The Mechanisms of Cerebral Vascular Dysfunction and Neuroinflammation by MMP-Mediated Degradation of VEGFR-2 in Alcohol Ingestion

P. M. Abdul Muneer, Saleena Alikunju, Adam M. Szlachetka, James Haorah

Objective—Blood-brain barrier (BBB) dysfunction caused by activation of matrix metalloproteinases (MMPs) is a pathological feature in vascular/neurological disease. We describe the mechanisms of BBB dysfunction and neuroinflammation as a result of MMP-3/9 activation and disruption of vascular endothelial growth factor (VEGF)-A/VEGFR-2 interaction, impairing effective angiogenesis.

Methods and Results—We investigate the hypothesis in human brain endothelial cells and animal model of chronic alcohol ingestion. Proteome array analysis, zymography, immunofluorescence, and Western blotting techniques detected the activation, expression, and levels of MMP-3 and MMP-9. We found that degradation of VEGFR-2 and BBB proteins, for example, occludin, claudin-5, and ZO-1 by MMP-3/9, causes rupture of capillary endothelium and BBB leakiness. Impairment of BBB integrity was demonstrated by increased permeability of dye tracers and Fluo-3/calcin-AM–labeled monocyte adhesion or infiltration and decrease in transendothelial electric resistance. Alcohol-induced degradation of endothelial VEGFR-2 by MMP-3/9 led to a subsequent elevation of cellular/serum VEGF-A level. The decrease in VEGF-A with subsequent increase in VEGF-A level led to apoptosis and neuroinflammation via the activation of caspase-1 and IL-1β release. The use of MMPs, VEGFR-2, and caspase-1 inhibitors helped to dissect the underlying mechanisms.

Conclusion—Alcohol-induced MMPs activation is a key mechanism for dysfunction of BBB via degradation of VEGFR-2 protein and activation of caspase-1 or IL-1β release. Targeting VEGF-induced MMP-3/9 activation can be a novel preventive approach to vascular inflammatory disease in alcohol abuse. (Arterioscler Thromb Vasc Biol. 2012;32:1167-1177.)

Key Words: alcohol • blood-brain barrier • matrix metalloproteinase • vascular endothelial growth factor • vascular endothelial growth factor-R2 • vascular inflammation

The blood-brain barrier (BBB) is consisted of highly specialized intercellular tight junction (TJ) proteins that regulate the trafficking of ions and molecules into the brain from blood circulation.¹ The TJ proteins such as occludin, claudins, and zona occludens (ZO-1–3) connect the brain endothelial cells to form a tight monolayer for ensuring structural integrity and selective permeability across the BBB.² Occludin and claudins act as sieve proteins that are linked to actin cytoskeleton via the intracellular anchoring protein ZO3.³ Disruption of BBB function/structure integrity is commonly observed in neurological diseases such as stroke,⁴ Alzheimer disease,⁵ HIV-1 encephalitis,⁶ and multiple sclerosis.⁷,⁸ The cellular and molecular mechanisms of BBB damage and neurovascular inflammation are not well known in these neurological diseases.

One common mechanism of BBB leakiness is regulated by activation of matrix metalloproteinases (MMPs, zinc-dependent endopeptidases) during oxidative stress.⁹ Activation of MMP-1, MMP-2, and MMP-9 has been shown to degrade BBB basement membrane and TJ proteins that lead to increased permeability and immune cell infiltration into the brain.⁵,¹⁰ Such an increase in MMPs activities and BBB damage are also demonstrated in animal model of ischemic stroke.¹¹,¹² Significantly, there is a strong association between activation of MMPs and degradation of BBB basement membrane proteins in human after ischemic and hemorrhagic stroke.¹³,¹⁴ Surprisingly, there is very little information as to why the inherent angiogenesis fails to repair the damaged basement membrane and TJ proteins of these capillaries.

Vascular endothelial growth factor (VEGF-A), an agonist to VEGFR-2 is a key regulator of angiogenic response and endothelium wound healing.¹⁵ The binding of VEGF-A to VEGFR-2 induces receptor dimerization and autophosphorylation, which promotes angiogenesis and repair of the damaged existing vasculature.¹⁶,¹⁷ Tyrosine kinase receptors such as VEGFR-1, VEGFR-2, and VEGFR-3 are expressed exclusively in endothelial cells.¹⁸ Out of this, the intracellular VEGFR-2 (also known as KDR/Flik-1) plays a central role in

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From the Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE.
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Correspondence to James Haorah, Laboratory of Neurovascular Oxidative Injury, Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198. E-mail jhaorah@unmc.edu
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the proliferation and vascular endothelium function. Thus, lack of VEGFR-2 can terminate the embryonic stage due to the absence of endothelium development. But, overexpression of VEGF in brain endothelium has been shown to diminish TJ proteins and BBB function. VEGF appears to exert protective as well as destructive effects on cerebral vascular function (BBB integrity). These biphase effects appear to depend on the physiological concentrations of VEGF. The mechanism by how VEGF signaling disrupts the BBB function in the context of VEGFR-2 and MMP activation remains elusive in brain vascular biology.

