

# Activation of Protein Tyrosine Kinases and Matrix Metalloproteinases Causes Blood-Brain Barrier Injury: Novel Mechanism for Neurodegeneration Associated with Alcohol Abuse

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## KEY WORDS

blood–brain barrier; brain endothelial cell; ethanol metabolism; matrix metalloproteinases; protein tyrosine kinase; protein tyrosine phosphatase

## ABSTRACT

Blood-brain barrier (BBB) formed by brain microvascular endothelial cells (BMVEC) regulates the passage of molecules and leukocytes in and out of the brain. Activation of matrix metalloproteinases (MMPs) and alteration of basement membrane (BM) associated with BBB injury was documented in stroke patients. While chronic alcoholism is a risk factor for developing stroke, underlying mechanisms are not well understood. We hypothesized that ethanol (EtOH)-induced protein tyrosine kinase (PTK) signaling resulted a loss of BBB integrity via MMPs activation and degradation of BM component, collagen IV. Treatment of BMVEC with EtOH or acetaldehyde (AA) for 2–48 h increased MMP-1, -2 and -9 activities or decreased the levels of tissue inhibitors of MMPs (TIMP-1, -2) in a PTK-dependent manner without affecting protein tyrosine phosphatase activity. Enhanced PTK activity after EtOH exposure correlated with increased phosphorylated proteins of selective receptor and nonreceptor PTKs. Up-regulation of MMPs activities and protein contents paralleled a decrease in collagen IV content, and inhibitors of EtOH metabolism, MMP-2 and -9, or PTK reversed all these effects. Using human BMVEC assembled into BBB models, we found that EtOH/AA diminished barrier tightness, augmented permeability, and monocyte migration across the BBB via activation of PTKs and MMPs. These findings suggest that alcohol associated BBB injury could be mediated by MMPs via BM protein degradation and could serve as a comorbidity factor for neurological disorders like stroke or neuroinflammation. Furthermore, our preliminary experiments indicated that human astrocytes secreted high levels of MMP-1 and -9 following exposure to EtOH, suggesting the role of BM protein degradation and BBB compromise as a result of glial activation by ethanol. These results provide better understanding of multifaceted effects of alcohol on the brain and could help develop new therapeutic interventions. © 2007 Wiley-Liss, Inc.

## INTRODUCTION

Alcohol is the most commonly used and abused drug in the United States. Deleterious alcohol-related health

effects attributed to the internal organ toxicity include irreversible brain tissue injury (Harper and Matsumoto, 2005). Brain tissue of chronic alcoholics features neurodegeneration (Harper, 1998) paralleling neuro-cognitive deficits in these patients (Parsons, 1998; Zeigler et al., 2005). White matter abnormalities seen in alcoholics (Mann et al., 2001) could be associated with blood-brain barrier (BBB) dysfunction detected in alcohol abusers (Pratt et al., 1990; Thomsen et al., 1994) and seen in animal models of chronic alcohol administration (Phillips and Cragg, 1982; Rosengren and Persson, 1979). The underlying mechanism of BBB dysfunction caused by alcohol abuse remains elusive. BBB formed by brain microvascular endothelial cells (BMVEC), pericytes, and astrocytes (Rubin and Staddon, 1999), and tight junctions (TJ) connecting BMVEC ensure the structural tightness of the BBB (Pardridge, 1983). Intracellular signaling process regulating phosphorylation of TJ proteins controls the TJ assembly and BBB integrity (Rubin and Staddon, 1999). Using primary human BMVEC, we previously demonstrated that ethanol (EtOH) increases activity and content of EtOH-metabolizing enzymes leading to the production of acetaldehyde (AA) and reactive oxygen species (ROS) in brain endothelium (Haorah et al., 2005b). AA and ROS then activate myosin light chain kinase (Haorah et al., 2005a) via stimulation of inositol 1,4,5-triphosphate (IP<sub>3</sub>R)-gated intracellular Ca<sup>2+</sup> release signaling pathway (Haorah et al., 2007a) resulting in phosphorylation of cytoskeletal/TJ proteins and BBB dysfunction.

Our recent findings suggested that oxidative stress activates matrix metalloproteinases (MMPs) via protein tyrosine kinase (PTK) signaling and resulted in degradation of basement membrane (BM) protein and BBB dis-

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ruption (Haorah et al., 2007b). Similar findings have been reported in mice and rats after ischemic stroke (Kim et al., 2003; Machado et al., 2006). Chronic alcohol administration has been shown to activate MMP-2 and -9 in *in vitro* and *in vivo* models (Aye et al., 2004; Lois et al., 1999); however, mechanisms leading to such effects remain undefined. We hypothesize that alcohol abuse disrupts BBB because of activation of MMPs via PTK signaling pathway leading to degradation of BM protein and tyrosine phosphorylation TJ proteins. To test this idea, we treated human BMVEC with EtOH and studied the activity of MMP-1, -2, -9, expression of tissue inhibitors of MMPs (TIMP-1, -2), BM degradation, activity of PTK and protein tyrosine phosphatase (PTP), expression of receptor and nonreceptor PTKs, phosphorylation of TJ proteins, and alterations of BBB function. Our results indicated that EtOH metabolism activated MMP-1, -2, -9 in a PTK dependent manner, and PTK/MMPs inhibitors prevented functional changes of BBB (decreased structural integrity, enhanced permeability, and leukocyte migration across the BBB).

## MATERIALS AND METHODS

### Cell Isolation and Culture

Primary human BMVEC were isolated from the temporal cortex of brain tissue obtained during surgical removal of epileptogenic foci in adult patients and were supplied by Dr. M. Witte (University of Arizona). BMVEC purity evaluation and culture condition were performed as described previously (Haorah et al., 2007a). Optimal concentrations determined by dose-dependent response were 50 mM EtOH (concentrations tested were 10–200 mM), 100  $\mu$ M AA (10–200  $\mu$ M), 1 mM 4-methylpyrrole (4-MP, inhibitor of EtOH metabolizing enzymes; 0.25–4 mM), 50  $\mu$ M uric acid (UA, anti-oxidant; 10–200  $\mu$ M), 100  $\mu$ M genistein (GS, PTK inhibitor; 10–200  $\mu$ M), 20  $\mu$ g/mL endostatin (ES, MMP-2 and -9 inhibitor; 1–40  $\mu$ g/mL), and 100  $\mu$ M phenylarsine oxide (PAO, PTP inhibitor; 10–200  $\mu$ M).

