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Eugen Brailoiu  
*Temple University*

Christine L. Barlow  
*Thomas Jefferson University*

Servio H. Ramirez  
*Temple University*

Mary E. Abood  
*Temple University*

G. Cristina Brailoiu  
*Thomas Jefferson University*

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## Effects of Platelet-Activating Factor on brain microvascular endothelial cells

Eugen Brailoiu<sup>1</sup>, Christine L. Barlow<sup>2</sup>, Servio H. Ramirez<sup>1,3</sup>, Mary E. Abood<sup>1</sup>, and G. Cristina Brailoiu<sup>2,\*</sup>

<sup>1</sup>Center for Substance Abuse Research, Lewis Katz School of Medicine, Philadelphia, PA 19140

<sup>2</sup>Department of Pharmaceutical Sciences, Jefferson College of Pharmacy, Philadelphia, PA 19107

<sup>3</sup>Department of Pathology & Laboratory Medicine, Lewis Katz School of Medicine, Philadelphia, PA 19140

### Abstract

Platelet-activating factor (PAF) is a potent phospholipid mediator that exerts various pathophysiological effects by interacting with a G protein-coupled receptor. PAF has been reported to increase the permeability of the blood-brain barrier (BBB) via incompletely characterized mechanisms. We investigated the effect of PAF on rat brain microvascular endothelial cells (RBMVEC), a critical component of the BBB. PAF produced a dose-dependent increase in cytosolic Ca<sup>2+</sup> concentration; the effect was prevented by the PAF receptor antagonist, WEB2086. The effect of PAF on cytosolic Ca<sup>2+</sup> was abolished in Ca<sup>2+</sup>-free saline or in the presence of L-type voltage-gated Ca<sup>2+</sup> channel inhibitor, nifedipine, indicating that Ca<sup>2+</sup> influx is critical for PAF-induced increase in cytosolic Ca<sup>2+</sup>. PAF produced RBMVEC depolarization; the effect was inhibited by WEB2086. In cells loaded with DAF-FM, a nitric oxide (NO)-sensitive fluorescent dye, PAF increased the NO level; the effect was prevented by WEB2086, nifedipine or by L-NAME, an inhibitor of NO synthase. Immunocytochemistry studies indicate that PAF reduced the immunostaining of ZO-1, a tight junction-associated protein, increased F-actin fibers, and produced intercellular gaps. PAF produced a decrease in RBMVEC monolayer electrical resistance assessed with Electric Cell-Substrate Impedance Sensing (ECIS), indicative of a disruption of endothelial barrier function. *In vivo* studies indicate that PAF increased the BBB permeability, assessed with sodium fluorescein and Evans Blue methods, via PAF receptor-dependent mechanisms, consequent to Ca<sup>2+</sup> influx and increased NO levels. Our studies reveal that PAF alters the BBB permeability by multiple mechanisms, which may be relevant for central nervous system (CNS) inflammatory disorders.

\*Address correspondence to G.C. Brailoiu, M.D., Department of Pharmaceutical Sciences, Jefferson College of Pharmacy, 901 Walnut St, Suite 901, Philadelphia, PA 19107, Phone: 215-503-7468; Gabriela.Brailoiu@jefferson.edu.

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## Keywords

blood-brain barrier; barrier disruption; calcium signaling; electrical; resistance; PAF

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## Introduction

Platelet-activating factor (PAF) is a bioactive phospholipid messenger, originally reported to produce platelet aggregation after being released from IgE-sensitized basophils (Benveniste et al., 1972). In addition to platelet activation, PAF has been ascribed to other physiological and pathophysiological functions (Prescott et al., 2000, Liu et al., 2016). PAF is a potent inflammatory factor, involved in pathogenesis of several diseases such as anaphylaxis, asthma, rheumatoid arthritis, atherosclerosis, glomerulosclerosis, and diabetes (Hilliquin et al., 1995, Prescott et al., 2000, Tsoupras et al., 2009, Liu et al., 2016). PAF also induces smooth muscle contraction, and increases vascular permeability (Kornecki and Ehrlich, 1988, Lacerda-Queiroz et al., 2012, Vadas et al., 2013, Jeewandara et al., 2015).

### PAF synthesis occurs via two pathways

*de novo*, and by the cytosolic phospholipase A<sub>2</sub>-dependent remodeling pathway, with the latter being predominant (Liu et al., 2016). Similarly to the eicosanoids, PAF is not stored preformed, but rapidly synthesized intracellularly in response to cell-specific stimuli (Prescott et al., 2000). Synthesis and release of PAF occur in a variety of cells, predominantly in endothelial cells and leukocytes (Aliberti et al., 1999, Mariano et al., 2003). The synthesis, distribution and degradation of PAF are tightly controlled, contributing to brief and selective responses (Prescott et al., 2000).

PAF exerts its effects by interaction with a G protein-coupled receptor, namely PAF receptor (Alexander et al., 2015). PAF receptor, the first cloned receptor for lipids, is expressed in a variety of cell types (Honda et al., 1991, Bito et al., 1994, Ihida et al., 1999), including brain microvascular cells (Predescu et al., 1996). Activation of the PAF receptor promotes inositol trisphosphate (IP<sub>3</sub>) formation via phospholipase C (PLC) stimulation; it also stimulates protein kinases, such as protein kinase C and tyrosine kinase (Barzaghi et al., 1989, Shukla, 1992). In addition, PAF was shown to produce Ca<sup>2+</sup> influx in cell lines such as NG108-15, mouse neuroblastoma-rat glioma cells, and PC12, rat pheochromocytoma cells (Kornecki and Ehrlich, 1988).

In the central nervous system (CNS), PAF was involved in the inflammatory response, pathogenesis of meningitis, ischemia-reperfusion injury, stroke, multiple sclerosis, Alzheimer's and Parkinson's diseases, or HIV-associated neurocognitive disorders (Feuerstein, 1996, MacLennan et al., 1996, Liu et al., 2016, Reiner et al., 2016). PAF has been reported to produce a transient increase in blood-brain barrier (BBB) permeability (Fang et al., 2011, Fang et al., 2014), however the effects of PAF on endothelial cells of the BBB remain incompletely characterized. The current study investigated the effects elicited by PAF on rat brain microvascular endothelial cells, an experimental model of BBB, on cytosolic Ca<sup>2+</sup> concentration, membrane potential, nitric oxide (NO) level and electrical

resistance. In addition, *in vivo* studies examined the contribution of the pathways identified for PAF *in vitro* on the rat BBB permeability.

