In vitro work confirmed that the presence of subendothelial VWF is inhibitory to claudin-5 expression.

Conclusions—VWF deficiency confers partial preservation of BBB integrity after hypoxia/reoxygenation and seizures. Surprisingly, this decrease in BBB permeability did not result in protection of animals because they demonstrated more severe pathology in both models compared with wild-type animals. These data suggest that a rigid BBB is detrimental (to the organism) during certain disease states and that VWF release may provide desired flexibility under stress. (Arterioscler Thromb Vasc Biol. 2013;33:2112-2120.)

Key Words: blood–brain barrier ■ blood vessels ■ claudin-5 ■ endothelium ■ hypoxia ■ seizures ■ von Willebrand factor

Blood–brain barrier (BBB) opening is a hallmark of many pathological conditions of the central nervous system, including hypoxia, epilepsy, multiple sclerosis, and ischemic stroke.1-5 Although it is known that von Willebrand factor (VWF) is expressed abundantly by cerebral endothelial cells,6 very little is known about the role of VWF, in general, and the regulation of BBB, in particular. VWF is a glycoprotein that is only synthesized by endothelial cells and megakaryocytes.7 Endothelial cell–derived VWF is secreted constitutively and stored in Weibel–Palade bodies (WPBs) from where it is released by regulated secretion into the plasma and basement membrane in response to endothelial activation.8 Interestingly, studies have shown that VWF protein is upregulated in the plasma of patients with neurological conditions as diverse as stroke,9 severe head injury,10 cerebral malaria,11 and cerebral venous sinus thrombosis.12 Although it has been published that all these conditions involve BBB disruption, there has been very little literature examining the potential role of VWF in BBB regulation under pathological conditions. Interestingly, a role for endothelial VWF in regulation of angiogenesis has recently been reported, indicating that it may play a role in endothelial biology.13

To study the role of VWF in conditions associated with increased BBB permeability, we subjected wild-type (WT) and VWF−/− mice to 2 models: hypoxia/reoxygenation (H/R) and pilocarpine-induced status epilepticus (SE). Generalized normobaric hypoxia is a pathological condition in which the body as a whole is deprived of adequate oxygen supply. Hypoxia occurs in healthy people when they ascend to high altitudes where it can cause altitude sickness, often manifested by headache,
leading to potentially fatal complications, such as high-altitude cerebral edema. Hypoxia followed by reoxygenation is also commonly used as a model to investigate pathology associated with ischemia/reperfusion, because the latter condition is present in several disease states, including stroke.

Epilepsy is a neurological disease afflicting 1.2% to 3% of the general population. Symptomatic epilepsy is associated with a higher risk for death compared with the general population. Death commonly occurs during SE, which is defined as a prolonged seizure state with no return to normal state of consciousness lasting ≥30 minutes and has a mortality rate of 20% to 40%. Several triggers of SE have been identified, such as hypoxia, infection, and trauma, and a complete mechanism of SE is unknown.

SE can be closely mimicked by intraperitoneal injection of pilocarpine, a model that was developed decades ago. This model first presents with an instantaneous period of SE followed by a latent period and, in a percentage of mice, spontaneous recurrent seizures. It has been shown that administration of pilocarpine induces seizures through the M1-cholinoreceptor M1; however, maintenance of SE involves another mechanism because blocking this receptor after the initiation of SE does not alleviate the condition.

In animal models, H/R and SE have both been shown to cause BBB abnormalities, such as tight junction alterations and increased BBB permeability. However, a complete mechanism by which these disease states lead to BBB opening and its importance are still undefined. In the present study, we demonstrated that administration of pilocarpine induces seizures in VWF−/− mice through the M1-cholinoreceptor M1; however, maintenance of SE involves another mechanism because blocking this receptor after the initiation of SE does not alleviate the condition. Pilocarpine-induced seizures can also be prevented by antiepileptic drugs used for epilepsy treatment in humans, further supporting the pathophysiological relevance of this model.

In animal models, H/R and SE have both been shown to cause BBB abnormalities, such as tight junction alterations and increased BBB permeability. However, a complete mechanism by which these disease states lead to BBB opening and its importance are still undefined. In the present study, we demonstrate that in VWF−/− mice, BBB permeability was significantly decreased compared with WT controls in mouse models of H/R and pilocarpine-induced SE. Interestingly, despite having decreased BBB permeability, VWF−/− mice were not protected and even fared worse in both of these models.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**VWF Is Released by H/R**

It has been reported that hypoxia induces WPB secretion. To determine the state of endothelial activation in our model of H/R, we analyzed WT mice for plasma VWF antigen levels. Mice that were exposed to 24 hours of hypoxia or 24 hours of hypoxia plus 1 hour of reoxygenation showed increased VWF antigen levels compared with normoxic controls (normoxia: 99.94±5.73 versus hypoxia: 128.2±12.37 P=0.03; normoxia versus H/R: 146.2±9.16; P<0.001; n=6–11 per group; Figure 1A). It is possible that the prolonged period of hypoxia may have enhanced VWF synthesis in addition to its known effect on WPB secretion.