Recently, we reported that selective phosphorylation of VEGFR, insulin receptor, and nonreceptor Src kinase was associated with MMP-1, MMP-2, and MMP-9 activation and BBB dysfunction during acute ethanol exposure and oxidative stress. We examined the effects of 50 mmol/L ethanol (0.2% vol/vol) as acute exposure in cell culture and 5% (vol/vol) ethanol liquid intake for 9 weeks as animal model of chronic alcohol intake on VEGF/VEGFR-2 regulation and MMP activation. The concentrations of ethanol use in this study is within the physiologically detectable range because in human up to 0.31% (68 mmol/L) blood alcohol levels are detected in moderate to severe intoxicated alcohol drinkers. The purpose of the study is to disentangle the molecular mechanisms of BBB disruption by ethanol exposure in acute condition and then validate the findings in animal model of moderately high alcohol ingestion in chronic condition. We propose the idea that physiological level of VEGF in acute alcohol intake triggers the activation of MMP-3/9 in brain endothelium via the autophosphorylation of VEGFR-2. The vasculature damage (BBB damage) caused by MMPs as a result of churning up the TJ proteins and basement membrane components is likely to be repaired by VEGF-mediated VEGFR-2 phosphorylation in BBB dysfunction. However, in chronic alcohol intake, we propose that a sustained MMPs activation causes the degradation of TJ and VEGFR-2 proteins. The inability of VEGF-2 to actively regulate the vascular repair process leads to chronic rupture of capillaries and leakiness of BBB, thereby initiating the neurovascular inflammation. We propose that this decrease in VEGFR-2 levels enhances VEGF-A in endothelium and in the blood serum, which appears to be responsible for the sustained activation of MMP-3/9 via the caspase-1 and IL-1β release pathway. We conclude that excess VEGF production in chronic alcoholism may be a detrimental risk factor for neurological disorders such as hypertension and stroke.

**Methods**

**Reagents and Cell Culture**

The source of antibodies, chemicals, primary human brain endothelial cells (hBECs), and cell culture techniques are described in detail in the online-only Data Supplement material. Briefly, primary hBECs were cultured in type I rat-tail collagen and fibronectin precoated plates as described previously.

**Animal Studies**

Five-week-old weight-match male rats were pair fed the Lieber-DeCarli control or 29% calorie intake (5% vol/vol) ethanol liquid diets for 9 weeks. Animals were euthanized at the 10th week on different days, depending on the nature of experiments with/without cell infusion. Particularly, experiments that involve infusion of fluo-3–labeled cells into the common carotid artery as previously described require a few days to complete the whole experimental conditions. The levels of alcohol, VEGF-A and IL-1β were determined in blood samples, and blood alcohol level was determined to be 8.8–24 mmol/L (0.05–0.11%) in alcohol diet ingested animals (determined by EtOH assay kit, Diagnostic Chemicals Ltd, Charlottetown, PEI, Canada). This huge variation in blood alcohol levels is commonly in animal studies due to nocturnal feeding habits of rats and variation in ethanol metabolic clearance rate. The detectable levels of blood alcohol can be increased and the variable range can be narrowed in this animal model. This can be achieved by starving the animal overnight follow by feeding them at about 5–6 am and then collect the blood samples at about 7–8 am. This was not done in the present study because we knew the outcome from our past experience.

**Immunofluorescence and Microscopy**

Changes in cell adhesion/migration, expression of TJ proteins, MMPs, caspase-1, and VEGFR2 were analyzed in intact external cerebral capillary vessels and brain tissue sections containing the internal capillaries, or hBECs cultured on cover slips by immunohistochemical stainings and fluorescent microscopy. For detail protocols and dilution of respective antibody, please see the online-only Data Supplement material.

**Western Blotting**

Changes in protein levels of MMP-3, MMP-9, occludin, claudin-5, ZO-1, VEGF-2, p-VEGF-2 (tyr1054), p-VEGF-2 (tyr1175), caspase-1, and IL-1β in intact capillary vessels, brain tissue sections, and hBECs culture were analyzed by Western blotting. See detail experimental protocol in the online-only Data Supplement.

**Dot Blot MMPs Array Analysis**

Human matrix metalloproteinases antibody array (Ray Biotech, Norcross, GA) was used to analyze the regulation of MMPs in control or EtOH-treated samples as per manufacturer’s instructions.

**Zymography of MMP Activity**

We determined the MMPs activities in hBECs culture, brain tissue, and microvessel homogenates by zymography as per well-established method. Gelatinolytic (for MMP9) or stromelysin (for MMP3) activities were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel containing 0.1% gelatin or a 12% gel containing 0.1% casein at 125 V for 90 minutes at 4°C and stained with 0.5% Coomassie brilliant blue R-250. See details of the experimental protocol in the online-only Data Supplement.

**Enzyme-Linked Immunosorbent Assay**

Using commercial enzyme-linked immunosorbent assay kits (ELISA), the levels of VEGF-A (R&D Systems, Minneapolis, MN) and IL-1β (BD Biosciences, San Jose, CA) were analyzed in cell culture media and blood serum as per manufacturer’s instructions.

**TUNEL and PARP Analyses**

Using the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL, Roche Diagnostics, IN) assay kit, cell apoptosis was determined in tissue sections as per manufacturer’s instructions. To confirm the TUNEL assay, Western blotting determined the breakdown of 113 kDa poly-ADP-Ribose-Polymerase (PARP) to 89 kDa and 24 kDa PARP fragments using rabbit anti-PARP primary antibody (BD Biosciences). Detection of 89 kDa PARP fragment is an early marker for apoptosis, which is mediated by caspase-1/3 signaling pathway.