## Experimental Procedures

### Ethical approval

Animal protocols were approved by the Institutional Animal Care and Use Committee from each institution.

### Chemicals and reagents

PAF (C-16 PAF) and WEB2086 were from Tocris Biosciences (Bristol, UK). Fura-2AM, DAF-FM, DiBAC<sub>4</sub>(3), and ActinRed555 were from Molecular Probes (ThermoFisher Scientific, Waltham, MA). Other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned.

### Cell Culture

Rat brain microvascular endothelial cells (RBMVEC), purchased from Cell Applications, Inc (San Diego, CA) were cultured as previously described (Altmann et al., 2015, Brailoiu et al., 2017). Cells were grown in endothelial basal medium enriched with endothelial growth supplement, in T75 flasks coated with attachment factor (Cell Applications, Inc). For immunocytochemistry studies, cells were plated on 12 mm diameter glass coverslips. For live imaging studies, cells were grown on 25 mm diameter glass coverslips coated with human fibronectin (Discovery Labware, Bedford, MA). For impedance measurements, cells were grown on 8W10E+ arrays (Applied BioPhysics, Inc., Troy, NY) coated with fibronectin.

### Cytosolic Ca<sup>2+</sup> measurement

Cytosolic Ca<sup>2+</sup> concentration, [Ca<sup>2+</sup>]<sub>i</sub>, was assessed in RBMVEC loaded with Fura-2 AM (Molecular Probes, ThermoFisher Scientific, Waltham, MA), as previously described (Altmann et al., 2015, Brailoiu et al., 2016, Brailoiu et al., 2017). Cells on coverslips were incubated with Fura-2 AM (5 μM, 1 hour, room temperature) in Hanks' Balanced Salt Solution (HBSS). Coverslips, after washing with dye-free HBSS, were mounted on the stage of a Nikon Eclipse TiE microscope (Nikon Inc., Melville, NY), in an open bath chamber. Fura-2 AM fluorescence (emission 510 nm), following alternate excitation at 340 and 380 nm, was recorded using a Photometrics CoolSnap HQ2 CCD camera (Photometrics, Tucson, AZ) and NIS-Elements AR software (Nikon). The ratio of the fluorescence signals (340/380 nm) was converted to Ca<sup>2+</sup> concentrations (Grynkiewicz et al., 1985).

### Measurement of membrane potential

Changes in RBMVEC membrane potential were assessed using a voltage-sensitive dye, bis-(1,3-dibutylbarbituric acid)-trimethine-oxonol, DiBAC<sub>4</sub>(3) (Molecular Probes), as reported (Brauner et al., 1984, Altmann et al., 2015). RBMVEC were incubated in DiBAC<sub>4</sub>(3) (0.5 μM in HBSS, 30 min) and the fluorescence (excitation/emission 480 nm/540 nm) monitored. Membrane depolarization produces an increase in fluorescence intensity consequent to

accumulation of the dye into the cytosol, (Brauner et al., 1984). Calibration of DiBAC<sub>4</sub>(3) fluorescence was performed as previously reported (Altmann et al., 2015).

### **NO measurement**

Intracellular NO was measured in RBMVEC loaded with DAF-FM [(4-amino-5-methylamino-2',7'-difluoro-fluorescein) diacetate] (Molecular Probes) as described (Kojima et al., 1998, Altmann et al., 2015). RBMVEC were incubated in DAF-FM (0.5  $\mu$ M in HBSS, 45 min, room temperature) (Leikert et al., 2001) and the DAF-FM fluorescence (excitation/emission 480 nm/ 540 nm) was monitored.

### **Immunocytochemistry**

Immunocytochemistry studies were performed as described earlier (Brailoiu et al, 2011, Brailoiu et al, 2017). RBMVEC grown on 12 mm diameter glass coverslips, were treated for 10 min with PAF (1  $\mu$ M), WEB2086 (5  $\mu$ M), L-NAME (100  $\mu$ M), nifedipine (1  $\mu$ M). In other experiments, cells were treated with WEB2086, L-NAME or nifedipine for 15 min, followed by PAF for 10 min; untreated cells served as control. After rinsing with phosphate buffer saline (PBS), cells were fixed in 4% paraformaldehyde. Cell fixation was followed by additional rinsing with PBS and PBS with 0.5% Triton X for 5 min, and incubation with normal goat serum. Cells were then incubated with primary antibody ZO-1 (rabbit IgG, Molecular Probes) overnight at 4°C, followed by incubation with secondary antibody (goat anti-rabbit, conjugated to Alexa 488, 2 hours, room temperature). Cells were washed in PBS and incubated with ActinRed 555 (30 min, room temperature). After washing in PBS, cells were mounted with DAPI Fluoromount G (SouthernBiotech, Birmingham, AL), sealed, and examined under a Leica DMI6000B fluorescence microscope.

### **Impedance Measurements**

Impedance measurements were carried out via electric cell-substrate impedance sensing (ECIS) method, using a Z $\theta$  controller, an 16W array holder station and 8W10E+ arrays, similarly with previous reports (Stolwijk et al, 2015, Stolwijk et al, 2016). RBMVEC were cultured at a density of 100,000 cells/cm<sup>2</sup> on 8W10E+ arrays treated with L-cysteine (10 mM, 200  $\mu$ l/well, 20 min, room temperature) and coated with fibronectin (50 $\mu$ g/ml, 200  $\mu$ l/well, 30 min, 37°C). Cells were grown on arrays for 48 h in an incubator (37°C, 5% CO<sub>2</sub>, humidified atmosphere) before drug treatment. To assess the change in paracellular currents in response to agonists (PAF and histamine), the resistance part of impedance, was normalized to the value before the addition of the agonist and plotted as function of time.