### MMPs Activity and TIMPs Expression

Following the manufacturer's instructions (Amersham, Piscataway, NJ), MMP-1, -2, or -9 activity was detected by biotrak activity assay system; whereas, the level of total protein contents of MMP-1, -2, -9 or TIMP-1, -2 was assayed by biotrak (human) ELISA system using different concentrations of respective MMP or TIMP as standard curves. MMP-1, -2, -9 or TIMP-1, -2 protein contents determined by this system is much more sensitive and faster than Zymography or Western blot analyses. Briefly, secreted proteins were collected from cell culture conditioned media at 2–96 h after treatment with EtOH or AA. Secreted proteins were immuno-conjugated with precoated anti-MMP-1, -2, -9, or anti-TIMP-1, -2 antibody in microplate wells. MMPs

activities and level of MMPs or TIMPs protein contents were calculated from standard curve run in parallel with samples detected at 450 nm in a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). The results were expressed as nmol or  $\mu$ mol/mg protein.

### Extracellular Matrix/BM Degradation Assay

We utilized the R&D (Minneapolis, MN) thin layer BM (4–6  $\mu$ m) coating system. Briefly, glass cover slips precoated with type I collagen (0.1 mg/mL) were layered with a mixture (1:1) of fluorescein-conjugated and unconjugated type IV collagen (0.2 mg/mL). BMVEC (10,000 cells/well) cultured on top of fluorescein-conjugated collagen IV were treated with test compounds for 72 h, followed by washes with phosphate buffered saline (PBS), fixation, and confocal microscopy analyses. In parallel, quantitative analysis of collagen IV degradation was performed with BMVEC cultured on the top of collagen layers in 96-well black wall clear bottom plates after activation of MMPs by EtOH or AA. Cells were washed after 72 h treatment with EtOH or AA, and fluorescence was detected at 488 nm excitation and 515 nm emission using a microplate reader spectrophotometer (Molecular Devices). Nondegraded collagen IV from each condition was calculated using the standard curve of fluorescein-conjugated collagen IV run in parallel with samples.

### PTK/PTP Activity Assay and PTK Array

PTK and PTP activities were assessed using the ELISA-based kits (Calbiochem, San Diego, CA). Briefly, cellular lysates protein (from 48 h treatment) was immuno-conjugated with precoated anti-PTK/anti-PTP antibody in microplate wells. PTK/PTP activity was calculated from standard curve run in parallel with respective samples, detected at 450 nm in a microplate reader-spectrophotometer. PTK/PTP activity results were expressed as pmol or mmol/mg protein. Activation of specific PTK after EtOH treatment was assessed by human phospho-receptor tyrosine kinase (RTKs) array (R&D Systems). This proteome profiler array kit identifies 42 different activated RTKs at a time. Briefly, cell lysates protein (100  $\mu$ g) derived from control or EtOH treatment was conjugated to 42 different anti-RTKs antibodies that were spotted in duplicate on nitrocellulose membranes. The conjugates were captured by horseradish peroxidase and were detected by chemiluminescence.

### TEER Measurement

Transendothelial electrical resistance (TEER) measurement by sensitive electric cell-substrate impedance-sensing (ECIS) model 1600R (Applied BioPhysics, Troy, NY) assessed the tightness of BMVEC monolayers. The

ECIS model 1600R system monitors the dynamic tightness of cell monolayers in culture with a live recording of TEER, mimicking closely physiological conditions. In the ECIS system, BMVEC were grown directly on gold film electrode surface (0.25 cm<sup>2</sup>) in culture wells (0.8 cm<sup>2</sup>) with cell culture medium serving as the electrolyte. A constant current source of 4 kHz was maintained between the TEER measuring electrode (0.25 cm<sup>2</sup>) and the counter electrode (0.8 cm<sup>2</sup>). TEER readings of fully confluent BMVEC monolayers were recorded after application of each test compound for 2 or 48 h. TEER values were calculated as: Resistance of a unit area = Resistance (Ohms) × effective membrane area (cm<sup>2</sup>), where, effective membrane area is 0.25 cm<sup>2</sup> (for ECIS system). Normalizing the resistance for 1.0 cm<sup>2</sup> defines the “Resistance” as: “Ohms × 1.0 cm<sup>2</sup> divided by 0.25 cm<sup>2</sup> (i.e., Ohms cm<sup>2</sup>). Thus, resistance is inversely proportional to the effective membrane surface area. Results were expressed as percent of controls.

### Dextran Permeability Assay

We determined the rate of permeability of different molecular weights of dextrans (low to high molecular weights, 4, 10, and 40 kDa) prior to studying the effects of test compounds in permeability assay. Here, we used the low molecular weight (4 kDa) FITC-labeled dextran (Molecular Probes), because our control optimization data showed that only 0.05–0.22% of the initial dextran concentration was permeated after 1–4 h permeability assay. After treatment for 48 h with test compounds, we removed 100 μL (out of 200 μL) of media from all upper chambers of 24-transwell tissue culture inserts (pore diameter 0.4 μm) and replaced with 100 μL of media containing 20 μM FITC-labeled dextran (10 μM final concentration) with or without test compounds. Samples were collected from lower chambers at 2 h after addition of dextran with or without test compounds for analysis of permeability rate across the BBB. Together, with various concentrations of FITC-labeled dextran standard, fluorescence intensity of each sample was read at 488 nm excitation and 525 nm emission. Permeability flux rate in nmol/h was calculated from the standard curve. Results were expressed as apparent permeability coefficient (P), defined as cm/h permeability. “P” is derived from the ratio of flux rate (nmol/h) to that of initial concentration (in nmol) and surface area of permeant constant at 0.32 cm<sup>2</sup>.

### Monocyte Migration and Western Blot

Migration of monocytes across the BBB was performed as previously described (Haorah et al., 2007b), and our methods for immunoconjugation, immunoprecipitation, and Western blot were also described (Haorah et al., 2005b). Antibodies to occludin, claudin-5, ZO-1, and phosphotyrosine were purchased from Zymed (Zymed, San Francisco, CA), and CD68 antibody was purchased

from Dako (macrophage marker, at 1:100 dilution, Dako, Carpinteria, CA).

### Statistical Analysis

Results were expressed as mean values (±SEM), and a value of  $P < 0.05$  was considered significant. Statistical significance was assessed by two-way ANOVA analyses with Newman–Keuls post-test for multiple comparisons. The specific or the nonselective inhibitors that we used did not significantly affect the activity, expression or the BBB functional integrity of the respective basal controls (data not shown).

## RESULTS

### Activity of MMP-1, -2, or -9

In the present study, we explored the idea that in alcohol abuse the BBB is disrupted via activation of MMPs because of alcohol metabolism in human brain endothelial cells. We analyzed the activity of endogenous free MMPs and total MMPs (free active and bound MMPs in its pro-form) in human BMVEC culture media after treatment with EtOH/AA for 2–96 h. Total MMPs were activated by *p*-aminophenylmercuric acetate (APMA) following the manufacturer’s instructions (Amersham). We found that MMP-1 or -9 was mainly represented by free active form while MMP-2 existed mostly in the pro-form and very little in inducible form. BMVEC treatment with EtOH/AA respectively enhanced the activity of MMP-1 by 119/141%, MMP-2 by 132/124%, and MMP-9 by 215/1,380% ( $P < 0.001$ ) compared with controls (Figs. 1A–C). Treatment of BMVEC with EtOH (for 2, 4, 8, 24, 48, 72, and 96 h) resulted in maximum MMP-1, -2, or -9 activity at 24–48 h, while AA showed highest MMPs activity at 2 h (tested at 2, 4, 8, 24, and 48 h). A potent stimulator of MMPs, IL-1β (used as a positive control) augmented the MMPs activities (MMP-1 by 725%, MMP-2 by 65%, and MMP-9 by 315%,  $P < 0.001$ ). Increase in MMPs activities after EtOH or AA treatment were inhibited by 4-MP (inhibitor of EtOH metabolizing enzymes), ES (inhibitor of MMP-2 or -9), GS (PTK inhibitor), or UA (antioxidant), suggesting that MMPs activation was mediated by EtOH metabolism and ROS production via PTK stimulation.