![Figure 1](http://atvb.ahajournals.org/)

**VWF−/− Animals Have Less BBB Permeability Than WT After H/R**

Cerebral hemispheres of WT and VWF−/− animals were processed to determine the amount of Evan’s blue dye bound to albumin that extravasated into the brain after 24 hours of 6% oxygen followed by 1 hour of reoxygenation (H/R). Two-way ANOVA indicated a statistically significant difference between genotype (P<0.001) and condition (normoxia or HR; P<0.001; n=6–9). Student t test revealed that there was significantly less BBB permeability in VWF−/− mice compared with WT controls after H/R (WT: 0.140±0.01; VWF−/−: 0.101±0.001; P<0.001; n=8 per group; Figure 1B). It was previously reported that VWF−/− mice have a defect in P-selectin translocation to the surface of the endothelium. To determine whether this defect, which could result in reduced early leukocyte recruitment, played a role in the tighter BBB in this model, we inhibited P-selectin in WT mice using a P-selectin aptamer that has been shown to inhibit P-selectin–mediated leukocyte rolling completely (Figure 1A in the online-only Data Supplement). Two-way ANOVA indicated a statistically significant difference between condition (normoxia or HR; P<0.001), but not aptamer treatment (n=4–6). Student t test also revealed that there was no difference between control aptamer–treated mice (WT+control aptamer: 0.184±0.007...
versus WT+P-selectin aptamer: 0.194±0.019; \( P=0.611 \); n=5–6 per group). Thus, we concluded that the tighter BBB in this model paradigm was not because of P-selectin defects in VWF−/− mice.

VWF−/− Mice Are Not Protected From H/R

The glial-specific S100B leakage into plasma has been previously reported to be an indicator of brain damage.34 We used Western blotting to assess the extent of S100B leakage into the plasma in WT mice and VWF−/− mice exposed to H/R (Figure 2A). Our results indicated that significantly more S100B protein was released into the plasma in VWF-deficient mice (\( P<0.01 \); n=3–5 per group; Figure 2B). Degradation of neurofilament proteins has been reported to occur as a result of ischemia.35,36 Interestingly, although VWF-deficient animals had much less BBB permeability than WT mice, we did not see protection of neuronal integrity after H/R as assessed by Western blot analysis of neurofilament-70 (NF-70) in VWF−/− mice (Figure 2C). Immunostaining showed that under normoxic conditions there was no obvious difference between WT normoxic and WT H/R (\( P=0.224 \); n=4 per group; Figure 2D), whereas we did not find a significant difference between WT normoxic and WT H/R (\( P=0.0224 \); n=5 per group; Figure 2C). Immunostaining showed that under normoxic conditions there was no obvious difference in NF-70 immunostaining in the hippocampal region (stratum lacunosum moleculare) of the brain of WT and VWF−/− mice, whereas exposure to H/R led to a decrease in staining in both WT and VWF−/− mice in this area of the hippocampus (Figure 2).

VWF Is Released by Seizures

We next sought to determine whether the tightness of BBB in VWF−/− mice exposed to H/R could be observed in a separate BBB permeability model. As increased BBB permeability has been proposed to promote the development of SE during pilocarpine-induced seizures,1 we, therefore, subjected WT and VWF−/− mice to this model. Plasma VWF levels were significantly increased only in mice that had experienced ≥1 seizure, whereas in mice that did not develop major seizures, VWF plasma content remained similar to control level (no pilo: 94.78±6.536 versus pilo no seizures 115.0±5.887; \( P=0.06 \); +pilo no seizures versus +pilo with seizures 168.7±13.56; \( P<0.001 \); n=6–15 per group; Figure 3A). Endothelial activation seems to be a result of seizure activity and not a side effect of scopolamine or pilocarpine administration, although there was a strong trend toward an increase in VWF levels with

Figure 3. von Willebrand factor (VWF) is released in wild-type (WT) mice after seizures, and VWF-deficient animals have less blood–brain barrier (BBB) permeability than WT during status epilepticus. A, Endothelial activation was assessed by comparison of plasma VWF levels, in WT mice pretreated with scopolamine, after PBS or pilocarpine administration with and without seizures (sz). B, BBB permeability to Evans blue dye in WT and VWF−/− mice pretreated with scopolamine that received either pilocarpine to induce status epilepticus or PBS as a control. Two-way ANOVA analysis revealed a significant difference between genotype (\( P<0.05 \)) and treatment (\( P<0.001 \)). Student t test was also used to compare only between genotypes. *\( P<0.05 \); **\( P<0.001 \). NMP indicates normal mouse plasma.
pilocarpine administration. All pilocarpine-administered animals used for these studies developed early behavioral signs of SE, such as mouth and facial movements and tail stiffness. These less severe behavioral signs may also lead to low-level endothelial activation.

**VWF−/− Mice Have Decreased BBB Permeability During SE Compared With WT**

To investigate changes in BBB permeability, we intravenously injected Evan’s blue dye 30 minutes before pilocarpine administration (Figure 3B). Two-way ANOVA indicated a statistically significant difference between genotype (P<0.05) and treatment (P<0.001; n=4–6). Student t test revealed that BBB permeability was significantly decreased in VWF−/− mice that had seizures compared with WT mice that had seizures (WT+pilo: 0.207±0.013 versus VWF−/+pilo: 0.175±0.005; P<0.05), whereas there was no difference between control WT and VWF−/−. In this experimental group, all VWF-deficient animals had ≥1 seizure, and all died within 120 minutes after pilocarpine administration (data not shown).