### **MTT assay**

We assessed the cell viability using a colorimetric assay with methylthiazolyldiphenyl-tetrazolium bromide (MTT). This assay is based on the conversion of the water-soluble tetrazolium salt (yellowish) by mitochondrial dehydrogenase of living cells to water-insoluble formazan (dark-blue). RBMVEC were cultured in 96-well plates at a density of 10,000 cells/well. After 48 hours in culture, cells were treated with PAF (0.1- 5  $\mu$ M) for one hour. Cells were treated with MTT (10  $\mu$ l/well) and incubated at 37°C for 3 hours. The

medium containing MTT was removed and DMSO (50  $\mu$ l/well) added. After gently mixing for 5 min, the absorbance at 570 nm was read using a Synergy plate reader.

### Permeability assay with sodium fluorescein

BBB permeability was assessed by measuring the sodium fluorescein content in the rat brain, similarly with previous reports (Lenzser et al., 2007). The drugs tested or saline (control) were administered i.v. in the tail vein 30 min after systemic injection of sodium fluorescein (2%, 4 mL/kg). One hour after drug administration, the rats were anesthetized and transcardially perfused with ice-cold PBS. The brain was removed, rinsed, weighed and homogenized in PBS. The homogenate was treated with trichloroacetic acid (80%), then centrifuged (20 mins, 10,000g). The supernatant was diluted with 5 M NaOH, and its fluorescence was determined (excitation/emission- 440 nm/525 nm) using a microplate reader. The concentration of sodium fluorescein in the brain was quantified and expressed per gram of tissue.

### Evans Blue extravasation method

BBB disruption was assessed quantitatively by measuring Evans Blue extravasation as previously reported (Uyama et al., 1988, Radu and Chernoff, 2013). Evans blue 2% in PBS (4 mg/Kg) was injected i.v. via tail vein, 30 min before the administration of drugs tested. One hour later, rats were anesthetized with ketamine (100 mg/kg) and perfused transcardially with PBS. The brain was dissected, weighed and homogenized in PBS. The homogenate was treated with trichloroacetic acid (80%), incubated at 4 °C, 1 h and centrifuged (20 min, 10,000 g). The supernatant's absorbance at 610 nm was determined using a plate reader. Brain Evans Blue concentration was quantified from a linear standard curve plotted from known amounts of Evans Blue dye.

### Statistical analysis

Data were expressed as mean  $\pm$  standard error of mean. One-way ANOVA followed by Bonferonni and Tukey tests was used to evaluate significant differences between groups;  $P < 0.05$  was considered statistically significant.

## Results

### PAF increases cytosolic $\text{Ca}^{2+}$ concentration, $[\text{Ca}^{2+}]_i$ , in RBMVEC

The cytosolic  $\text{Ca}^{2+}$  concentration,  $[\text{Ca}^{2+}]_i$ , before and after treatment with PAF was assessed in Fura 2-AM-loaded RBMVEC cells. PAF (1  $\mu$ M) increased the RBMVEC fluorescence ratio  $F_{340}/F_{380}$  (Fig. 1A). The increase in fluorescence was converted to  $\text{Ca}^{2+}$  concentration (Grynkiewicz et al., 1985). PAF produced a fast and transient increase in  $[\text{Ca}^{2+}]_i$ , that was inhibited by the PAF receptor antagonist, WEB2086 (5  $\mu$ M). Cells were pretreated with WEB2086 for 15 min before PAF treatment. Examples of averaged  $\text{Ca}^{2+}$  responses induced by PAF, and by PAF in the presence of WEB2086 are shown in Fig 1B. The comparison of the amplitude of  $\text{Ca}^{2+}$  responses produced by different concentrations of PAF (0.1-5  $\mu$ M) is shown in Fig. 1C. PAF (0.1  $\mu$ M, 0.5  $\mu$ M, 1  $\mu$ M and 5  $\mu$ M) produced an increase in  $[\text{Ca}^{2+}]_i$  by  $73 \pm 1.9$  nM,  $146 \pm 2.7$  nM,  $579 \pm 4.16$  nM, and  $638 \pm 5.09$  nM, respectively (n= 35-65 cells) (Fig. 1C).

### PAF promotes Ca<sup>2+</sup> influx in RBMVEC

In Ca<sup>2+</sup>-free saline, or in the presence nifedipine (1 μM), a L-type Ca<sup>2+</sup> channels blocker the Ca<sup>2+</sup> response to PAF (1 μM) was absent. Cells were pretreated with nifedipine 15 min before PAF treatment. To verify the functionality of internal Ca<sup>2+</sup> stores in RBMVEC, we tested the Ca<sup>2+</sup> response produced by ATP, an agonist known to generate inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and consequent Ca<sup>2+</sup> release from endoplasmic reticulum Ca<sup>2+</sup> stores. In Ca<sup>2+</sup>-free saline, ATP (50 μM) induced a fast and transient increase in [Ca<sup>2+</sup>]<sub>i</sub>. Typical examples of Ca<sup>2+</sup> responses are shown in Fig. 1D and the comparison of the amplitude in [Ca<sup>2+</sup>]<sub>i</sub> increase in each condition, is shown in Fig. 1E. In Ca<sup>2+</sup>-free saline, PAF (1 μM) produced a minimal increase in [Ca<sup>2+</sup>]<sub>i</sub> by only 18 ± 2.23 nM (n = 45 cells), as compared to an increase of [Ca<sup>2+</sup>]<sub>i</sub> by 579 ± 4.16 in Ca<sup>2+</sup>-containing saline. ATP (50 μM) increased Ca<sup>2+</sup> by 248 ± 4.36 nM (n = 53 cells) in Ca<sup>2+</sup>-free saline. Pre-treatment with nifedipine (1 μM, 15 min), similarly to Ca<sup>2+</sup>-free saline condition, inhibited the response to PAF; [Ca<sup>2+</sup>]<sub>i</sub> = 11 ± 1.97 nM (n = 43 cells), indicating that the PAF-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> was produced by Ca<sup>2+</sup> influx.

### PAF depolarized RBMVEC

The effect of PAF on membrane potential was monitored in RBMVEC loaded with DiBAC<sub>4</sub>(3). PAF (1 μM) produced a depolarization with an average amplitude of 8.27 ± 0.38 mV (n = 35 cells); in the presence of WEB2086, the effect of PAF was markedly diminished; ΔV = 1.35 ± 0.24 mV. Pretreatment with nifedipine (1 μM, 15 min), prevented the PAF-induced depolarization (ΔV = 0.81 ± 0.21 mV), while L-NAME did not affect the response to PAF (ΔV = 8.03 ± 0.41 mV). Averaged changes in membrane potential produced by PAF in each condition are shown in Fig. 2A, and the comparison of the amplitude of depolarization produced in each condition is shown in Fig. 2B.