**Transendothelial Electric Resistance**

To determine the integrity of BBB function, a highly sensitive 1600R ECIS system (Applied Biophysics, Troy, NY) determined the...
changes in transendothelial electric resistance (TEER) across the BBB as previously described. See detail protocol in the online-only Data Supplement.

Cell Adhesion and Migration Across BBB

Immune cells adhesion on the endothelium and migration across the BBB in an in vivo and in vitro were analyzed by fluorescence-labeled cells as described previously. See detail protocol in the online-only Data Supplement.

Data Analysis

All the results are expressed as the mean±SEM. Statistical analyses of the data were performed using Graphpad Prism V5 (Sorrento Valley, CA). Two-way ANOVA with Dunnett post hoc tests determined the differences between control and experimental conditions, and a probability value of <0.05 was considered significant.

Results

Activation of MMP-3/9 Causes BBB Leakiness

Activation of MMPs Diminishes TJ Protein Expression in Brain Endothelium and Microvessels

Degradation of extracellular matrix and basement membrane proteins is the key function of MMPs. We have shown that VEGFR is involved in activation of MMPs and BBB damage in acute alcohol exposure. To identify the specific types of MMPs, we first examined the changes in the expression of 10 different types of MMPs by proteome array in primary hBECs culture after exposure to 50 mmol/L ethanol for 24 hours. Out of the 10 MMPs, ethanol exposure significantly elevated the levels of MMP-3 and MMP-9 compared with untreated hBECs in culture (Figure IA–IC in the online-only Data Supplement), and these observations were confirmed by immunofluorescence staining and Western blotting. Further, detection of MMPs activity by zymography validated the changes in expression and protein levels of MMPs in brain endothelial cells and rat brain tissues and microvessels. Thus, we found that alcohol increased the gelatinolytic (for MMP9) or stromelysin (for MMP3) activity with/without VEGF treatment in cell culture or in brain tissue and microvessels (Figure II in the online-only Data Supplement). Thus, in this study, MMP-3 and MMP-9 were targeted as the causative factors for disruption and leakiness of neurovascular function (the BBB). As expected, colocalization of MMPs and TJ proteins revealed that increase in the expression of MMP-3 and MMP-9 reciprocally diminished the expression of occludin and ZO-1 by alcohol treatment compared with untreated control cells (Figure 1A through 1C and Figure III in the online-only Data Supplement). In parallel, we also analyzed the colocalization of MMP-3 or MMP-9 and occludin in intact rat brain microvessels from control and chronic alcohol intake. In agreement with the findings in primary hBEC culture, we observed that increase in MMP-3 or MMP-9 expression correlated with a decrease in occludin expression in intact capillary vessels (Figure 1D) and in microvessels of brain tissue section (Figure IV in the online-only Data Supplement) from alcohol ingested animal compared with controls.

Reduction in TJ Proteins by MMPs Disrupts the Integrity of BBB Interface

Changes in the expression of MMP-3, MMP-9, and TJ proteins were validated by Western blot analyses in lysate protein from hBEC culture, intact brain microvessels, and brain tissue homogenates. Similar to microscopy imaging data, Western blot analyses confirmed that alcohol in deed enhanced the levels of MMP-3 and MMP-9 proteins (Figure 1E) as well as decreased the levels of occludin, claudin-5 and ZO-1 proteins (Figure 1F) in hBEC culture, intact capillary vessels, and in brain tissue. Degradation of occludin, claudin-5 and ZO-1 by addition of exogenous MMP-3 or MMP-9 proved the point that BBB is targeted by MMPs activation. The attenuating effect of TIMP1 on TJ proteins in the absence or presence of ethanol further validated the notion that activation of MMPs lead to disruption and leakiness of the BBB integrity.

Loss of TJ Integrity Enhances Permeability and Leakiness of BBB

As functional assays, we assessed the integrity of BBB by TEER, permeability of BBB by sodium fluorescein/Evan blue (NaFl/EB) tracers, and infiltration of immune cells across the BBB and into the brain by calcein-AM–labeled monocytes. Assessing the changes in real-time TEER by a highly sensitive ECIS system showed a significant reduction in BBB electric resistance by EtOH or in combination with MMP-3 or MMP-9, which exacerbated the effect of EtOH in a time-dependent manner (Figure 2A). The fact that TIMP1 prevented the effect of EtOH on TEER reduction reinforced the direct role MMPs on BBB dysfunction. The role of VEGFR-2 in the integrity of BBB was come from the treatment of Ki8751 (VEGFR-2 kinase inhibitor), where TEER value was significantly reduced and with EtOH the effect was exacerbated (Figure 2A). Disruption of this BBB integrity was further supported by increased permeability of small molecular weight tracer NaFl (MW=376) and large molecular weight tracer EB (MW=961) into the brain of chronic alcohol intake rats compared with respective controls (Figure 2B).