**Deficiency of Endothelial VWF Increases Susceptibility to Seizures and SE-Induced Mortality**

Consistent with previous literature, ≥20% of WT mice did not survive SE.8 Surprisingly, ≥100% of VWF−/− mice died within 120 minutes after pilocarpine administration. Mice heterozygous for VWF, which have 50% of WT VWF levels, also had a 100% mortality rate (Figure 4A). There was a trend toward a decrease in the time to first seizure after pilocarpine administration in VWF−/− and VWF+/− animals compared with WT (Figure 4B). All VWF−/− and VWF+/− experienced ≥1 seizure compared with ≥50% in the WT mice, suggesting that mice with decreased VWF levels are more susceptible to pilocarpine-induced seizures and seizure-induced mortality (WT versus VWF−/−, WT versus VWF−/+; P<0.001; Figure 4C). Platelets can interact with activated endothelium through VWF–glycoprotein Ibα binding,38 thus we hypothesized that decreased platelet recruitment to the endothelium during SE might play a role in mortality in VWF−/− mice. To test this hypothesis, we depleted platelets in WT mice. We found no difference in survival time (Figure 4D), time to first seizure (Figure 4E), or seizure susceptibility (Figure 4F) between platelet-depleted and nondepleted mice, indicating that the protective role of VWF in this model is independent of platelet VWF and VWF–platelet interactions. On endothelial stimulation, VWF is released to the endothelial surface and cleaved to circulate in the plasma. To determine whether plasma VWF was protective in this model, we intravenously administered recombinant murine VWF (rmVWF) to VWF−/− mice at the time of pilocarpine administration and again 30 minutes later, which resulted in ≥400% of normal VWF plasma levels (Figure 4J). Infusion of rmVWF did not affect survival time (Figure 4G), time to first seizure (Figure 4H), or seizure susceptibility (Figure 4I) in VWF−/− mice. These data indicate that plasma VWF is not protective against SE-induced mortality.

The role of glutamate and N-methyl-D-aspartate receptors is well established in epilepsy, and it has been reported in both mice and humans that N-methyl-D-aspartate receptor antagonism using low dosing of ketamine is protective.39-41 To ensure that mortality in VWF−/− mice was the result of seizure activity, we used ketamine to determine whether VWF−/− mice could be rescued during SE. We found that 100% of WT and VWF−/− mice treated with ketamine 20 minutes after pilocarpine survived SE (Figure 4A). These data further indicate that mortality in VWF−/− mice is because of seizure activity and is most likely glutamate dependent.

**VWF Plays an Inhibitory Role in Claudin-5 Expression**

To determine the state of cerebral endothelial tight junction proteins in WT and VWF−/− and VWF+/− mice, we used Western blot analysis of isolated cerebral microvessels. Interestingly, we found a significant increase in claudin-5 expression under baseline conditions in VWF−/− compared with WT (P<0.001; n=5 per group; Figure 5A and 5B). We found no difference in the expression of occludin (Figure 5A and 5C). Using a different method of extracting proteins from a separate microvessel preparation from WT, VWF−/−, and VWF+/−, we again found a significant increase in claudin-5 expression in VWF−/− mice compared with WT (P<0.05; n=3–4 per group), a strong trend toward an increase in expression between WT and VWF−/+ (P=0.09; n=3–4 per group), and no difference between VWF−/− mice and VWF−/+ (Figure IIIF in the online-only Data Supplement). Quantification is representative of the average values obtained from 2 separate blots probed for claudin-5 and GAPDH. No differences were found among WT, VWF−/−, and VWF−/+ in the levels of occludin expression (Figure IIIG in the online-only Data Supplement) in these preparations. On comparison of microvessels isolated from normoxic and H/R–exposed VWF−/− mice, we observed that protein levels of claudin-5 persisted (Figure IIE in the online-only Data Supplement; P=0.69; n=4–5 per group). As vascular endothelial-cadherin has been shown to play a role in the regulation of claudin-5 expression,42 we also examined levels of this protein in the samples. We did not find any difference of vascular endothelial-cadherin protein levels among WT, VWF−/−, and VWF+/− (WT versus VWF−/−; P=0.692; WT versus VWF−/+; P=0.278; n=3 per group; Figure IIIF in the online-only Data Supplement). We next used in vitro culture techniques to determine whether rmVWF treatment of plates (to mimic basement membrane VWF) would affect claudin-5 expression in VWF−/− brain endothelial cells (BECs). VWF−/− BECs were grown on glass plates pretreated with collagen IV (Figure 5D), collagen IV+rmVWF (Figure 5E), rmVWF (Figure 5F), or PBS (Figure 5G). Contrary to collagen IV and collagen IV+rmVWF, which grew to confluence in the same time frame, VWF−/− BECs did not grow to confluence on rmVWF, and there were very few adherent cells on glass alone. Collagen IV− and collagen IV+rmVWF–treated plates were kept for 2 days past confluency before being processed for immunostaining and Western blot analysis. Immunostaining for claudin-5 and platelet endothelial cell adhesion molecule-1 revealed that junctional staining of claudin-5 was more intense in the cultures grown on collagen IV alone (Figure 5H and 5J) compared with cultures grown on collagen IV+rmVWF (Figure 5I and 5K). We next determined protein levels of claudin-5 and occludin in the cultures treated
with collagen IV and collagen IV+rmVWF and found that there was significantly less claudin-5 in cultures grown in the presence of rmVWF (Figure 5L; \( P = 0.025; n=5 \) per group). Culture experiments were repeated, and claudin-5 levels were found again to be significantly lower (col IV: 1.171±0.130 versus col IV+rmVWF: 0.690±0.054; \( P = 0.026; n=3 \) per group). We found no difference in occludin protein levels in either experiment (Figure 5M; \( P = 0.45; n=5 \) per group; col IV: 0.582±0.131 versus col IV+rmVWF: 0.394±0.079; \( P = 0.29; n=3 \) per group).