### PAF increases NO in RBMVEC

In cells loaded with DAF-FM, a NO-sensitive dye (Kojima et al, 1998), PAF (1 μM) increased DAF-AM fluorescence ratio (Fig. 3). The response was abolished by WEB2086, a PAF receptor antagonist, by L-NAME (100 μM), NO synthase inhibitor, or by nifedipine, a L-type Ca<sup>2+</sup> channel antagonist (Fig. 3A). PAF (1 μM) increased the fluorescence by about 30% (ΔDAF-FM = 0.32 ± 0.027). WEB2086, L-NAME and nifedipine reduced the response to PAF to levels similar to control; ΔDAF-FM = 0.02 ± 0.011 in cells pretreated with WEB2086, 0.016 ± 0.008 in cells pretreated with L-NAME, and 0.057 ± 0.011 in RBMVEC treated with nifedipine (Fig. 3B).

### PAF alters RBMVEC cytoskeleton and tight junctions

We examined the distribution of ZO-1, a regulatory protein used as a marker for tight junctions complex (Abbott et al., 2010, Molino et al., 2014) before and after treatment of RBMVEC with PAF (1 μM). Treatment with PAF reduced the peripheral ZO-1 staining indicative of disruption of tight junctions, and increased the formation of F-actin fibers, suggesting cytoskeletal changes (Fig. 4A). Moreover, PAF promoted the intercellular gap formation (Fig. 4A). Treatment of RBMVEC with WEB2086, L-NAME and nifedipine while did not affect the ZO-1 and actin staining, but prevented the changes elicited by PAF.

### PAF disrupts endothelial barrier function

ECIS experiments indicate that treatment of RBMVEC monolayers with PAF (1  $\mu\text{M}$ ) produced a transitory decrease in the resistance part of impedance (Fig. 4B), indicative of barrier function disruption. Blockade of PAF receptor with WEB2086 (5  $\mu\text{M}$ ) prevented the response to PAF. The barrier-disruptive effect of histamine (10  $\mu\text{M}$ ) was used as a positive control (Stolwijk et al., 2015, Stolwijk et al., 2016).

### PAF did not affect RBMVEC viability

Since earlier studies (Fang et al., 2011, Predescu et al., 2013) indicate that PAF at concentration higher than  $10^{-7}$  M had cytotoxic effects, we examined the effect of PAF on RBMVEC viability, in our experimental conditions. Treatment with PAF (0.1  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 1  $\mu\text{M}$  and 5  $\mu\text{M}$ ) for one hour, did not significantly affect the viability of RBMVEC, assessed with MTT assay (Fig. 4C)

### PAF increases BBB permeability

*In vivo* studies examined the effect of systemic administration of PAF on the BBB permeability by determining the concentration of sodium fluorescein and Evans Blue in the rat brain. In control rats, the brain concentration of sodium fluorescein was  $386 \pm 47$  ng/mg ( $n = 6$ ). Treatment with PAF (0.01 mg/kg) increased the brain concentration of sodium fluorescein to  $703 \pm 84$  ng/mg ( $n = 6$ ). On the other hand, administration of WEB2086 (0.5 mg/kg), L-NAME (20 mg/kg), or nifedipine (0.3 mg/kg) 10 min before PAF reduced the sodium fluorescein concentration in the brain to  $426 \pm 39$  ng/mg,  $402 \pm 53$  ng/mg and  $447 \pm 61$  ng/mg ( $n = 6$ ), respectively (Fig. 5A). In experiments where BBB permeability was assessed using Evans Blue, in control rats, the brain concentration of Evans Blue as  $471 \pm 54$  ng/mg ( $n = 6$ ). Treatment with PAF (0.01 mg/kg) increased the brain concentration of Evans Blue to  $1138 \pm 74$  ng/mg ( $n = 6$ ). On the other hand, administration of WEB2086 (0.5 mg/kg), L-NAME (20 mg/kg), or nifedipine (0.3 mg/kg) 10 min before PAF reduced the Evans Blue concentration in the brain to  $557 \pm 61$  ng/mg,  $582 \pm 64$  ng/mg and  $541 \pm 67$  ng/mg ( $n = 6$ ), respectively (Fig. 5B).

## Discussion

Platelet-activating factor (PAF) is a phospholipid mediator involved in various cardiovascular and CNS disorders (Prescott et al., 2000). While low basal PAF level was detected in the brain (Kumar et al., 1988), increased brain PAF levels occurred after hypoxia and reoxygenation injury (Deng et al., 2009), in response to convulsant stimuli (Kumar et al., 1988) or in pathological conditions like multiple sclerosis (Callea et al., 1999) or HIV-1 infection (Gelbard et al., 1994). In addition to the synthesis in inflammatory cells, PAF can also be produced by endothelial cells (Liu et al., 2016). In a porcine model, cultured brain microvascular endothelial cells were reported to produce smaller amounts of PAF than aortic endothelial cells (Sato et al., 1995). As previous reports indicate that PAF produced an increase in blood-brain barrier (BBB) permeability (Fang et al., 2011, Fang et al., 2014), we examined the effect of PAF on rat brain endothelial cells (RBMVEC), an essential component of the BBB.