### Expression of MMPs/TIMPs Protein

EtOH/AA-mediated increase in MMPs activity correlated with the significant up-regulation of protein content for MMP-1 (by 30–47%), MMP-2 (by 17–18%), and MMP-9 (by 111–211%,  $P < 0.001$ ) compared with controls (Figs. 2A–C). Treatment of BMVEC with 4-MP, UA, ES, or GS prevented the EtOH/AA-induced increase expression of MMPs protein, suggesting that EtOH metabolism and ROS production initiated the activation of MMPs via PTK signaling pathway. We expected that

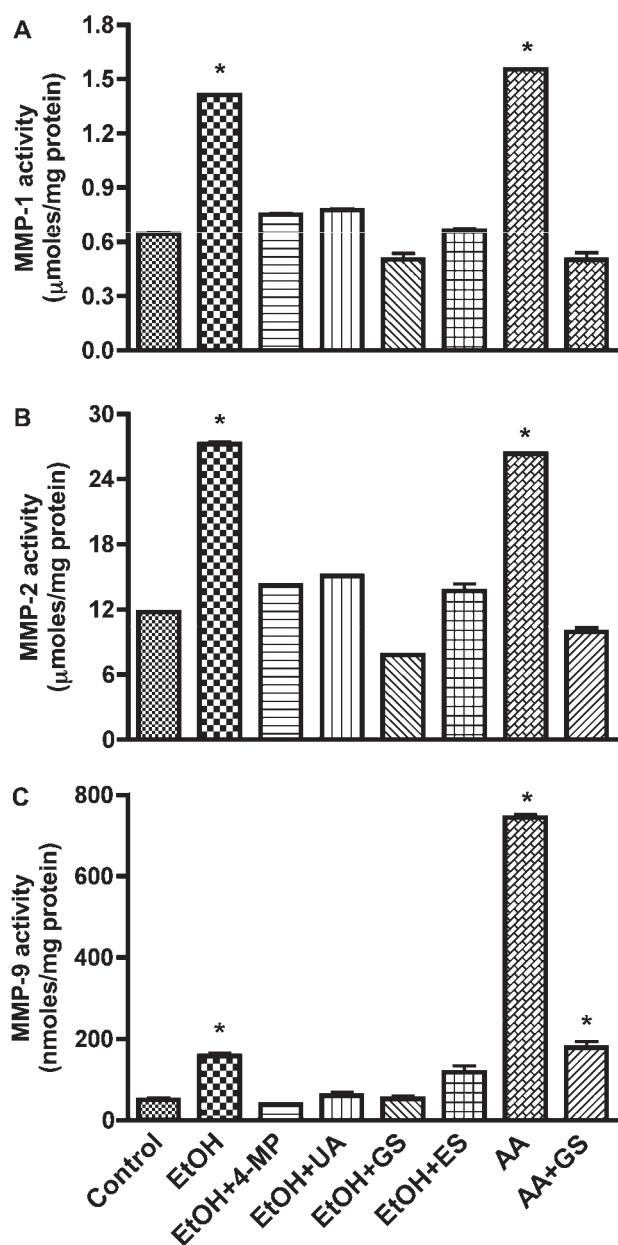


Fig. 1. Secreted proteins from BMVEC cultured media after treatment with EtOH 48 h or with AA for 2 h were assayed for activity of (A) MMP-1, (B) MMP-2, and (C) MMP-9. Results presented are inducible endogenous active form for MMP-1 and -9, total activity for MMP-2. MMPs activities were calculated from standard curve, and results were expressed as nmol or μmol/mg protein ( $\pm$ SEM;  $n = 4$ ). \*Indicates  $P$ -values  $< 0.01$  compared with control. 4-MP (inhibitor of ADH/CYP2E1), UA (antioxidant), GS (PTK inhibitor), ES (inhibitor of MMP-2/-9).

increased MMPs activity and expression after BMVEC treatment with EtOH/AA would diminish the levels of TIMP-1 and -2 proteins, natural inhibitors of MMPs. Indeed, EtOH significantly decreased TIMP-1 and -2 protein contents by 22 and 36% ( $P < 0.01$ ) respectively, and AA also lowered TIMP-1 and -2 levels by 21 and 36% ( $P < 0.01$ ) respectively, compared with controls (Figs. 3A,B). Application of 4-MP, UA, ES, or GS prevented decrease in TIMPs levels, indicating the role of