To evaluate claudin-5 expression within vessels, we labeled blood vessel preparations with fluorescein lycopersicon esculentum (tomato) lectin and immunostained for claudin-5 (Figure IIH–IIJ in the online-only Data Supplement). Claudin-5 distribution was similar among the genotypes. Although nonquantitative, the staining was stronger in VWF−/− and VWF+/− vessels.

To determine whether increased claudin-5 expression leads to overt abnormal arrangement of tight junction proteins in cerebral microvessels of VWF−/− mice, we immunostained for claudin-5 and another tight junction protein, zona occluden-1, in microvessel smears (Figure IIK–IIM in the online-only Data Supplement).

Figure 4. Deficiency of endothelial von Willebrand factor (VWF) is pathological during status epilepticus. Wild-type (WT), VWF−/−, VWF+/−, WT+ketamine, and VWF−/−+ketamine survival time (A), time to first seizure (B), and percentage of mice that had ≥1 seizure (C). Normal WT and platelet-depleted WT mice survival time (D), time to first seizure (E), and percentage of mice that had ≥1 seizure (F). VWF−/− mice treated with recombinant murine VWF (rmVWF) survival time (G), time to first seizure (H), percentage of mice that had ≥1 seizure (I), and plasma VWF levels in VWF−/− mice reconstituted with rmVWF (J). **\( P < 0.01 \), ***\( P < 0.001 \).
We found that claudin-5 staining was similar in pattern to zona occluden-1 in VWF−/− mice because no gross abnormalities were found. Confirmation of VWF deficiency in the endothelial cells is shown with immunostaining for VWF expression in VWF−/− mice at baseline and a tighter BBB than WT during the disease process were not protective. Inhibition of VWF decreases neutrophil extravasation and permeability in peripheral tissues. Furthermore, a recent study has suggested that VWF plays a regulatory role in cerebrovascular permeability because intraventricular administration of VWF leads to increased BBB permeability as assessed by Evans blue dye leakage. These previous observations and those made here in our work on BBB permeability are in contrast to a report showing increased BBB permeability in VWF−/− mice as a result of immunization with complete Freund’s adjuvant and pertussis toxin. We propose that the mechanism of BBB opening that occurs as a result of immunization could be different than that occurring in the models used in our work, resulting in opposite phenotypes. Previously, it has been reported that hypoxia alone can induce WPB release from endothelial cells. Our results confirm these reports and show that VWF levels remain increased after 1 hour of reoxygenation. New in this study is the finding that seizures activate the endothelium and lead to VWF secretion. One possible mechanism behind this increase is that a WPB secretagogue, such as epinephrine, may be released during seizures. From our results, it seems likely that the VWF released to the basement membrane during seizures has a protective effect for the organism. It is known that VWF is pivotal for the formation of WPBs in the endothelium. Mice deficient in VWF do not form WPBs, and therefore storage of P-selectin in these mice is impaired.
which could potentially play a role in the phenotypes we are reporting.32 We did not see a difference in BBB permeability between control aptamer–treated and inhibitory P-selectin aptamer–treated WT mice, indicating that P-selectin does not play a major role in BBB permeability in our model of H/R. Also, mortality in the SE model in mice treated with the P-selectin inhibiting aptamer was similar to that of control-treated animals. Another component of WPBs that is associated with BBB permeability is angiopoietin-2.52 Because of the lack of WPB formation in VWF−/− mice, it is plausible to speculate that decreased BBB permeability in these models is caused by lack of angiopoietin-2 secretion. We do not think that this is the case for 2 reasons. First, it was recently reported that endothelial cells in which VWF is silenced actually secrete more angiopoietin-2 into circulation, possibly because of the lack of storage in WPB.53 Second, VWF−/− mice have normal WPB formation and demonstrated the same phenotype as VWF null mice during SE. Furthermore, the in vitro effect of recombinant VWF on claudin-5 expression in VWF-deficient endothelial cells also argues against an important role for another WPB component in the claudin-5 regulation process.

We initially anticipated that decreased BBB permeability after H/R would have a protective effect on the brain. Instead, we found increased S100B protein in plasma after H/R in VWF−/− mice. S100B is a Ca2+-binding protein that is primarily expressed by astrocytes.53 Detection of S100B in circulation has been used to reflect brain damage after traumatic brain injury.34,54 It has also been suggested that S100B levels in serum may be used to assess BBB permeability.55 Our finding that claudin-5 is also localized to the junction in VWF−/− mice. The role of basement membrane proteins in tight junction protein expression is to date underexplored. Importantly, Osada et al43 have recently reported that the interaction between β1-integrins on endothelial cells and collagen IV in the extracellular matrix plays a role in claudin-5 expression. When this interaction was inhibited, claudin-5 expression was decreased, and BBB permeability to 40 and 150 kDa dextran, as well as IgG, was increased. VWF is a component of the extracellular matrix, and, on endothelial activation, VWF is largely secreted to the basement membrane matrix. We hypothesized that subendothelial VWF may interfere with interactions of collagen IV and endothelial cells, leading to less expression of claudin-5. Indeed, when we cultured VWF−/− BECs on plates treated with collagen IV or collagen IV+rmVWF, we found significantly less claudin-5 protein when VWF was present.