We found that in RBMVEC, PAF (0.1 - 5  $\mu\text{M}$ ) increased cytosolic  $\text{Ca}^{2+}$  concentration,  $[\text{Ca}^{2+}]_i$  in a dose-dependent manner. Lower concentrations of PAF tested here (0.1  $\mu\text{M}$ , 0.5  $\mu\text{M}$ ) mimic those found physiologically, while higher concentrations (>1  $\mu\text{M}$ ) were similar to those found in inflammatory conditions (Reiner et al., 2016). The effect of PAF was inhibited by the PAF receptor antagonist, WEB2086 (Ukena et al., 1988), furthering the implication of PAF receptor activation as the impetus for the cytosolic  $\text{Ca}^{2+}$  increase observed. Similarly, PAF increased  $[\text{Ca}^{2+}]_i$  in several other cell models like human monocytic leukemic U-937 cells (Barzaghi et al., 1989), hippocampal neurons (Bito et al., 1992), N1E-115 neuroblastoma cells (Diserbo et al., 1995), human and canine aortic endothelial cells (Bkaily et al., 1993), bovine cerebral microvascular endothelial cells (Lin and Rui, 1994), oviductal cells (Tiemann et al., 1996, Tiemann et al., 1999) and rat brain microvessel endothelial cells (Deng et al., 2009). Earlier studies indicate that PAF produced phosphoinositide hydrolysis, synthesis of inositol phosphates and release of  $\text{Ca}^{2+}$  from endoplasmic reticulum stores, suggestive of  $G_q$ -protein signaling (Shukla, 1992, Kamata et al., 1993b). PAF receptor has been reported to signal also via  $G_o$  and  $G_i$  proteins (Alexander et al., 2015).

In RBMVEC,  $\text{Ca}^{2+}$  influx and not  $\text{Ca}^{2+}$  release from internal stores was responsible for the increase in  $[\text{Ca}^{2+}]_i$  produced by PAF. Significant differences were reported in different cellular models between  $\text{Ca}^{2+}$  sources mobilized by PAF. While release of  $\text{Ca}^{2+}$  from internal stores was responsible for part of the  $\text{Ca}^{2+}$  increase,  $\text{Ca}^{2+}$  influx was considered the predominant mechanism elicited by PAF in platelets (Hallam et al., 1984), and U-397 cells (Barzaghi et al., 1989). In human airway epithelial cells (Stoll et al., 1994), or N1E-115 neuroblastoma cells (Diserbo et al., 1995) PAF produced both a  $\text{Ca}^{2+}$  release from internal stores and  $\text{Ca}^{2+}$  influx. On the other hand, in NG108 cells (Kornecki and Ehrlich, 1988), human platelets (Avdonin et al., 1991), bovine oviductal cells (Tiemann et al., 1996, Tiemann et al., 1999) similarly to RBMVEC, PAF produced  $\text{Ca}^{2+}$  influx. However, in oviductal cells, PAF-induced  $\text{Ca}^{2+}$  influx was not affected by verapamil (Tiemann et al., 1996), but blocked by flufenamic acid or TMB-8 (Tiemann et al., 1999), indicating the involvement of non-selective cationic channels. In bovine cerebral microvascular endothelial cells, PAF increased  $[\text{Ca}^{2+}]_i$  via phospholipase C-dependent mechanism, while blockade of  $\text{Ca}^{2+}$  influx with diltiazem or verapamil did not affect the response (Lin and Rui, 1994). On the other hand, in RBMVEC, PAF produced  $\text{Ca}^{2+}$  influx was sensitive to nifedipine, indicating the participation of L-type voltage-gated  $\text{Ca}^{2+}$  channels, and supporting different  $\text{Ca}^{2+}$  influx mechanisms between different cell types and/or species differences.

An increase in  $[\text{Ca}^{2+}]_i$  may lead to an increase in NO production consequent to the  $\text{Ca}^{2+}$ -dependent activation of endothelial NO synthase (eNOS) (Fleming et al., 1997). In RBMVEC, PAF produced an increase in NO levels; the effect was sensitive to the PAF receptor antagonist and NO synthase inhibitor. In addition, the increase in NO produced by PAF was abolished by a L-type  $\text{Ca}^{2+}$  channel blocker, indicating that the increase in  $[\text{Ca}^{2+}]_i$  by  $\text{Ca}^{2+}$  influx was a determinant of NO production. PAF has been reported to increase NO production via eNOS phosphorylation in other endothelial cells such as mouse pulmonary artery and lung microvascular endothelial cells (Predescu et al., 2013) and bovine coronary postcapillary venular endothelial cells (Sanchez et al., 2008) and produced subsequent increase in permeability. While these earlier studies examined the structural and functional

relationship of PAF receptor and eNOS at the plasma membrane microdomains, we examined the PAF-induced NO production in the context of the effect of PAF on  $[Ca^{2+}]_i$  concentration, membrane potential and their significance on endothelial permeability *in vitro* and *in vivo*.

We also identified that PAF depolarized RBMVEC loaded with a slow response voltage-sensitive fluorescent dye, DiBAC4(3); the response was significantly diminished by antagonism of PAF receptor or by inhibition of  $Ca^{2+}$  influx. Similarly, earlier studies reported that PAF depolarized human or rabbit neutrophils (Naccache et al., 1986, Lerner et al., 1988) human platelets (Avdonin et al., 1991), bovine oviductal cells (Tiemann et al., 1999) or rat stomach fundus (Kamata et al., 1993a). Depolarization of vascular endothelial cells was associated with NO production and increased cellular stiffness via reorganization of cortical actin cytoskeleton (Callies et al., 2011).

Brain microvascular endothelial cells are characterized by low paracellular permeability due to the presence of tight junctions between adjacent cells (Abbott et al., 2010). Tight junctional complex comprises of occludin, claudins and regulatory zonula occludens (Coeffier et al.) proteins (Aijaz et al., 2006). ZO-1, an intracellular regulatory protein, links the tight junctions molecules claudin and occludin to intracellular actin and the cytoskeleton and is widely used as a marker for tight junctions (Hawkins and Davis, 2005, Abbott et al., 2010). Our results indicate that, in RBMVEC, PAF reduced ZO-1 staining. Similarly, PAF reduced ZO-1 expression in human umbilical vein endothelial cells (HUVEC) (Jeewandara et al., 2015). A decrease in ZO-1 expression or immunoreactivity has been associated with an increase in BBB permeability (Abbruscato et al., 2002, Hawkins and Davis, 2005, Reinhold and Rittner, 2017). PAF also produced an increase in F-actin staining and induced intercellular gaps. Similar changes were produced in microvascular endothelial cells by other agonists such as bradykinin (Liu et al., 2008), thrombin (Stolwijk et al., 2016, Brailoiu et al., 2017) and glutamate (Andras et al., 2007) or by hypoxia (Brown and Davis, 2005). In human dermal microvascular endothelial cells (HDMEC), actin fibers were found to redistribute to the cytosol 2-5 min after thrombin stimulation and toward the cell membrane after 30 min (Stolwijk et al., 2016). PAF increased actin cytoskeleton in rabbit neutrophils (Naccache et al., 1986) and increased contractility of rat stomach fundus (Kamata et al., 1993a).