Leakiness of BBB Enhances Immune Cell Infiltration Into the Brain

Migration of calcein-AM–labeled monocytes across the endothelium showed a significant 2- to 2.5-fold increments of immune cell migration in EtOH or EtOH in combination with MMP-3 or MMP-9 compared with controls (Figure 2C). The effect of EtOH on cell migration was comparable to that of lipopolysaccharide (LPS), which was used here as positive control. As expected, TIMP1, an inhibitor of MMPs attenuated the effect of EtOH on monocyte migration. VEGF-A alone did not cause robust cell migration. However, inhibition of VEGFR-2 by Ki8751 exacerbated the effect of EtOH on cell adhesion and migration across the BBB. Thus, we further investigated the involvement of VEGFR-2 on BBB integrity in hBEC culture. Western blot analyses revealed that treatment of hBECs with Ki8751 selectively downregulated the levels of occludin, claudin-5, and ZO-1 proteins either in the presence or absence of alcohol exposure, suggesting the synergistic association between VEGFR-2 and brain vascular function (Figure 2D). Moreover, addition of VEGF-A (100 ng/mL) exogenously decreased the level of occludin, claudin-5 and ZO-1 proteins (Figure 2D), suggesting that
antagonizing the VEGFR-2 function by a high concentration of VEGF-A can impair the integrity of BBB.

Finally, utilization of our animal model of chronic alcohol intake allowed us to validate the notion that neuroinflammatory process was initiated by BBB damage. We found that infusion of bone marrow derived rat monocytes and macrophages labeled with Fluo-3 into the right common carotid artery demonstrated an increase in cell adhesion and infiltration at the damage sites of the brain capillary alcohol intake animal compared with pair-fed controls (Figure 2E). Further, infiltration of immune cells into brain was analyzed by staining with antibody to CD68 in brain tissue sections from rats that were not subjected to cell infusion. Our data confirmed that there was more number of infiltrated immune cells in the brain of alcohol intake than the pair-fed controls (Figure 2F). Taken together, these results indicate that activation of MMPs and diminished repair function of VEGFR-2 may lead to vascular leakiness and enhanced cell adhesion/infiltration for initiation of cerebral inflammatory disease in alcohol abuse.

Figure 1. Activation of MMPs by ethanol degrades TJ proteins. A through D, Immunofluorescent staining of A, MMP-9 (red), occludin (green), and DAPI (blue); B, MMP-3 (red), ZO-1 (green), and DAPI (blue); C, MMP-9 (red), ZO-1 (green), and DAPI (blue) in hBECs; and D, MMP-3 (red), occludin (green), and DAPI (blue) in intact brain microvessels of rat. E and F, Western blot analyses of MMP-3, MMP-9, and actin (E); occludin, claudin5, ZO-1 and actin (F) in hBEC lysate protein, whole brain tissue homogenates, and in protein extract from isolated microvessels. For all hBECs culture work, cells were treated with or without EtOH (50 mmol/L) in the presence or absence of MMP-3 or MMP-9 (100 ng/mL each) or TIMP1 (100 ng/mL) for 24 hours. Bar graphs show the results that are expressed as ratio of MMP-3/9, occludin, claudin5, or ZO-1 to that of β-actin bands. Values are mean±SEM; n=4. *P<0.05; **P<0.01 versus control in E and F; **P<0.01 versus EtOH in E or MMP-3/9 in F. Scale bar=40 µm in A, B, and C; and 5 µm in D.
Alcohol-induced reduction in VEGFR-2 protein was validated by Western blot in protein samples from brain tissue and microvessel of rats (Figure 3C). To prove that degradation of VEGFR-2 protein by MMPs was a possible mechanism for endothelium dysfunction, we treated hBECs with exogenous MMP-3 and MMP-9 in the presence or absence of EtOH or TIMP1. Addition of MMP-3 or MMP-9 significantly reduced the level of VEGFR-2 protein either in the presence or absence of EtOH compared with untreated control (Figure 3D). Importantly, TIMP1 mitigated the MMP-mediated degradation of VEGFR-2 protein, supporting the hypothesis that MMP-3/9 activation and VEGFR-2 reduction was involved in alcohol-induced leakiness of the BBB function.

Degradation of VEGFR-2 Elevates Cellular and Serum VEGF-A Levels

The decrease in VEGFR-2 protein that we observed is expected to elevate the cellular and circulatory VEGF-A levels because the platelets and endothelial cells will continue to secrete VEGF-A, whereas VEGFR-2/VEGFR-1 is unable...
Alcohol-induced degradation of VEGFR-2 causes VEGF-A to bind to VEGF-A inefficiently. Thus, we quantified the levels of VEGF-A in blood serum samples and supernatants of hBECs cultured media by ELISA. Our results show that alcohol elevated the levels of VEGF-A in serum and cellular culture media compared with respective controls (Figure 4A and 4B). Interestingly, MMP-3 and MMP-9 also increased the levels of VEGF-A either in the presence or absence of alcohol, whereas TIMP1 (inhibitor of MMPs) decreased the effect of alcohol on VEGF-A regulation. These data suggest that MMP-3/9 was involved in the malfunctioning of VEGFR-2 causing increase extracellular VEGF-A levels. To address the idea that this excess level of VEGF-A in turn triggers the idea that this excess level of VEGF-A in turn triggers the phosphorylation of VEGFR-2.