A tighter BBB in VWF−/− mice also did not confer protection in the pilocarpine model of SE. Peripheral inflammation has been proposed to play a role in BBB permeability and seizure activity in this model.1,3 However, whether seizures cause peripheral inflammation or the opposite is still heavily debated.41 We have ruled out the potential role for plasma VWF and platelets in protecting mice from mortality associated with VWF deficiency. In light of these data, we propose that it is endothelial, likely subendothelial, VWF that plays a role in decreased permeability in this model. It is known that BBB regulates glutamate transport between the plasma and brain and that epileptics have an increase in brain glutamate.43,58 It is evident from our ketamine studies that antagonism of N-methyl-D-aspartate receptors is protective from seizures and mortality in these VWF−/− mice during SE, suggesting that seizure activity is indeed the cause of pathology in VWF−/− animals. Our observation that VWF−/− mice have a tighter BBB and increased baseline levels of claudin-5 led us to propose that their glutamate-dependent mortality in the SE model is because of cerebrovascular dysfunction. Potentially toxic substances produced by the brain during seizure activity, such as glutamate, may be trapped in the brain parenchyma of VWF−/− mice, leading to increased excitation, seizures, and death. Interestingly, hypoxia also leads to excess glutamate release in the brain, and N-methyl-D-aspartate receptor antagonism has been shown to reduce ischemia-induced neuronal cell death.59,60 An interesting possibility is that hypoxia contributes to BBB permeability and pathology in mice during seizures. It has been postulated that hypoxia occurs during SE in a rat model and has been shown in a clinical study that oxygen desaturation occurs during seizures.61,62

The finding that claudin-5 protein is increased at baseline in VWF−/− animals is intriguing and may explain the lack of BBB flexibility in both models because changes in claudin-5 levels have been shown to alter BBB permeability.41,63,64 The claudin-5 staining is similar to that of zona occludens-1, suggesting that claudin-5 is also localized to the junction in VWF−/− mice. Also, another WPB component in the claudin-5 regulation process.

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Role of VWF in Blood–Brain Barrier Regulation

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Disclosures

None.

References

The role of von Willebrand factor (VWF) in hemostasis is well established, but little is known about its effects on endothelial biology. VWF is abundantly expressed at the blood–brain barrier (BBB). Here, we report that VWF-deficient mice have a tighter BBB than wild-type mice when subjected to hypoxia/reoxygenation and status epilepticus, 2 models in which increased permeability is thought to be implicated in the disease process. Surprisingly, the partial preservation of BBB integrity in the VWF-deficient mice did not confer protection in either of these models and was detrimental to the animal. We found that VWF influences expression of tight junction protein claudin-5, important in BBB formation transiently increases the permeability of brain capillary endothelial cells through translocation of claudin-5. Neurochem Res 2013;38:1641–1647.


Significance

The role of von Willebrand factor (VWF) in hemostasis is well established, but little is known about its effects on endothelial biology. VWF is abundantly expressed at the blood–brain barrier (BBB). Here, we report that VWF-deficient mice have a tighter BBB than wild-type mice when subjected to hypoxia/reoxygenation and status epilepticus, 2 models in which increased permeability is thought to be implicated in the disease process. Surprisingly, the partial preservation of BBB integrity in the VWF-deficient mice did not confer protection in either of these models and was detrimental to the animal. We found that VWF influences expression of tight junction protein claudin-5, important in BBB function. Our study suggests that subendothelial VWF regulates BBB permeability via claudin-5 downregulation and that BBB opening can be protective under stressful conditions. Thus, tightening of BBB may not always be a desirable therapeutic approach.
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Supplemental Figure I- BBB permeability assessed by EBD leakage into the brain in control aptamer-treated and P-selectin aptamer-treated WT mice exposed to normoxia or hypoxia/reoxygenation (A). Leukocyte rolling in mesenteric venules after calcium ionophore stimulation at baseline (B), with administration of control aptamer (C) and P-selectin aptamer (D). Survival times of WT mice administered with either P-selectin or control aptamer and subjected to pilocarpine model of status epilepticus (E).
Supplemental Figure II-Immunostaining for neurofilament-70 with Hoechst counterstain to label DNA in WT (A) and VWF−/− (B) under normoxic conditions and WT (C) and VWF−/− (D) mice after H/R. (DG=Dentate Gyrus; SLM= stratum lacunosum moleculare;). Scale bar = 50µm. NF-70 images were taken in the same region in every condition and acquired using identical exposure time and microscope settings. Claudin-5 levels persisted in microvessels isolated from normoxic and H/R exposed VWF−/− mice (E). Western blot and quantification of claudin-5 (F), occludin (G) normalized to GAPDH in a second group of WT, VWF+−/−, and VWF−/− mice. Immunostaining for claudin-5 and labeling for blood vessel marker lectin (H, I, J), immunostaining for tight junction proteins ZO-1 and claudin-5 (K, L, M) and immunostaining for VWF and PECAM-1 (N, O, P). Western blot quantification of VE-Cadherin (Q) normalized to GAPDH in WT, VWF+−/−, and VWF−/−. Scale bar = 10µm. * P<0.05
Materials and Methods