In endothelial cells, an increase in  $[Ca^{2+}]_i$  modulates junctional and cytoskeletal proteins, promotes phosphorylation of myosin light chain kinase, actin-myosin interaction and increases paracellular permeability (De Bock et al., 2013). Our ECIS experiments indicate that PAF decreased the electrical resistance, indicative of an increase in paracellular current flow. Similarly, PAF decreased electrical resistance in mouse pulmonary artery and lung microvascular endothelial cells (Predescu et al., 2013). A previous report indicates that the decrease in the trans-endothelial resistance (TEER) of HUVECs produced by PAF from the serum of patients with dengue fever played a critical role in the increased vascular permeability (Jeewandara et al., 2015). PAF was involved in the increase vascular permeability in experimental cerebral malaria (Lacerda-Queiroz et al., 2012) and anaphylaxis (Vadas et al., 2013). Other GPCR agonists, such as thrombin or histamine, were shown to

produce endothelial barrier dysfunction subsequent to morphological changes, rearrangement of actin cytoskeleton and junctional proteins (Stolwijk et al, 2016).

To further determine the significance of our *in vitro* studies, we investigated the *in vivo* effects of PAF on BBB permeability in rat, assessed with sodium fluorescein and Evans Blue. Earlier *in vivo* studies reported the PAF (1  $\mu$ M, 1 h) induced transient and reversible BBB opening (Fang et al, 2014), while longer exposure to PAF (1  $\mu$ M, 24h) increased permeability of RBMEC by upregulating intercellular adhesion molecule-1 (ICAM-1) and had cytotoxic effect (Fang et al, 2011). Our *in vivo* studies indicate that PAF increased the BBB permeability; the effect was sensitive to antagonism of PAF receptor. This finding is in agreement with a relatively recent study reporting a preserved integrity of blood-brain barrier in mice lacking the PAF receptor, PAFR(-/-) (Toscano et al, 2016). Blockade of  $Ca^{2+}$  influx or of NO formation also prevented the increase in BBB permeability produced by PAF, indicating a central role of the increase in  $[Ca^{2+}]_i$ ; and subsequent NO formation in this effect. Taken together, our studies identify multiple mechanisms by which PAF modulates the function of the brain microvascular endothelial cells and ultimately increases the permeability of the blood-brain barrier, relevant for the cerebrovascular inflammatory disorders.

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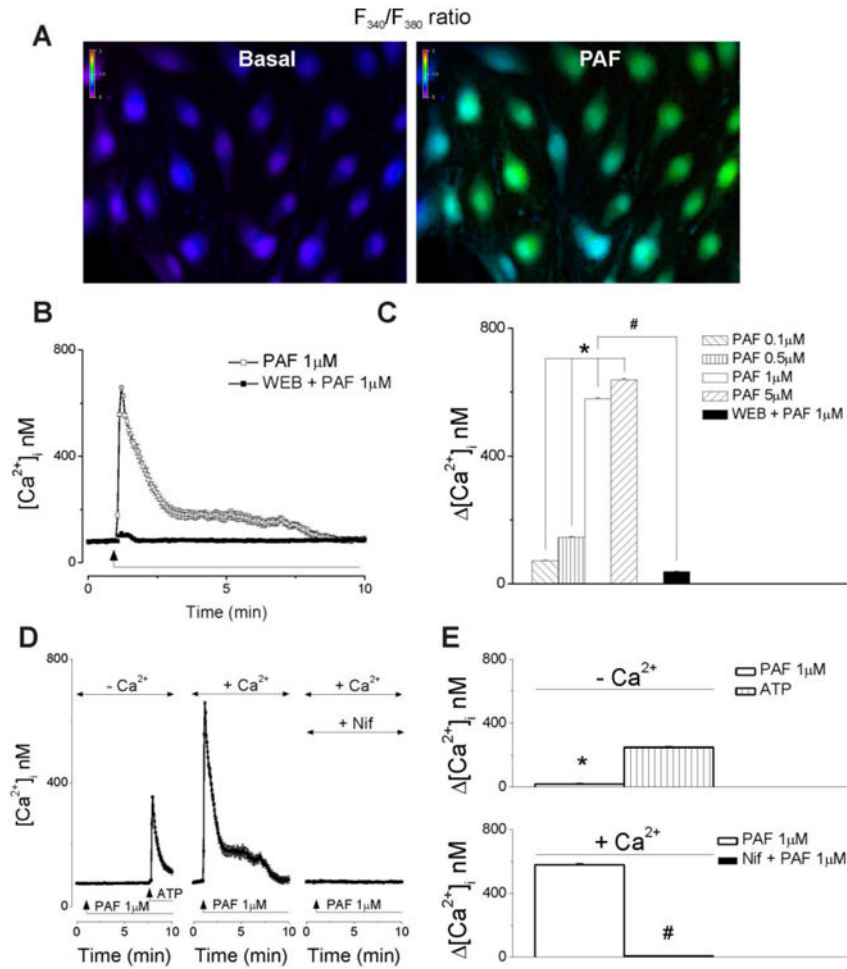
## Abbreviations

<b>BBB</b>	blood-brain barrier
<b>[Ca<sup>2+</sup>]<sub>i</sub></b>	cytosolic Ca <sup>2+</sup> concentration
<b>ECIS</b>	Electric Cell-Substrate Impedance Sensing
<b>HBSS</b>	Hanks' Balanced Salt Solution
<b>NO</b>	nitric oxide
<b>PBS</b>	phosphate buffer saline
<b>RBMVEC</b>	rat brain microvascular endothelial cells