Caspase-1 Activation Controls the VEGF Signaling Loop

To test the hypothesis whether alcohol-induced increase in VEGF-A (due to VEGFR-2 reduction) could activate caspase-1, we analyzed the expression of caspase-1 in hBECs exposed to EtOH, Ki8751, VEGF-A, z-YVAD-fmk (caspase-1 inhibitor), and TIMP1. It was apparent that EtOH, VEGF-A, or inhibition of VEGFR-2, respectively, increased the expression of caspase-1 accompanied by an abrupt decrease in VEGF-A protein expression (Figure 5A). These changes in caspase-1 protein expression were validated by the changes in protein levels in total protein extracts from hBECs, brain tissue, and microvessel homogenates by Western blot analyses (Figure 5B). EtOH, VEGF-A, or Ki8751, respectively, increased caspase-1 protein without significantly affecting the levels of the procaspase-1. As expected, z-YVAD-fmk inhibited the maturation of procaspase-1 to caspase-1. Interestingly, TIMP1 was able to inhibit procaspase-1 maturation, suggesting that sparing the VEGFR-2 degradation by MMPs can reinforce the VEGF–VEGFR-2 interaction for vascular repair process. The effects of EtOH on caspase-1 activation in brain tissue and microvessel of chronic alcohol intake animal and pair-fed control are further demonstrated with a colocalization of brain endothelial marker protein von Willebrand factor (Figure V in the online-only Data Supplement).

Activation of Caspase-1 Matures IL-1β

Because activation of caspase-1 leads to production of interleukin 1 beta (IL-1β), we then analyzed the level of IL-1β in...
cell-cultured media and in blood serum from chronic animal studies by ELISA. We observed that treatment of hBECs with exogenous MMP-3, MMP-9, VEGF-A, or Ki8751 significantly increased the levels of IL-1β in the presence or absence of EtOH; however, TIMP1 and z-YVAD-fmk prevented the effects of EtOH on IL-1β elevation (Figure 6A). These in vitro studies findings were further validated by our in vivo studies, where the levels of IL-1β in the blood serum of EtOH-diet consumed animal had about 7-fold higher than the pair-fed control (Figure 6B). These findings were further substantiated by the induction of 89 kDa PARP in hBECs (Figure 6G) and in rat brain tissue or microvessel homogenates (Figure 6E). Taken together, these data indicate that MMP-mediated loss of VEGFR-2 function and subsequent elevation of VEGF-A levels in alcohol intake lead to activation of caspase-1 and release of proinflammatory IL-1β. Thus, release of this cytokine via the VEGF signaling pathway may be the reversible loop mechanism for the activation of MMP-3/9 by VEGF-A, which was observed in data presented in Figure 4A and 4B.

**Caspase-1 and IL-1β Causes Cell Apoptosis**

As a functional read out assay for caspase-1 activation and IL-1β release, we next evaluated the extent of cell apoptosis by TUNEL and Poly-ADP-Ribose-Polymerase (PARP) induction Western blot in hBECs culture and in animal studies. It was obvious that there was much higher positive staining of TUNEL in brain tissue from alcohol-diet ingested animal than the pair-fed control brain tissue section (Figure 6F). These findings were further substantiated by the induction of 89 kDa PARP in hBECs (Figure 6G) and in rat brain tissue or
microvessel homogenates (Figure 6H) after alcohol exposure. Similar to IL-1β release data, Ki8751 with or without EtOH upregulated the level of 89 kDa PARP, whereas TIMP1 or z-YVAD-fmk decreased the levels of 89 kDa PARP induction. These results suggest that downregulation of VEGFR-2 mediated caspase-1 activation and that IL-1β release causes cell apoptosis.

**Discussion**

It is now widely accepted that MMP-induced leakiness of the BBB is a key event for the development of vascular and neurological disease. Here, we demonstrated that activation of MMP-3/9 by ethanol impaired the TJ proteins of the BBB in hBECs culture and brain capillary of animal compared with respective controls. Alcohol-induced decrease in real-time transendothelial electric resistance supported the loss of BBB integrity, which was validated by enhanced permeability of Na-Fl/EB tracers across the capillary and into the brain. These findings suggested that leakiness of BBB might be susceptible for initiation of inflammatory neurological disease such as atherosclerosis and stroke. As a proof-of-concept to this inflammatory process, our in vitro and in vivo study findings indicated an increase immune cell (calcein-AM/Fluo3 labeled monocytes) adhesion and infiltration into the brain. Interestingly, TIMP significantly but not completely reversed the effect of EtOH on TEER unlike that of monocyte infiltration. This may be attributed to the fact that TIMP is an inhibitor of MMPs but not an inhibitor of ethanol metabolism. Thus, TIMP is unable to prevent the effect of acetaldehyde and oxidative damage of the BBB. Degradation of the BBB basement by MMPs plays a significant role for migration of immune cells into the brain, thus, inhibition of MMPs by TIMP was effective in reversing the effect of ethanol for infiltration of monocytes. Because activation of MMPs is involved in BBB leakiness, inhibition of MMPs has been proposed as beneficial approach to alleviate vascular inflammatory diseases. It is now evident that disruption of BBB and neurovascular components correlates with inflammatory disease such as multiple sclerosis and ischemic stroke.
Our idea is that a sustained activation of MMPs by chronic alcohol intake leads to degradation of VEGFR-2 protein, thereby unable to repair the leakiness of the capillary. Our data supported this argument because exposure of hBECs to exogenous MMP-3 or MMP-9 led to the reduction of VEGFR-2 protein, which was further validated by the fact that activation of MMP-3 or MMP-9 in brain microvessel correlated with a downregulation of VEGFR-2 protein. We have not examined the cleavage site(s) of VEGFR-2 by MMPs; however, it has been shown that cleavage of VEGFR-2 by MMPs occur at multiple positions of the extracellular domain of the endothelium VEGFR-2.37,38 There appears to be a strong association between VEGFR-2 signaling and activation of MMPs in vascular biology, because it has been shown that inhibition of VEGFR-2 phosphorylation by propranolol blocked the secretion of MMP-2 in endothelial cells.39 Conversely, interaction of VEGF and VEGFR-2 (ie, phosphorylation of VEGFR-2) appears to downregulate MMP-9 through a STAT1 activation pathway.40 In this study, the authors suggest that tyrosine phosphorylation of STAT1 by VEGF may responsible for downregulation of MMP-9. Thus, Ito et al (2009) demonstrated that degradation of VEGFR-1 by MMP-7 exploited the VEGF function to deleterious health effects.41 We propose that acute exposure of alcohol activates MMP-3/9 via the phosphorylation of VEGFR-2, whereas chronic exposure of alcohol activates MMPs by a sustained VEGF-A loop pathway at the expense of TJ/VEGFR-2 protein degradation by MMPs.