Animals
All animal experiments were approved by the Animal Care and Use Committee of the Immune Disease Institute and Boston Children’s Hospital. WT (C57BL/6J) were purchased from the Jackson Laboratory (Bar Harbor, ME). VWF−/− and VWF+/+ mice used for these studies were on C57BL/6J background and bred in-house under standard pathogen free conditions. VWF−/− and VWF+/+ mice were extensively backcrossed on the C57BL/6J background and continue to be routinely crossed to C57BL/6J mice from the Jackson Laboratory to avoid genetic drift. VWF and Claudin-5 genes are located on separate chromosomes with VWF being on chromosome 12 and Claudin-5 on chromosome 22, thus deletion of VWF was not likely to affect claudin-5 regulation directly.

Hypoxia/Reoxygenation model
Female mice, 6-8 weeks of age, were exposed to normobaric hypoxia at 6.0 ± 0.2% oxygen for 24 hours in a controlled atmosphere animal chamber (A-15274-P, Biospherix, Lacona, NY). Hypoxia was achieved by substituting nitrogen for oxygen using a Pro:ox model 110 compact oxygen controller (Biospherix). After hypoxic exposure, mice were reoxygenated in normal room air for one hour. Control (normoxic) animals were housed in normal air room. Mice had access to dry food, gel and water ad libitum. For plasma collection, animals were bled via the retro-orbital sinus into EDTA-containing tubes. Plasma was stored at -80°C.

Pilocarpine model of status epilepticus and observations
Male mice, 6-8 weeks of age (weighing 18-25 g) were administered scopolamine (10 mg/kg; Sigma, St. Louis, MO) via i.p. injection. Thirty minutes later, mice were administered pilocarpine hydrochloride (300 mg/kg; Sigma) via i.p. injection. Mice were monitored for 120 minutes during which they were assessed for Racine scale behavioral signs of SE such as mouth and facial movements, tail stiffness, salivation, rearing, whole body tremors, rearing and falling and loss of postural control. One hundred and twenty minutes after pilocarpine administration, all surviving mice were euthanized by isoflurane overdose. Only animals exhibiting signs of SE in accordance with the Racine scale were included in these studies. Times were recorded for seizure activity that involved rearing and falling (termed "seizures") and for survival up to 120 minutes after pilocarpine administration. For N-methyl-D-Aspartate (NMDA) receptor antagonism experiments, ketamine (50 mg/kg) was administered i.p. 20 minutes after pilocarpine injection. For plasma collection, animals were bled via the retro-orbital sinus into EDTA-containing tubes at the times indicated. Plasma was stored at -80°C.

Determination of VWF antigen
Overnight, microtiter plates (96-well; Nunc, Thermo Fisher Scientific, Rochester, NY) were coated with 100 µL/well of polyclonal anti-VWF solution at 1:1000 in phosphate-buffered saline (PBS) buffer (Dako, Carpinteria, CA) at 4°C. Plates were blocked with 250 µL/well 3% milk powder in PBS for 2 hours at room temperature. Plasma samples were diluted in 0.3% milk powder with PBS in increments ranging from 1/10 to 1/640 dilutions. Samples were incubated at 37°C for 90 minutes. Samples then incubated
with 100 µL/well of anti-VWF-HRP at 1:3000 in 0.3% milk powder in PBS for 1 hour at room temperature. Adhering VWF was detected using tetramethylbenzidine substrate (Sigma) with 0.5 M H₂SO₄ to stop the reaction. Absorbance was acquired at 450 nm on a plate reader. Following each incubation period, plates were washed in PBS containing 0.1% Tween-20 (Sigma). The reference standard for VWF levels was determined using pooled plasma from 20 WT mice.

### BBB assay

The collection of brains in this study is highly time-sensitive therefore the brains could not be perfused. This is acceptable because the level of Evan’s blue dye (EBD) in the normal brains reflects brain volume and is the same in the VWF⁻/⁻ and WT mice. Furthermore, the architecture of their brain vasculature is also highly similar.⁵,⁶ To determine BBB permeability in the SE model, 2% EBD (Sigma) made in PBS was administered to the mice at the time of scopolamine administration via i.v. injection into the retro-orbital venous sinus. EBD binds albumin and cannot cross an intact blood-brain barrier. Brains were collected immediately after SE-induced death or 120 minutes after pilocarpine administration and frozen on dry ice and stored at -20°C until processing. To determine BBB permeability in the hypoxia model, mice were injected with EBD immediately after hypoxia and brains were collected after one hour of reoxygenation. To quantify extravasation of EBD bound to albumin into the brain, cerebral hemispheres were first weighed and then incubated in 50% Trichloroacetic acid (TCA) made in PBS in a 3:1 µL/mg ratio for 30 minutes at room temperature. Brains were then sonicated for 15 pulses and centrifuged at 10,000 rpm for 30 minutes. Supernatant (100 µL for H/R, 200 µL for SE) was plated on a 96-well plate and absorbance measured at 630 nm.