### Highlights

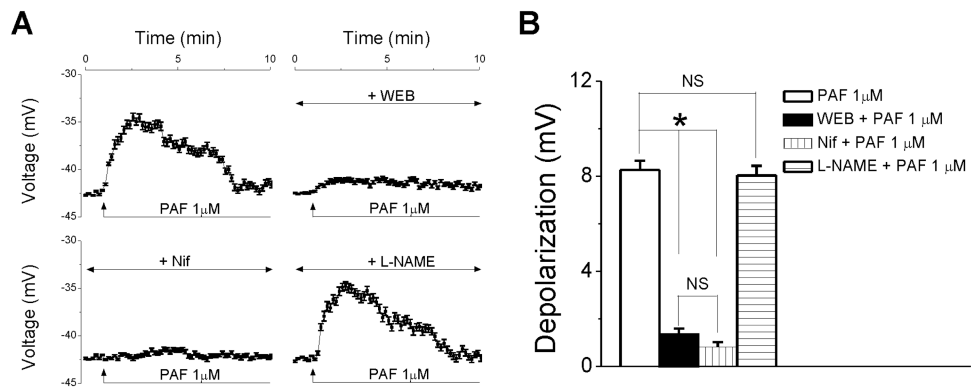
- We examined the effect of PAF on rat brain microvascular endothelial cells (RBMVEC) using *in vitro* and *in vivo* assays
- In addition to increasing cytosolic  $\text{Ca}^{2+}$  and NO production, we show for the first time that PAF depolarized RBMVEC
- PAF-induced changes in F-actin and tight junctions converged in a reduced electrical resistance and increased permeability
- Our results reveal multiple mechanisms by which PAF increases BBB permeability, relevant to CNS inflammatory disorders





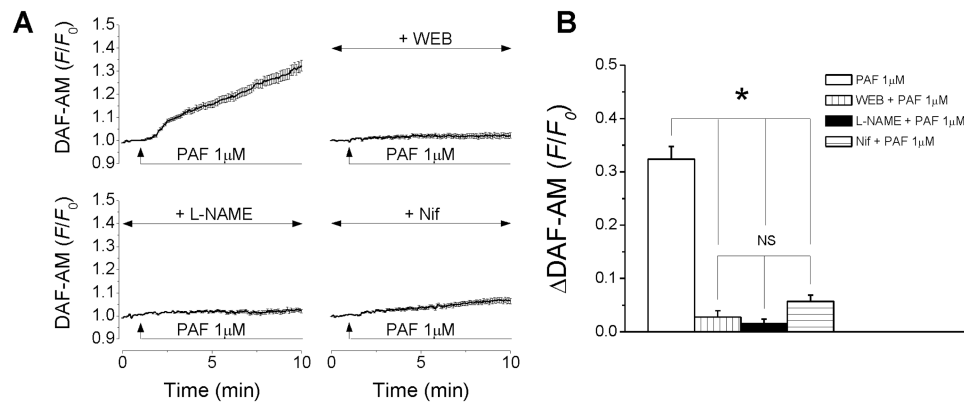
**Figure 1. PAF increases cytosolic  $\text{Ca}^{2+}$  concentration,  $[\text{Ca}^{2+}]_i$ , by promoting  $\text{Ca}^{2+}$  influx in RBMVEC**

**A**, Examples of fura-2 AM fluorescence ratio ( $F_{340}/F_{380}$ ) in RBMVEC before (basal) and after treatment with PAF (1  $\mu\text{M}$ ). Cold colors represent low ratios and hot colors represent high ratio (scale 0-2). **B**, Representative examples (average  $\pm$  SEM) of  $[\text{Ca}^{2+}]_i$  increases produced by PAF (1  $\mu\text{M}$ ) and PAF (1  $\mu\text{M}$ ) in the presence of PAF receptor antagonist WEB2086 (5  $\mu\text{M}$ ). PAF produced a fast and transient increase in  $[\text{Ca}^{2+}]_i$ ; the response was inhibited by WEB2086. **C**, Comparison of the amplitude of  $[\text{Ca}^{2+}]_i$  increase produced by each concentration of PAF tested (0.1  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 1  $\mu\text{M}$  and 5  $\mu\text{M}$ ) and by PAF (1  $\mu\text{M}$ ) in cells pretreated with WEB2086 (5  $\mu\text{M}$ ).  $P < 0.05$  as compared to the response to the other concentrations of PAF tested (\*), or to the response produced by PAF 1  $\mu\text{M}$  (#). **D**, In  $\text{Ca}^{2+}$ -free saline, PAF (1  $\mu\text{M}$ ) did not elicit an increase in  $[\text{Ca}^{2+}]_i$ , while ATP (50  $\mu\text{M}$ ) increased  $[\text{Ca}^{2+}]_i$ , indicating the integrity of internal  $\text{Ca}^{2+}$  stores. Pretreatment with the L-type  $\text{Ca}^{2+}$  channels blocker, nifedipine (1  $\mu\text{M}$ ), prevented the increase in  $[\text{Ca}^{2+}]_i$  produced by PAF. **E**, Comparison of the amplitude of  $[\text{Ca}^{2+}]_i$  responses elicited in  $\text{Ca}^{2+}$ -free saline by PAF (1  $\mu\text{M}$ ) and ATP (50  $\mu\text{M}$ ) (top) and in  $\text{Ca}^{2+}$ -containing saline (bottom) by or PAF (1  $\mu\text{M}$ ) alone or by PAF (1  $\mu\text{M}$ ) in cells pretreated with nifedipine (1  $\mu\text{M}$ ) (bottom).  $P < 0.05$  as compared to ATP-induced response in  $\text{Ca}^{2+}$ -free (\*), or to PAF-induced response in  $\text{Ca}^{2+}$ -containing saline (#).