In the present study, we demonstrated that high concentration of VEGF initially increased the expression of VEGFR-2 protein but decreased the VEGFR-2 protein time-dependently, with a gradual increase in MMP-3 or MMP-9. These findings indicate that binding of VEGF to VEGFR-2 initially caused the dimerization of VEGFR-2 and increased the expression of receptor protein. Because the binding sites of VEGFR-2 get saturated, the excess VEGF level began to activate MMP-3/9 via the caspase-1 activation loop. In turn, MMPs activation degraded VEGFR-2 protein and further elevated the extracellular and intracellular VEGF levels. Thus, activation of MMPs by VEGF not only degrades VEGFR-2 but also has been shown to decrease the BBB TJ proteins occludin and claudin-5 in autoimmune disease.21,42 similar to our present findings. In physiological condition,
platelets and endothelial cells constantly produce VEGF-A. As such impairment of VEGFR-2/VEGFR-1 is expected to increase the levels of VEGF-A in the blood circulation. The present studies showed that impairment of VEGFR-2 was accompanied by high levels of VEGF-A (270 pg/mL) in the serum of alcohol intake animal compared with pair-fed controls (125 pg/mL). However, the levels of VEGF-A detected in hBEC culture with or without exposure to EtOH or MMP was in the range of 9–22 ng/mL, which was much higher than the VEGF-A levels detected in the serum. This high level of VEGF-A in cell culture supernatants could be contributed by the presence of exogenous VEGF-A in the culture media in addition to EtOH-stimulated secretion by the brain endothelial cells. Similar to these findings, others have reported the elevation of serum VEGF levels in human alcoholics and alcohol-exposed endothelial cells. Interestingly, the serum level of VEGF-A detected in the present studies was comparable to the serum VEGF levels of diabetic patients (257–365 pg/mL), where the mean VEGF-A level in serum of healthy subjects was 216 pg/mL. Similarly, the level of mean VEGF-A in serum of cancer patients was 291–380 pg/mL compared with the mean VEGF level of 219 pg/mL in healthy subjects. All these findings clearly indicate that detection of abnormal VEGF levels in blood samples can be a standard diagnostic marker for neurovascular disease, diabetes and cancers. It must be noted here that normal physiological level of VEGF is an essential growth factor required for endothelial cell growth and vasculogenesis. Interruption of this physiological homeostasis such as by impairing the VEGF receptors can lead to elevation of VEGF, which in turn may promote activation of MMPs and growth of cancer cells. We describe for the first time that alcohol-induced decrease in VEGFR-2 raises the VEGF above the physiological level, and this sustained increase in VEGF level in turn activates the MMP-3/9 through caspase-1 activation and IL-1β release. Activation of caspase-1 and release of IL-1β release were validated by TUNEL cell apoptosis and induction of PARP in hBECs and in animal studies.

In summary, the alteration of the normal VEGF/VEGFR-2 interaction appears to dictate the outcome of this vascular disease, because VEGF is a promoter of angiogenesis (BBB repair process) at physiological level and an activator of MMPs at high level. We discuss the mechanism of switch by VEGF for causing BBB leakage in the context of MMP activation and VEGFR-2 reduction in alcohol abuse. We found that activation of MMP-3/9 by alcohol not only altered the TJ proteins and basement membrane components but also degraded the VEGF-2 protein. Although disruption of TJ proteins and basement membrane led to leakiness of BBB and vascular inflammation, the impairment of VEGFR-2 function led to inefficient repair process of the leaky BBB as well as elevation of VEGF levels in the circulation. This sustained elevation of VEGF-A above the physiological level triggered the activation of MMP-3/9 through a caspase-1 and IL-1β release to cause inflammation and brain cell death. Figure VI in the online-only Data Supplement depicts the schematic presentation of our findings.

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Disclosures

None.

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The Mechanisms of Cerebral Vascular Dysfunction and Neuroinflammation by MMP-mediated Degradation of VEGFR-2 in Alcohol Ingestion

P. M. Abdul Muneer, Saleena Alikunju, Adam M. Szlachetka and James Haorah*

Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198.