### Administration of recombinant murine VWF (rmVWF) into VWF-deficient mice

rmVWF (kind gift from Archemix, Cambridge, MA) or PBS was administered i.v. via the right retro-orbital plexus at the time of pilocarpine administration and again in 30 minutes. This allowed for maintenance of high VWF plasma levels (about 400% of normal values) in VWF⁻/⁻ mice during up to 60 minutes after infusion. Due to the estimated short half-life of rmVWF in circulation, we only monitored the animals for 60 minutes and sacrificed any animal that was still surviving after 60 minutes. We found this to be an acceptable time point as the majority of VWF⁻/⁻ and VWF⁺/⁻ animals died within 60 minutes in prior experiments. Blood was taken via retro-orbital bleeding from the left eye immediately after death or 60 minutes after pilocarpine administration to determine VWF antigen levels in the plasma.

### Induction of thrombocytopenia

Thirty WT male mice were administered via i.v. injection with either anti-GPIbα (2.5 µg/g; Emfret, Eibelstadt, Germany) to deplete platelets or rat IgG (Emfret) as a control. Thrombocytopenia was verified by flow cytometry using anti-GPIX conjugated to FITC (BD Biosciences, San Jose, CA). Platelets were depleted to less than 5%.

### Microvessel isolation

Microvessel isolation was adapted from previously described methods.⁷ Briefly, brain hemispheres were separated and homogenized in microvessel isolation buffer, 26%
dextran (TCI America, Portland, OR) was added and samples were vortexed and centrifuged for 10 minutes at 5600 x g at 4°C (Avanti J-25 centrifuge, Beckman Coulter, Indianapolis, IN). Supernatants were discarded and the pellet was resuspended in microvessel isolation buffer and passed through a 70 µm filter. Samples were centrifuged at 5000 x g for 10 minutes at 4°C. Pellets used for western blotting were resuspended in CelLytic M Lysis Reagent (Sigma C2978) (for Figure 5 A, B, C and Supplemental Figure II E) or protein was extracted using the TRIzol method of protein extraction per the manufacturer's protocol (for Supplemental Figure II F, G) and frozen at -80°C. Both methods included protease and phosphatase inhibitors. For immunostaining, microvessel pellets were smeared onto slides and stored at -20°C.

**Brain endothelial cell (BEC) culture**

Twenty-four hours prior to BEC preparation, glass slides were pre-treated with either: rmVWF (40 µg/mL), collagen Type IV from Engelbreth-Holm-Swarm murine sarcoma basement membrane (66 µg/mL; Sigma C0543), collagen IV + rmVWF or nothing for 2 hours at 37°C and then stored overnight at 4°C. Plates were thoroughly washed and allowed to dry before cells were seeded. BEC isolation was adapted from a previously described method. Briefly, brains were harvested from 6-8 week old male and female VWF−/− mice, minced in dissociation buffer (20 U/mL Papain, 250 U/mL DNase I type IV, MEM-HEPES) and incubated for 1 hour at 37°C. After incubation, brains were vigorously pipetted until all tissue was dissociated. Dissociated tissue was added to 22% BSA, inverted twice to mix and centrifuged at 1000 x g for 20 minutes at 4°C. The supernatant was discarded and the pellet washed in MEM-HEPES. Viable cells were counted on a hemacytometer. BECs were resuspended in Endothelial Cell Growth Medium (EGCM) (10% FBS (Life Technologies, Grand Island, NY), 1 µg/mL ascorbic acid (Sigma), 1X Endothelial growth supplement containing porcine heparin (Sigma) and plated. Cultures were incubated at 37°C in 5% CO₂ overnight. The next day media was removed and EGCM containing puromycin (3 µg/mL; Sigma) was added. Media was changed every 1.5 days thereafter. Collagen IV- and collagen IV + rmVWF –treated slides grew to confluence in the same time frame. rmVWF- and non-coated slides did not reach confluence but were grown for the same amount of time as other treatments. After two days of confluence, cells for western blot were removed from culture plates with trypsin-EDTA which was then neutralized with FBS-containing media. Cells were spun down and resuspended in CelLytic M lysis reagent (Sigma) containing protease and phosphatase inhibitor cocktails.