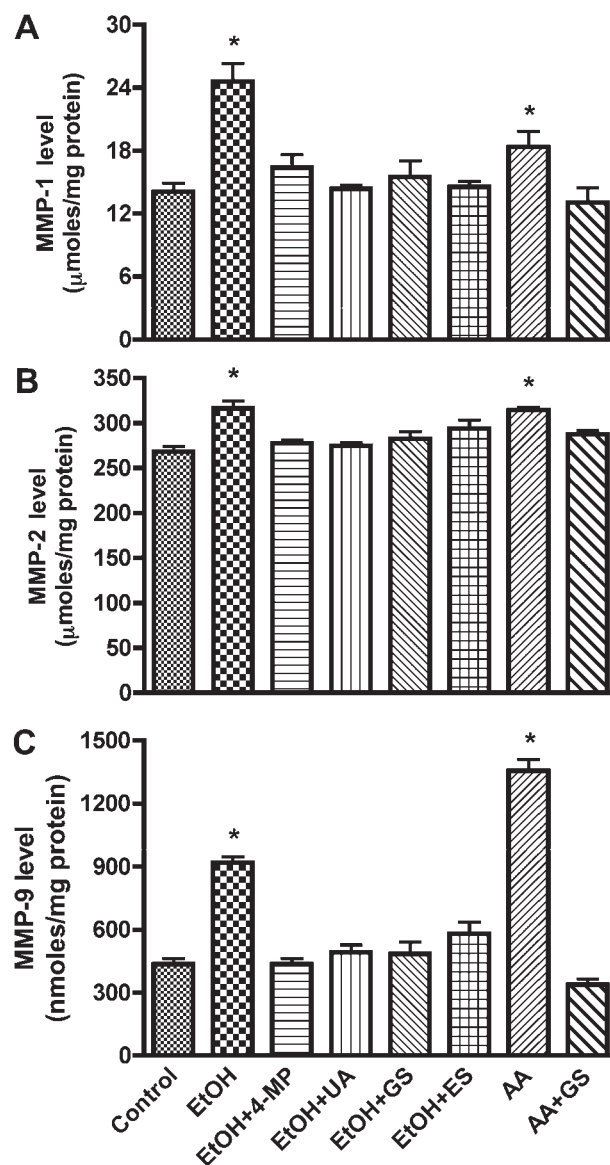


Fig. 2. Secreted proteins from BMVEC cultured media after treatment with EtOH 48 h or with AA for 2 h were assayed for the content of (A) MMP-1, (B) MMP-2, and (C) MMP-9. Total MMP-1, -2, and -9 protein contents were calculated from standard curve, and results were expressed as ng/μg/mL condition media ( $\pm$ SEM;  $n = 4$ ). \*Indicates  $P$ -values  $< 0.01$  compared with control. Inhibitors did not significantly affect MMPs activity or expression of respective basal controls.

EtOH metabolism, ROS production, and activation of MMPs via PTK signaling pathway.

### BM Protein Degradation

To determine whether MMPs activation could result in degradation of BM proteins, we assessed the changes in fluorescein-conjugated collagen IV after EtOH/AA exposure. EtOH/AA-induced degradation of collagen IV was prevented by GS or ES, suggesting that MMPs activation was regulated by PTK signaling in BMVEC (Fig. 4A1-9). In the absence of BMVEC monolayers (without

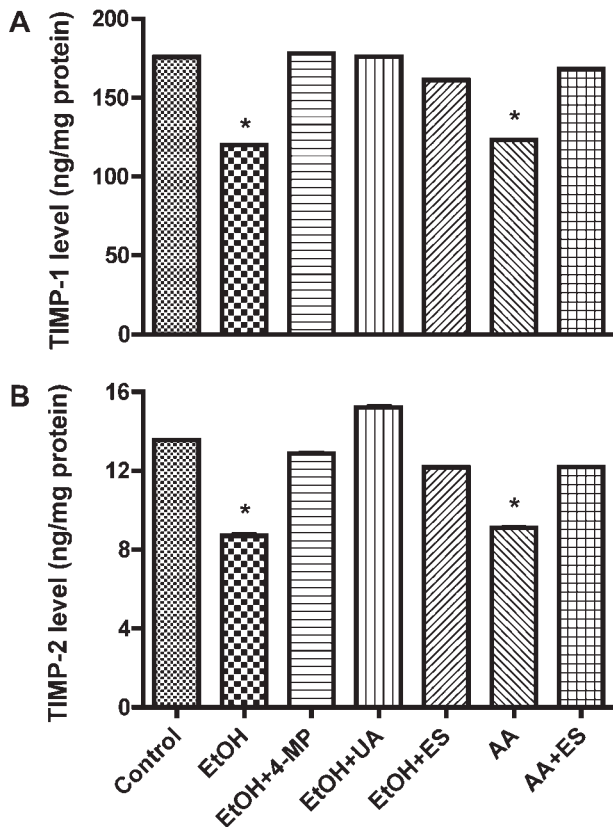


Fig. 3. Secreted proteins from BMVEC cultured media after a 48 h treatment with EtOH or 2 h with AA were analyzed for levels of (A) TIMP-1 or (B) TIMP-2 expression using the ELISA kits. TIMPs levels were calculated from standard curve and results were expressed as ng/mg protein ( $\pm$ SEM;  $n = 4$ ). Respective inhibitor did not change the TIMPs expression of basal controls.

CYP2E1/ADH), EtOH had no effect on collagen IV degradation (cell-free + EtOH), supporting the idea that EtOH metabolism was essential for MMPs activation and BM protein degradation (Fig. 4 A4 and A7 compared with control, A3). These qualitative results were further confirmed by quantitative analysis. Exposure of BMVEC with EtOH or AA showed 42 or 62% reduction in collagen IV levels compared with control (Fig. 4B). GS or ES prevented the effects of EtOH or AA on type IV collagen degradation, indicating that PTK-mediated activation of MMPs led to BM protein degradation. We observed 14% of fluorescein-conjugated collagen IV initially bound to collagen I prior to BMVEC culture (14  $\mu$ g/mL bound out of 100  $\mu$ g/mL applied). After 72 h of BMVEC (control) culture at 37°C, there was 42% drop in amount of collagen IV compared with cell-free, suggesting that the cells digested the bound IV collagen. Wells without BMVEC featured only 4% loss of collagen IV after 72 h in culture media at 37°C compared with initial 14% bound collagen IV.

#### PTK/PTP Activity and RTK Array

We proposed that PTK signaling could mediate MMPs activation after BMVEC exposure to EtOH. Therefore,

we next assayed the PTK activity in protein extracts from EtOH/AA treated BMVEC. We also examined PTP activity, because PTK activation would result in diminished PTP activity as PTK and PTP function in reciprocal fashion. EtOH and AA treatment increased PTK activity by 949 and 555% respectively, compared with controls without affecting PTP activity (Figs. 5A,B). The increase in PTK activity was inhibited by 4-MP or GS, suggesting that EtOH metabolism mediated the PTK activation. As expected, PAO (inhibitor of PTP) completely inhibited PTP activity (Fig. 5B). Using a proteome profiler array, we observed that EtOH activated four receptor PTKs (insulin receptor/insulin-like growth factor-1 receptor, IR/IGF1-R; ephrinB2 receptor, EphB2-R; vascular endothelial growth factor receptor, VEGFR; epidermal growth factor receptor, ErbB2-R) and two nonreceptor PTKs (cellular Sarcoma, c-Src kinase; focal adhesion kinase, FAK) in BMVEC (Fig. 5C). Results of EtOH effects on PTKs are shown in descending order of magnitude, compared with controls. The specific role of the individual PTK in EtOH mediated BMVEC alterations will be studied in our future experiments.

#### TJ Protein Phosphorylation

Primary function of PTK activation is the phosphorylation of cellular proteins at tyrosine residues. Therefore, we explored the idea that PTK activation would lead to an increase of phosphotyrosine levels of TJ proteins. Indeed, 48 h EtOH treatment of BMVEC augmented the phosphotyrosine levels of occludin by 171%, claudin-5 by 80%, and ZO-1 by 75% ( $P < 0.01$ ) compared with respective controls (Fig. 6A-I). Similarly, AA up-regulated the tyrosine phosphorylation of TJ proteins (occluding by 168%, claudin-5 by 82%, and ZO-1 by 62%, respectively,  $P < 0.01$ , Fig. 6A-I) supporting the idea that EtOH metabolites initiated the PTK activation and TJ protein phosphorylation. EtOH/AA-mediated increase in TJ protein phosphorylation was accompanied by diminution of total occludin and claudin-5 contents (18 and 28%, respectively,  $P < 0.01$ ) without affecting total ZO-1 level. All these changes were prevented by alcohol metabolizing enzyme inhibitor (4-MP) or by PTK inhibitor (GS), suggesting that PTK activation is due to EtOH metabolism phosphorylated TJ proteins.