MATERIALS AND METHODS

Reagents: Antibodies to MMP-3, MMP-9, Claudin-5, von Willibrand factor (vWF), VEGFR-2, caspase-1, CD68 were purchased from Abcam (Cambridge, MA). Antibody to Occludin was from Invitrogen (Carlsbad, CA), IL-1β was from Abgent (San Diego, CA), ZO-1 was from US Biological (Massachusetts, MA), p-VEGFR-2tyr1054 and β-actin was from Millipore (Billerica, MA) and, p-VEGFR-2tyr1175 was from Cell Signaling (Danvers, MA). All secondary Alexa Fluor antibodies were purchased from Invitrogen. Human recombinant VEGF, MMP-3 and MMP-9 were purchased from R & D System (Minneapolis, MN), TIMP1 (MMP inhibitor) was from Calbiochem (Gibbstown, NJ), Ki8751 (VEGFR-2 kinase inhibitor) was from Sigma-Aldrich (St. Louis, MO), and zYVAD-fmk (caspase-1 inhibitor) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture: Primary human brain endothelial cells (hBECs) were obtained from Dr. Persidsky’s Lab, Temple University School of Medicine, and hBECs were cultured as described previously 1. Briefly, all cell culture plates and glass cover slips were pre-coated with type 1 rat-tail collagen (0.09 mg/mL in double distilled sterile water), aspirated the excess collagen, dried the plates and layered with fibronectin overnight in sterile hood. For immunocytochemistry, cells were plated on 12-well glass cover slips (40,000 cells/well) and
for protein extractions cells were cultured in T75 cm$^2$ flasks (1 X 10$^6$ cells/flask). Cell culture media (DMEM/F-12) contains 10 mM Heps, 13 mM sodium bicarbonate (pH 7), 10% fetal bovine serum, penicillin and streptomycin (100 µg/ml each, Invitrogen) that are changed on alternate days for 6-8 days. Once tight monolayers were formed, cells were treated with or without EtOH (50 mM) and VEGF (100 ng/mL), MMP-3/9 (100 ng/mL), TIMP1 (MMPs inhibitor, 100 ng/mL), Ki8751 (VEGFR-2 kinase inhibitor, 10 µM) and zYVAD-fmk (caspase-1 inhibitor, 5 µg/mL).

**Animal studies:** Five-week old male Sprague-Dawley rats were purchased from Charles River Laboratory (Wilmington, MA) and maintained in sterile cages under pathogen-free conditions in accordance with the institutional ethical guidelines for care of laboratory animals, National Institutes of Health (NIH) guidelines and the Institutional Animal Care Use Committee of the University of Nebraska Medical Center. Rats were acclimated to Lieber–DeCarli control or 29 % calorie intake (5% v/v) ethanol liquid-diets from Dyets Inc (Bethlehem, PA) for 5 days prior to weight-match pair feeding regimens for 9 weeks. Pair feeding was based on the amount of liquid-diets consumed by ethanol group. Animals were sacrificed at 10th week as described in the main text.

**Immunofluorescence and microscopy:** Intact external cerebral capillary vessels were surgical removed from the brain and smeared onto the slides. Adhesion and migration of Fluo-3 labeled immune cells were detected in these vessels directly under fluorescent microscope. For immunohistochemical stainings, intact external capillary, brain tissue sections (8 µm thickness) containing the internal capillaries, or hBECs cultured cover slips were washed with PBS and fixed in acetone-methanol (1:1 v/v) fixative for 10 minutes at 95°C, followed by incubation with 3% formaldehyde in PBS for 10 minutes at 25°C. Washed
tissue slides were then blocked with 3% bovine serum albumin at 25°C for 1 hr in the presence of 0.1% Triton X-100, or in the absence of 0.1% Triton X-100 for occludin, claudin-5 and ZO-1. Tissues slides were incubated with primary antibody to rabbit anti-MMP-3, anti-MMP-9, anti-occludin, anti-claudin-5, anti-ZO-1, anti-caspase-1, anti-CD68 (1:250 dilution for all), sheep anti-vWF (1:150 dilution), and mouse anti-VEGFR-2 (1:250 dilution) for overnight at 4°C. After washing with PBS, tissue slides were incubated with AlexaFluor 488 or 594 conjugated with anti-mouse or anti-rabbit or anti-sheep immunoglobulin G (IgG) for 1 hr, mounted with immunomount containing DAPI (Invitrogen), and fluorescence microphotographs were captured by fluorescent microscopy (Eclipse TE2000-U, Nikon microscope, Melville, NY) using NIS elements (Nikon, Melville, NY) software.

**Western blotting:** The hBECs cultured in T-75 cm² flasks, cortical brain tissues and brain microvessels were lysed with CellLytic-M (Sigma) for 30 min at 4°C, centrifuged at 14000g, and then total cell lysates protein concentrations were estimated by bicinchoninic acid (BCA) method (Thermo Scientific, Rockford, IL). Protein load was 20 μg/lane in 4-15% SDS-PAGE gradient gels (Thermo Scientific). Molecular size separated proteins were then transferred onto nitrocellulose membranes, blocked with superblock (Thermo Scientific), and incubated overnight with respective primary antibody to MMP-3, MMP-9, occludin, claudin-5, ZO-1, VEGFR-2, p-VEGFR-2tyr1054, p-VEGFR-2tyr1175, caspase-1 and IL-1β at 4°C, followed by incubation with horse-radish peroxidase conjugated secondary antibodies for 1 hr. Immunoreactive bands were detected by West Pico chemiluminescence substrate (Thermo Scientific). Data were quantified as arbitrary densitometry intensity units using the Gelpro32 software package (Version 3.1, Media Cybernetics, Marlow, UK).
Zymography of MMP Activity: To determine MMPs activities in the endothelial cell cultures with different treatments and in the brain tissue and brain microvessels, zymography was performed using a method similar to that previously described. For gelatin or casein zymography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by loading 40 μg protein in a 10% polyacrylamide gel containing 0.1% gelatin or a 12% gel containing 0.1% casein (Bio-rad, Hercules, CA) at 125 V for 90 minutes at 4°C. The gels were soaked in renaturing buffer (Invitrogen) for 30 minutes at room temperature and incubated in developing buffer (Invitrogen) for 30 minutes at room temperature and for overnight at 37°C. Gels were rinsed in distilled water, stained with 0.5% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for 1 hour, and destained with 40% methanol and 10% acetic acid. Proteolytic activities appeared as clear bands of lysis against a dark background of stained gelatin or casein.