**Immunostaining**

Brains were extracted from normoxic and H/R mice. Brains were fresh frozen on dry ice and stored at -20°C until sectioning. Ten µm sections were cut using a cryostat and heat-mounted to slides, then fixed in 95% ethanol and washed in PBS. Next, sections were incubated in blocking buffer (10% normal goat serum with 0.3% triton X-100 in PBS) for 60 minutes and then incubated overnight at 4°C in rabbit anti-neurofilament-70 at 1:400 made in blocking buffer (Millipore, Billerica, MA). The next day, sections were incubated in Alexa anti-Rabbit 488 at 1:500 (Invitrogen, Grand Island, NY) for 60 minutes at room temperature. Sections were then washed in PBS and mounted with
Fluorogel containing Hoechst stain at 1:1000 (Hoechst 33342, Invitrogen) to visualize nuclei. For claudin-5, zona occludens-1 (ZO-1), blood vessel carbohydrate marker, VWF and PECAM-1 visualization, microvessel smears were fixed in 95% ethanol for 10 minutes and washed with PBS. Next, smears were incubated in 5% normal goat serum made in PBS with 1% bovine serum albumin and 0.2% Tween-20 for 30 minutes. Smears were incubated overnight at 4°C in rabbit anti-claudin-5 (1:400; Invitrogen), mouse anti-ZO-1 (1:400; Invitrogen), fluorescein lycopersicon esculentum (tomato) lectin (blood vessel marker) (1:200; Vector, Burlingame, CA), rabbit anti-VWF (1:200; Dako) or rat anti-PECAM-1 (1:100; BD Biosciences) made in blocking buffer. The next day, smears were washed in 1% bovine serum albumin and 0.2% Tween-20 made in PBS and incubated for 60 minutes at room temperature in Alexa anti-rabbit 488, Alexa anti-mouse 488, Alexa anti-rabbit 555 or Alexa anti-rat 555 at 1:500 (Invitrogen). For immunostaining of BECs, cultures were fixed in 2% paraformaldehyde (PFA) for 1 hour at room temperature, permeabilized (0.1% Triton X-100, 0.1% sodium citrate) and blocked with 3% BSA. Cultures were incubated in rabbit anti-claudin-5 (1:400) and rat anti-PECAM-1 (1:500) for 2 hours at 37°C followed by the appropriate Alexa secondary antibody at 1:500. Claudin-5 staining appeared at the cell membrane (junction) as well as in the cytosol as has been reported under normal conditions in endothelial cultures fixed in 2% PFA with use of this claudin-5 antibody. Slides were washed in PBS and mounted with Fluorogel. Images were taken with a Zeiss epifluorescent Axiovert wide field microscope.

**Western blot analysis**

Hippocampi were rapidly dissected and immediately flash frozen on dry ice after exposure to normoxia or H/R. Protein concentration of hippocampi or microvessel isolations was determined using a BSA standard curve. Microvessels were normalized and either 15 µg (for Figure 5 A) or 7 µg (for Supplemental Figure II F, G) were loaded per well. Hippocampi homogenates, microvessel lysates and BEC lysates were added to Laemmli buffer (Bio-rad, Hercules, CA) containing 2-mercaptoethanol (BME) and boiled for 5 minutes. Samples were run on 4-20% SDS-PAGE gels and transferred onto Immobilon PVDF membranes. For tight junction analysis, blots were blocked in 5% milk and probed using mouse anti-GAPDH at 1:1000 (Fitzgerald Industries International, Concord, MA), rabbit anti-occludin at 1:400 (Invitrogen) or rabbit anti-claudin-5 at 1:400 (Invitrogen). Goat anti-rabbit conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-mouse conjugated to horseradish peroxidase (Invitrogen) was used to detect primary antibody at 1:10,000. For microvessel lysates, blot was cut and probed for claudin-5, GAPDH and Occludin. For BEC lysates, blot was cut and probed for claudin-5 and GAPDH and then stripped with 0.2N NaOH and reprobed for occludin. For neurofilament-70 detection in hippocampi, 30 µg of protein were loaded onto 4-20% SDS-PAGE gels and transferred to Immobilon PVDF membranes. Blots were blocked in 10% milk and probed using anti-neurofilament-70 at 1:1000 (Millipore) or anti-GAPDH. For S100B analysis, plasma in Laemmli buffer containing BME was boiled and loaded onto 10% SDS-PAGE gels and transferred onto PVDF membranes. The blot was blocked in 10% milk and incubated overnight at 4°C in mouse anti-S100B at 1:1000 (BD Biosciences). Ponceau S staining was utilized as a loading control for plasma. The membrane was incubated in Ponceau S staining solution (0.1% Ponceau S in 5% acetic acid) (Sigma) for 30 minutes at room
temperature. Background staining was removed by washing the membrane in deionized water. Western blots and Ponceau S staining were quantified using Image J64 software (NIH, Bethesda, MD). For all quantification of western blots, values for the protein of interest were divided by the value of either Ponceau S (loading control for plasma) or GAPDH (loading control for homogenates). Data are represented as the mean ± SEM.

**Inhibition of P-selectin (supplemental data)**

P-selectin was inhibited in WT animals using ARC 3690, a 30-mer oligonucleotide P-selectin “aptamer.” A “scrambled” version of the aptamer termed ARC 3694 was used as a control. Both aptamers were injected i.v. via the retro-orbital plexus immediately prior to experimentation at 1 mg/kg (generous gift from Dr. Robert Schaub, Archemix, Cambridge, MA)\(^{10}\). We verified the efficacy and duration of P-selectin inhibition by ARC 3690 by assessing leukocyte rolling after 24 hours of 6% oxygen (hypoxia) on calcium ionophore A23187-stimulated mesenteric venules using previously described methods (Supplemental Figure I B, C, D).\(^{11}\) We determined that inhibition of P-selectin occurs immediately following i.v. administration of ARC 3690 and lasts for at least 25 hours, whereas ARC 3694 did not affect leukocyte rolling.

**Statistical Analysis**

Chi-squared test was used to determine significance for survival and seizure susceptibility studies in the pilocarpine model of SE. Two-way ANOVA analysis was performed to determine statistical significance with respect to genotype and condition for the BBB assays followed by unpaired student’s t-test. Unpaired student’s t-test was performed for all other statistical evaluations. All statistics were performed using Prism 4 software (Graphpad Software, Inc. San Diego, CA). Significance is indicated as follows: * P<0.05, ** P<0.01, *** P<0.001.

**References**