#### EtOH Diminished BBB Integrity

The functional significance of MMPs activation via PTK signaling resulting in BM degradation and TJ phosphorylation were further investigated by assessing TEER, permeability, and monocyte migration across the BMVEC monolayers. BMVEC monolayers treated with EtOH/AA showed a 30% decrease in TEER compared with control (Fig. 7A). Inhibitors of EtOH-metabolizing enzymes (4-MP), PTK (GS), or MMP-2 and -9 (ES) partially restored the EtOH-induced decrease in TEER, indicating that EtOH metabolism and MMPs activation

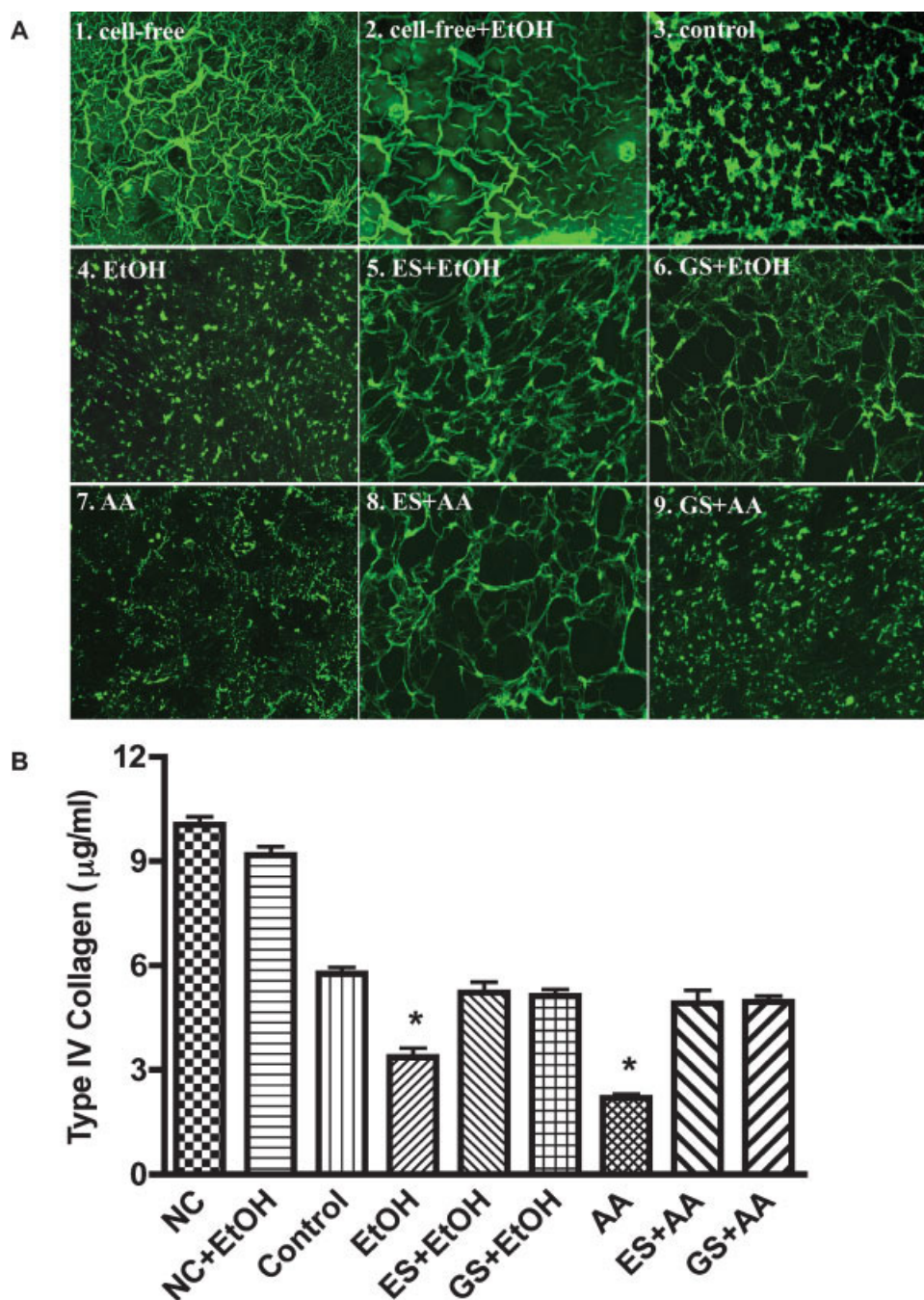


Fig. 4. (A) Confocal microscopy analysis of (1) fluorescein-conjugated collagen IV bound to collagen 1 (cell-free), (2) cell-free + EtOH incubated for 72 h. BMVEC monolayers cultured on top of collagen I and IV layers after 72 h exposure were (3) control, (4) 50 mM EtOH, (5) 20 µg/mL ES + EtOH, (6) 100 µM GS + EtOH, (7) 100 µM AA, (8) 20 µg/mL ES + AA, (9) 100 µM GS + AA. Original magnification  $\times 600$ . (B) Deg-

radation of collagen IV by MMPs activation after EtOH/AA stimulation. BMVEC cultured on top of collagen I and IV layers were treated with EtOH/AA for 72 h followed by quantitative analysis of nondegraded collagen IV amount. Results were expressed as mean µg/mL of undigested collagen IV (+SEM;  $n = 4$ ).

caused the BBB injury via PTK signaling (Fig. 7A). Treatment of BMVEC with EtOH for 48 h or with AA for 2 h augmented FITC-labeled dextran permeability (by 110 or 112%, respectively, Fig. 7B) and enhanced monocyte migration across the BMVEC monolayers (by 178 or 136%, respectively, Fig. 7C), compared with respective controls. Inhibition of alcohol metabolism, PTK

or MMP-2/-9 prevented increased in permeability and monocyte migration indicating that BBB injury was mediated by MMPs activation via PTK signaling. Taken together, these data suggested that EtOH associated BBB dysfunction was caused by PTK activation leading to MMPs stimulation, BM degradation, and TJ protein modifications. Figure 8 summarized these findings.