Transendothelial electrical Resistance (TEER): To determine the integrity of BBB function, changes in trans-endothelial electrical resistance (TEER) across the BBB were analyzed by a highly sensitive 1600R ECIS system (Applied Biophysics, Troy, NY) as previously described. The ECIS system provides real-time monitoring of changes in TEER. Briefly, hBECs plated on collagen type I coated 8W10E electrode arrays (Applied Biophysics) formed a tight cell monolayers. Stable electrical resistance across the BBB was first monitored for 2 hr prior to treatment. Exposure of cell monolayers with EtOH (50 mM), VEGF (100 ng/mL), MMP-3/9 (100 ng/mL), TIMP1 (MMPs inhibitor, 100 ng/mL), or Ki8751 (VEGFR-2 kinase inhibitor, 10 μM) altered the stable TEER values. TEER readings were monitored continuously for 24 hr at 400 hz with 10 min intervals.

In vitro cell adhesion and migration across BBB: To quantitatively analyze the changes
in cell adhesion and migration across the BBB, hBECs (2.5 X 10^4 cells/insert) were cultured on type I collagen-coated FluoroBlok tinted tissue culture inserts (with 3 μm pores, BD Biosciences). After forming the tight monolayers, cell cultures were treated with test compounds for 24 hr, then a cell tracker Calcein-AM (5 μM, Invitrogen) labeled primary human monocytes were introduced into the endothelial monolayers (1 X 10^6 monocytes/insert). Cells were then allowed to infiltrate into the monolayers for 2 hr at 37°C in tissue culture incubator in the absence of test compounds. The relative fluorescence intensity of the migrated cells at the lower chamber and those cells that were stuck between the porous membranes and the lower chambers were detected by fluorescence-based assay using the M5 fluorescence plate reader as previously described ⁴. The actual numbers of migrated cells were calculated from the internal standard curve of the labeled cells, and the data were presented as the fold difference of the untreated control.

**In vivo cell adhesion and infiltration in brain endothelium:** Rat femurus bone marrow cells were isolated under sterile condition, differentiated to monocytes with specific cell differentiating media containing MCSF, labeled the cells with Fluo-3, and labeled cells (2 X 10^6 cells per rat) in suspension were into the right common carotid artery using 27.5 G needle (see ⁵ for detail protocol). Adhesion and infiltration of these labeled cells were detected in intact brain microvessels under fluorescent microscope.
Supplementary Figures

Supplementary Figure I: Up-regulation of MMP-3/9 by alcohol: A-B, Dot blot analyses of 7 MMPs and 3 TIMPs in hBEC lysates protein by using human matrix metalloproteinases antibody array; (A) Untreated cells, (B) significant up-regulation of MMP-3 and MMP-9 (yellow rectangles) by 50 mM EtOH treatment for 24 hours. Pos: positive controls; Neg: negative controls. C, Bar graphs show the fold induction of MMPs and TIMPs in EtOH treated cells (black bar) compared with respective untreated cells (grey bar). The results
presented as the mean values (± SEM, n=3). Statistically significant *p<0.05 and **p<0.01 is compared with respective controls.

**Supplementary Figure II: Zymograms:** The gelatinolytic activity of MMP-9 (top) and caseinolytic activity of MMP-3 (bottom) were demonstrated by gelatin or casein zymography in the endothelial cell extracts different treatments and in brain tissue and microvessels.
Supplementary Figure III: Up-regulation of MMP-3 by alcohol down-regulates occludin expression in hBECs: Immunofluorescent analysis of MMP-3 (red) and occludin (green) merged with DAPI (blue), in untreated and 24 hr EtOH (50 mM) treated hBECs. Scale bar indicates 40 μm in all panels.
Supplementary Figure IV: Up-regulation of MMP-9 by alcohol reduces occludin expression in brain microvessel: Immunofluorescent analysis of MMP-9 (red) and occludin (green) merged with DAPI (blue) in the cross section of intact brain microvessels of control and chronic alcohol treated animals. Scale bar indicates 5 µm in all panels.
Supplementary Figure V: Alcohol activates and up-regulates caspase-1 expression in brain microvessels: Immunofluorescent analysis of caspase-1 (red) and vWF (green) merged with DAPI (blue) in the cross section of intact brain microvessels of control and chronic alcohol treated animals. Scale bar indicates 5 µm in all panels.
Supplementary Figure VI: Schematic presentation of the underlying mechanisms of MMP-3/9 activation by ethanol, degradation of TJ proteins/VEGFR-2, and subsequent activation of caspase-1/IL-1β via VEGF signaling loop.
Supplementary References