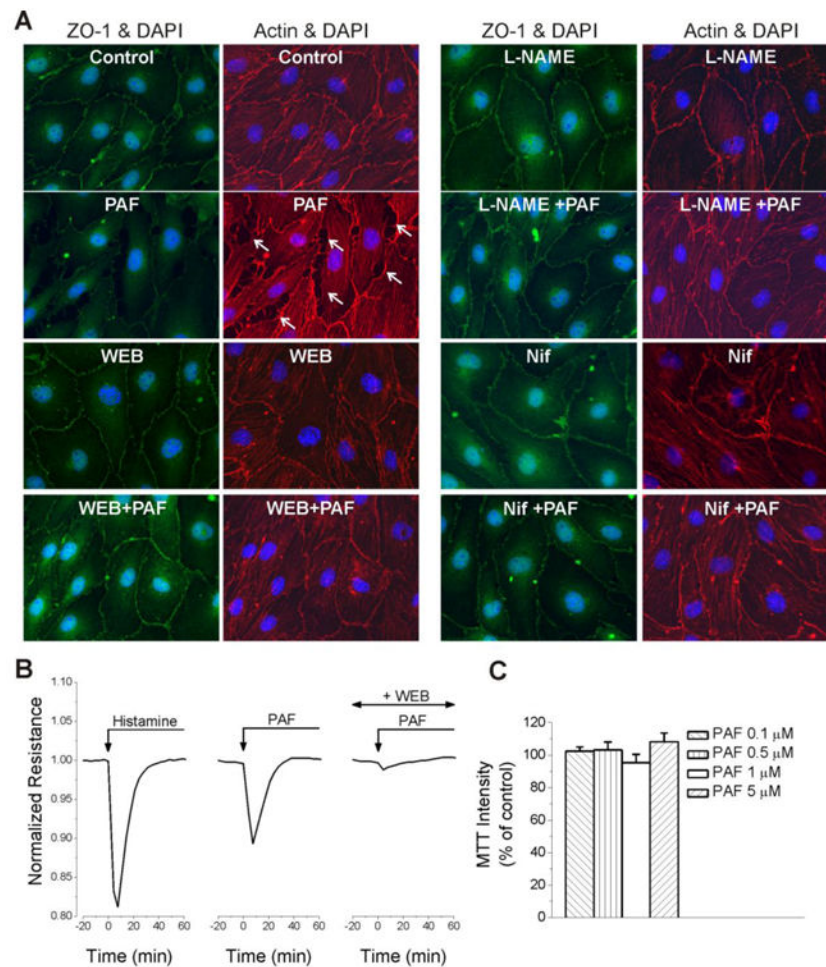
**Figure 2. PAF elicits RBMVEC depolarization**

**A**, Averaged changes in membrane potential  $\pm$  SEM in response to PAF (1  $\mu$ M), PAF in the presence of the PAF antagonist, WEB2086 (5  $\mu$ M), nifedipine (Nif, 1  $\mu$ M) or L-NAME (100  $\mu$ M). Treatment of RBMVEC with PAF (1  $\mu$ M) induced a depolarization that was prevented by WEB2086 (5  $\mu$ M), and nifedipine (1  $\mu$ M) but it was not affected by L-NAME. **B**, Comparison of the average amplitude of the depolarization produced by PAF alone or in the presence of WEB2086, nifedipine or L-NAME. \* $P < 0.05$  as compared to the effect of PAF in presence of PAF antagonist, WEB2086, or nifedipine. NS,  $P > 0.05$  between treatment groups.



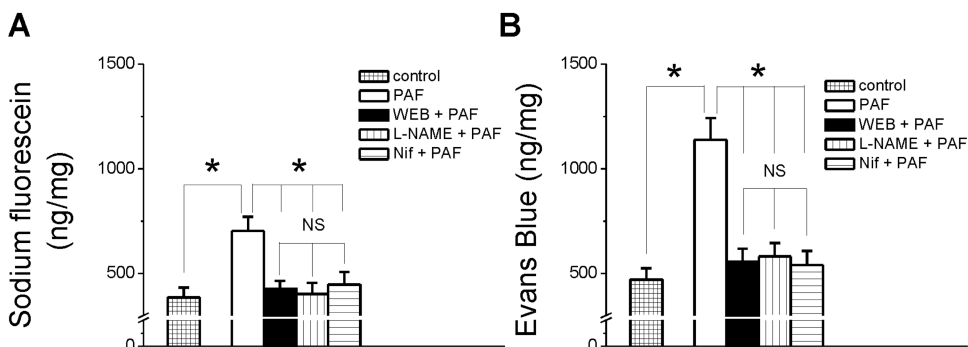
**Figure 3. PAF increases NO production in RBMVEC**

**A**, Averaged DAF-FM fluorescence ratios  $\pm$  S.E.M. in cells treated with PAF (1  $\mu$ M) (top left trace), PAF (1  $\mu$ M) in the presence of WEB2086 (top right trace), PAF (1  $\mu$ M) in the presence of L-NAME (bottom left trace) or PAF (1  $\mu$ M) in the presence of nifedipine (bottom right trace). **B**, Comparison of  $\Delta$ DAF-FM in cells treated with PAF (1  $\mu$ M) in the absence and presence of WEB2086, L-NAME, or nifedipine (Nif). (\*)  $P < 0.05$  as compared to response to PAF alone. NS,  $P > 0.05$  as compared to the other treatment groups.



**Figure 4. PAF produced cytoskeletal and tight junction changes and barrier dysfunction in RBMVEC**

**A**, Distribution of ZO-1 (green), a component of tight junctions and F-actin (red), a component of cytoskeleton, in control RBMVEC, and cells treated with PAF (1  $\mu$ M) in the absence and presence of PAF receptor antagonist, WEB2086. WEB2086, L-NAME and nifedipine (Nif) did not have any effect on ZO-1 or actin staining, but prevented the effect of PAF. Nuclei are stained with DAPI (blue). Treatment with PAF produced a reduction in ZO-1 staining, increased actin fibers, and intercellular gaps (arrows). **B**, Examples of changes in normalized electrical resistance of confluent RBMEC monolayer after histamine (10  $\mu$ M), PAF (1  $\mu$ M), PAF + WEB (5  $\mu$ M). PAF, similarly to histamine, decreased electrical resistance; the response to PAF was abolished by WEB2086. **C**. PAF (0.1  $\mu$ M – 5  $\mu$ M) did not significantly affect RBMVEC viability assessed with MTT assay.



**Figure 5. PAF produced an increase in the permeability of the blood-brain barrier (BBB) *in vivo***  
**A**, PAF (0.01 mg/kg) increased the permeability of the BBB assessed using the sodium fluorescein assay. **B**, PAF (0.01 mg/kg) increased the BBB permeability assessed using the Evans Blue method \* $P < 0.05$  as compared to control, or to the effect of PAF in presence of PAF antagonist, WEB2086, L-NAME or nifedipine. NS,  $P > 0.05$  between treatment groups.

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