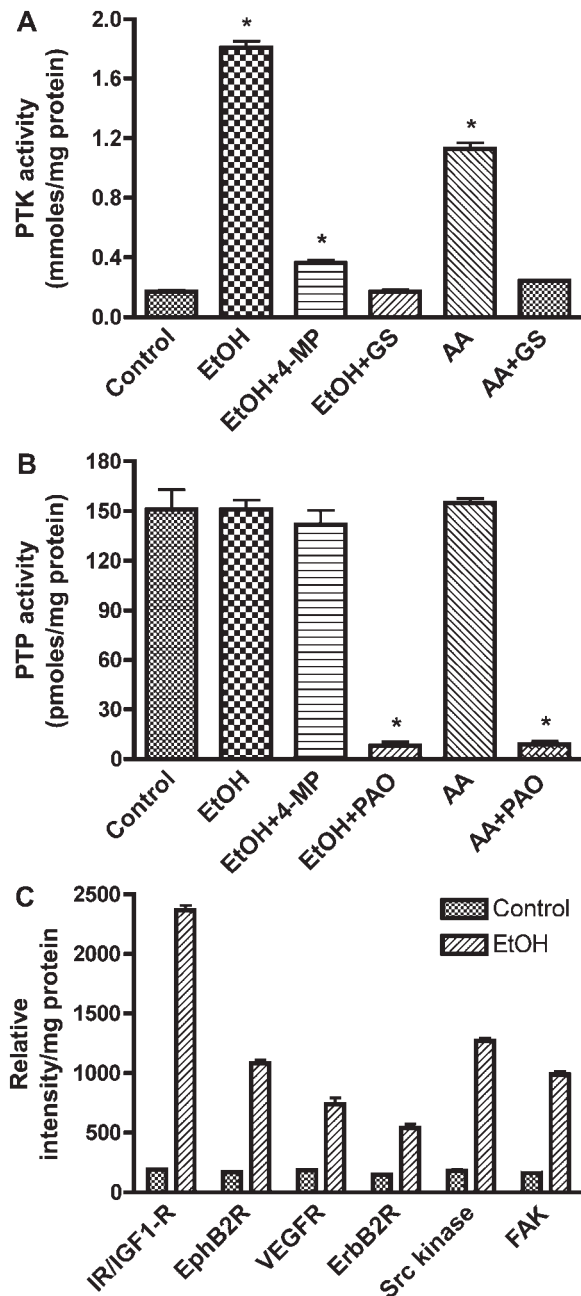


Fig. 5. Lysate proteins (20  $\mu$ g/well) derived from human BMVEC treated with EtOH for 48 h or with AA for 2 h were assayed for PTK/PTP activity. Activity of (A) PTK or (B) PTP was calculated from respective standard curve. Results were expressed as mmol or pmol/mg protein ( $\pm$ SEM;  $n = 4$ ). \*Designates significant decrease ( $P < 0.01$ ) compared with control. PAO is an inhibitor of PTP. (C) Using 100  $\mu$ g of lysate protein, EtOH-activated receptor and nonreceptor PTKs detected by human proteome profiler array kit compared with untreated controls ( $\pm$ SEM;  $n = 4$ ).

## DISCUSSION

Accumulated evidence acknowledges that chronic alcohol consumption increases the risk factor for developing cerebral vascular diseases such as stroke (Hillbom and Kaste, 1990; Regan, 1990). The putative mechanisms of such effects still remain undefined. Our results provided

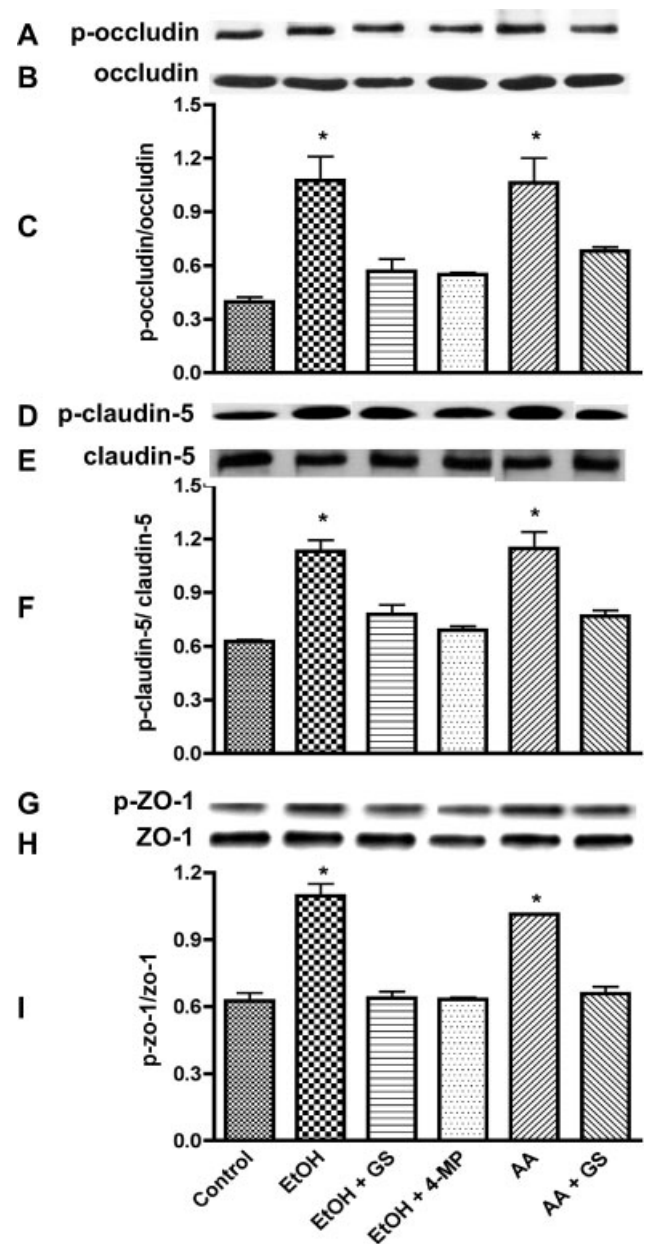


Fig. 6. Lysate proteins derived from human BMVEC treated with EtOH for 48 h or with AA for 2 h in the presence or absence of 4-MP or GS were subjected to Western blot analysis after immunoprecipitation. Representative immunoreactive bands of (A) occludin-phosphotyrosine, (B) total occludin, (C) ratio of occludin-phosphotyrosine/total occludin, (D) claudin-5-phosphotyrosine, (E) total claudin-5, (F) ratio of claudin-5-phosphotyrosine/total claudin-5, (G) ZO-1-phosphotyrosine, (H) total ZO-1, (I) ratio of ZO-1-phosphotyrosine/total ZO-1. \*Indicates statistical differences ( $P < 0.01$ ) compared with control. Respective inhibitor did not change the expression of phosphorylated TJ protein of the basal controls.

the first evidence that EtOH at pathophysiological doses activated several receptor and nonreceptor PTKs in primary human BMVEC leading to the diminished BBB tightness. Alcohol-induced PTK activation up-regulated activities of MMPs, led to BM degradation, and phosphorylation of TJ proteins (occludin, claudin-5, and ZO-1) at tyrosine residues, causing cumulative disruption of BBB

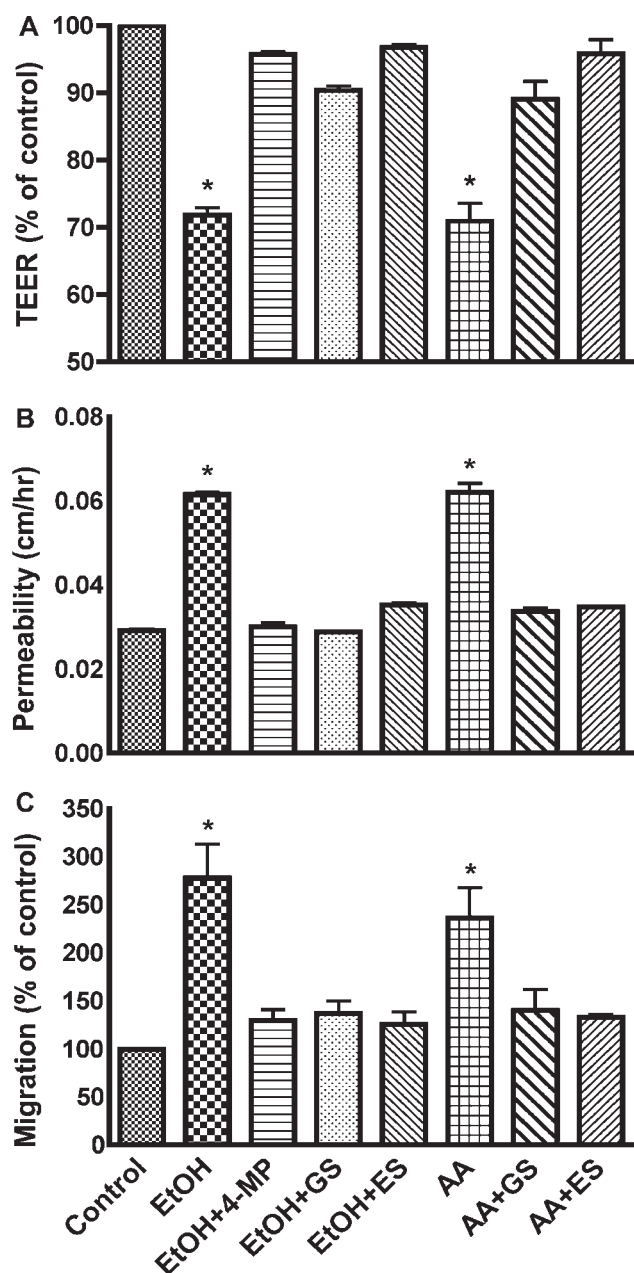


Fig. 7. Effects of 50 mM EtOH or 100  $\mu$ M AA on the BBB functional assays (A) Changes in TEER, (B) 4 kDa dextran permeability, and (C) monocyte migration across the BBB. Prior to TEER, permeability or migration assay, cell monolayers were treated with EtOH for 48 h or with AA for 2 h in the presence or absence of specific inhibitor. Results were expressed as percent of controls or apparent permeability coefficient "P" ( $\pm$  SEM;  $n = 3$ ). Respective inhibitor alone did not change the BBB functional integrity of the basal controls.

integrity. Consistent with our previous findings, enhanced tyrosine phosphorylation of TJ proteins paralleled decrease in TEER, increased permeability to low molecular tracer, and monocyte migration across the BBB (Haorah et al., 2007b). These events may trigger BBB breakdown commonly observed in stroke and neurodegenerative disorders, conditions frequently associated with oxidative damage and MMPs activation (Kim et al., 2003; Rosell et al., 2006).

Importantly, 4-MP or anti-oxidant prevented the EtOH-mediated increase in MMPs activity/protein level and subsequent degradation of BBB essential BM proteins, suggesting that EtOH metabolism and ROS production initiated the MMPs activation. Treatment of BMVEC with AA also activated PTK and MMPs confirming the role of EtOH metabolism in this process. PTK signaling regulated this redox-sensitive mechanism because PTK inhibitor prevented the EtOH-mediated MMPs-2/-9 activation, BM degradation, tyrosine phosphorylation of TJ proteins, and BBB dysfunction even in the absence of 4-MP or UA. These results suggest that PTK activation occurred prior to MMPs protein expression, which was also confirmed by the fact that PTK activation started within 24 h of EtOH treatment, whereas increase in MMPs activity or protein expression occurred after 24 h of EtOH exposure. Activation of PTK signaling by oxidative stress has been demonstrated in human smooth muscle cells and in gerbil hippocampus (Du et al., 1999; Whisler et al., 1994; Zalewska et al., 2003). Others and we have shown that increase in PTK activity was accompanied by inactivation of PTP after ROS exposure in different cell types, including BMVEC (Chen et al., 2006; Haorah et al., 2007b; Tao et al., 2005). This concurrent event was due to transient oxidation of catalytic cysteine on PTP and trans-activation of PTK because of an autophosphorylation of PTK receptor in a reversible manner (Meng et al., 2002). Such alterations were associated with MMPs activation and phosphorylation of occludin and ZO-1 (Lohmann et al., 2004). Contrary to these findings, EtOH metabolism in BMVEC up-regulated PTK activity and protein level without affecting PTP activity.

The neurovascular unit composed of BMVEC, BM, pericytes, microglia, astrocytes, and neurons are integral components of the BBB assembly. In particular, *in vitro* studies of endothelial-astrocytes coculture model support the idea that astrocytes are essential for the formation of BBB (Holash et al., 1993). Direct contact between BMVEC and astrocytes are necessary for optimal tightness of the barrier (Rubin et al., 1991), as such, astrocytes end-feet envelop about 99% of the outer surface of the CNS capillaries (Hawkins and Davis, 2005). Astrocyte-BMVEC interaction promotes the integrity of TJ assembly of brain endothelium (Abbott, 2002). The fact that coculture of astrocytes and BMVEC decreases permeability and increases TEER across BBB via increased expression of ZO-1 (Siddharthan et al., 2007), while coculture of GFAP-deficient astrocytes and BMVEC impairs the induction of these BBB properties (Pekny et al., 1998) clearly indicate the importance role of glial cells in the maintenance of BBB function. In addition to regulation of TJ proteins expression, astrocytes also improve barrier function through enhanced expression of P-glycoprotein (Gaillard et al., 2000). Conversely, secretion of IL-1 $\beta$  by astrocytes mediates endothelin-1 and TNF- $\alpha$  induced BBB breakdown during CNS inflammation (Didier et al., 2003). Recent findings implicate the involvement of toll-like receptors (TLRs) in neuroinflammation and neurodegeneration in the CNS, in which



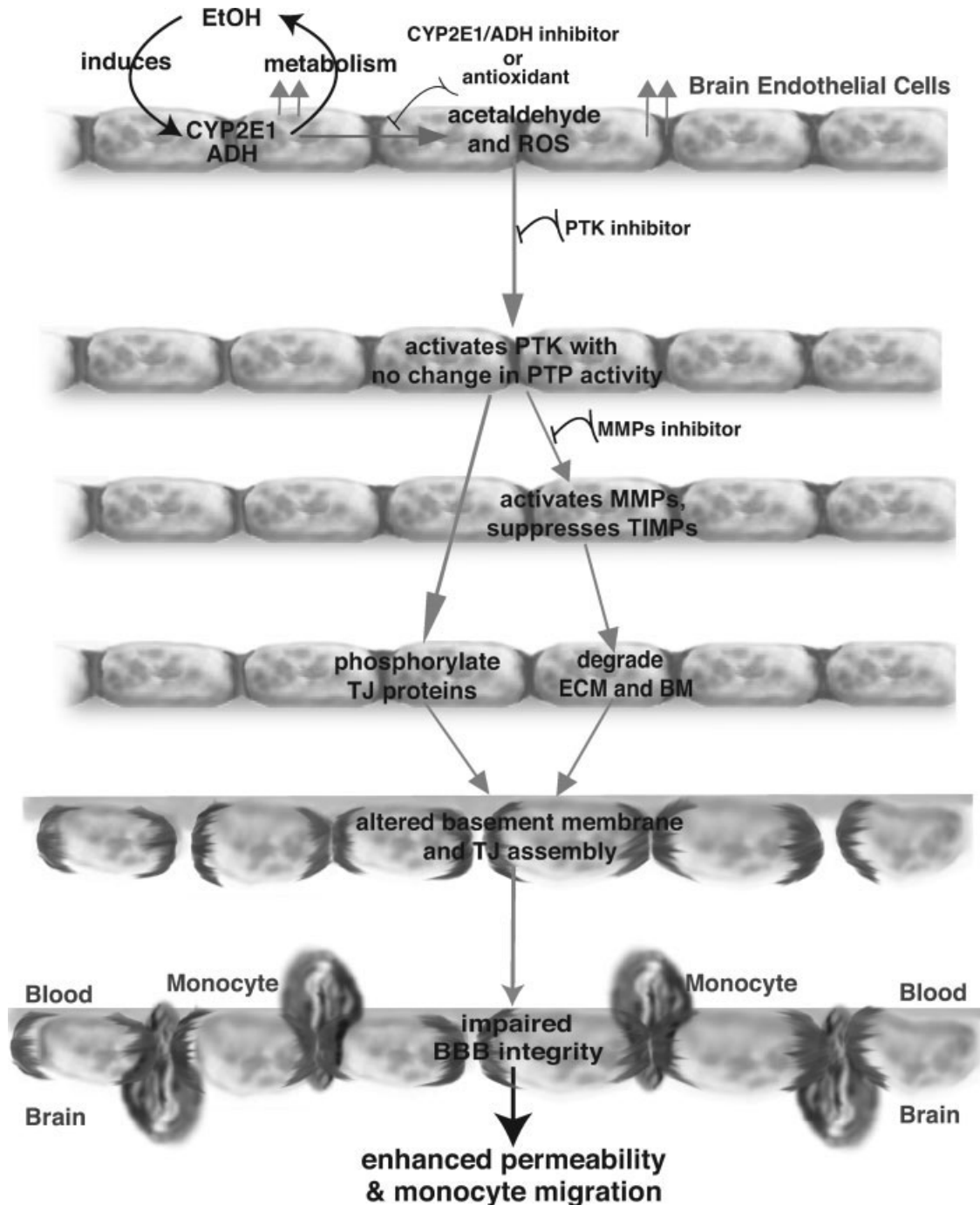


Fig. 8. Schematic representation of BBB dysfunction due to MMPs activation via PTK signaling pathway stemming from EtOH metabolism in human BMVEC as delayed effects. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

alcohol-induced astrocytes or microglia indicate the enhanced expression of TLR2 and TLR4 via IRAK/MAPK activation pathway (Blanco and Guerri, 2007). Further, our unpublished data indicate that human astrocytes secreted high levels of MMP-1 and -9 following exposure to EtOH, suggesting the role of BM protein degradation and BBB compromise as a result of glial activation by ethanol.

Using proteome profiler array for human PTKs, we observed that 50 mM EtOH selectively increased the phosphorylation/activation of receptor PTKs (IR/IGF1-R, EphB2-R, VEGFR, and ErbB2-R) and non-receptor PTKs (c-Src kinase, FAK). These events were associated with MMPs activation in human BMVEC. Trans-activation of VEGFR caused MMP-2 and -9 activation in pressure-induced resistance in mouse mesenteric arteries

(Lucchesi et al., 2004) was associated with MMP-2 and -9 activation. High EtOH concentration (100–200 mM) inhibited the tyrosine autophosphorylation of IGF-IR in neuroblastoma cells (Seiler et al., 2001); and chronic ethanol administration decreased the ligand binding properties, protein content, and the synthesis of the IGF-IR in rat hepatocytes (Haorah et al., 2002, 2003). The differential effects of EtOH on IGF-IR autophosphorylation could be related to EtOH concentration, because 100–200 mM EtOH inhibited MMP-2 activity; whereas, lower concentrations (<100 mM) activated MMP-2 in an ErbB2-R dependent manner in breast cancer cells (Aye et al., 2004; Luo, 2006).

EtOH-mediated increase in MMPs activity and protein level correlated with enhanced degradation of BM, the essential BBB component. Notably, ES prevented the increases in MMPs activity and BM degradation, indicating direct involvement of VEGFR in MMPs activation. Vascular endothelial growth factor (VEGF, a potent mediator of angiogenesis or tumorigenesis) binds to its receptor KDR/Flk-1 (VEGFR) and augments the receptor tyrosine phosphorylation leading to activation of the downstream extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and p125<sup>FAK</sup> pathways (Ferrara, 1996). The molecular mechanism is that direct binding of ES to KDR/Flk-1 prevents VEGF-KDR/Flk-1 interaction (causes VEGFR inactivation); thus, ES acts as an effective anti-angiogenic and anti-tumorigenic agent (Kim et al., 2002). ES can form a stable complex with pro-MMPs by directly binding to the catalytic site of MMPs preventing tumor-associated angiogenesis (Kim et al., 2000; Nyberg et al., 2003). We proposed that EtOH-induced MMPs activation via PTKs signaling was also regulated by downstream p38 MAPK or p125<sup>FAK</sup> pathway, because our results demonstrated a selective activation of both receptor (VEGFR, IR) and nonreceptor (Src kinase, FAK) PTK. VEGFR-mediated MMPs activation and BM disruption were demonstrated in stroke patients (Fukuda et al., 2004; Rosell et al., 2005). Ethanol consumption was shown to induce hemorrhagic and ischemic strokes in both experimental studies and epidemiological observations (Yang et al., 2001). Interestingly, PTK inhibition ameliorated deleterious effects of EtOH on arterial wall. Importantly, ES prevented MMPs activation in stroke patients (Wang et al., 2006). We conclude that MMPs activation by EtOH metabolism in the brain endothelium could be a major risk factor for stroke. We proposed that ES and specific inhibitor to selective PTK could be a beneficial therapeutic candidate for the treatment of stroke and patients with other neurological disorders.

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