Experimental Review on the Substance P-Enhanced Endothelial Permeability in Human Umbilical Vein Endothelial Cells (HUVECS)

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***Abstract* -- Inflammation is the immediate response to tissue damage or harmful stimuli. Though inconvenient, its role is significant and important as the protective and physiological response of our body. It directly sets the stage for tissue repair particularly increasing endothelial permeability which then contributes to the healing process. However, in some cases inflammation may progress out of control causing various inflammatory diseases. Neurogenic inflammation is a sub-set of inflammation and is characterized by an increase in neuronal chemical mediators such as Substance P (SP). In this study, we investigated the involvement of SP in enhancing endothelial permeability on HUVECs monolayer. Neurogenic inflammation was induced through the administration of SP (1 nM to 100 nM) on HUVECs monolayer inserts, and incubated with varying short (10, 20 and 30 minutes) and longer (6, 12 and 24 hours) time-points. FITC-Dextran were finally added to cell culture inserts for 5 minutes to let the fluorescence molecule pass through the gaps. Endothelial permeability is directly proportional with extravasation of FITC-Dextran, determined by fluorescence intensity reading. Based on our data, there were no significant differences between control group (non-treated cell) and the different concentrations of SP at different time-points. Our current findings suggest that SP was unable to increase the endothelial permeability on HUVECs monolayer inflammatory experimental model. Experiments that use this model to mimic vascular inflammation in laboratory settings may require further elucidation in the future.**

***Keywords* -- Substance P, Neuropeptide, Neurogenic Inflammation, HUVECs, FITC-dextran, endothelial permeability**

I. INTRODUCTION

Separating two compartments, the inner space of the blood vessels to the surrounding tissues is the main function of the endothelial barrier. It is also vital to control and facilitate the exchange of cells and fluids particularly plasma between the two compartments. However the endothelial layer is not static in nature but corresponds to both physiological and pathological stimuli.

In an inflammatory condition, hyper permeability of the endothelial barrier allows crucial passage for leucocytes to the injured or inflamed areas. This in turn causes oedema and swelling due to the excessive fluid migration from the blood vessels. Exaggerated vascular hyper permeability can cause life threatening situations as in asthma, lung injury and acute respiratory distress syndrome (Bogatcheva & Verin, 2008).

The impairment of the endothelial layer following tissue injury in certain disorders mentioned above causes leakage or extravasation of blood components to surrounding tissues. This process is further exaggerated by inflammatory mediators, vasoactive agents as well as vascular endothelial growth factors (van Nieuw Amerongen, Draijer, Vermeer, & van Hinsbergh, 1998; Van Hinsbergh & van Nieuw Amerongen, 2002). One prominent vasoactive agent is the SP. SP can induce rapid (<20 s) and transient (5–10 min) increase in endothelial permeability (Van Hinsbergh & van Nieuw Amerongen, 2002) when released from surrounding nerve cells leading to neurogenic inflammation.

Neurogenic inflammation is caused by the release of substances from primary sensory nerve terminals (Moussaoui, Montier, Carruette, Blanchard, Laduron, & Garret, 1993; Richardson & Vasko, 2002). Neurogenic inflammation is mediated by cross-talks between peripheral sensory fibres, leucocytes and endothelial cells at the site of injury (Gallicchio, Rosa, Benetti, Collino, Dianzani, & Fantozzi, 2006). Stimulation of peripheral terminals of sensory neurons causes the release of bioactive substances like SP, which also known as neuropeptides from C-fibre terminals (O'Connor, O'Connell, O'Brien, Goode, Bredin, & Shanahan, 2004).

SP in turn may act on target cells at the periphery such as mast, immune, as well as vascular smooth muscle cell evoking inflammatory effects such as vasodilatation, plasma extravasation and leukocyte activation (Gallicchio, Rosa, Benetti, Collino, Dianzani, & Fantozzi, 2006; Holzer, 1998). These events play a role in the pathogenesis of various diseases such as arthritis, asthma, and inflammatory bowel disease (Payan, 1989). Generation of neurogenic inflammation strictly depends on the production, release and binding of SP to its receptors as well as the activation of NK-1 receptor (Chen, Tsai, Wang, Yu, & Chang, 2007).

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According to Donnerer and Amann (1993), the presence of neurogenic and non-neurogenic inflammation are based on the inflammatory processes involving denervated versus the intactly innervated tissues. The occurrence of neurogenic inflammation must not be recognised as different isolated processes but it occur in a network of sequential events and reflex pathway besides it is primarily mediated by afferent neurons.

This study investigates the suitability of using monolayer Human Umbilical Vein Endothelial Cells (HUVECs) as an experimental model to mimic the SP induced-neurogenic inflammation, *in vitro*.

II. MATERIALS AND METHODS

Cells and Culture

Primary Human Umbilical Vein Endothelial Cells (Cascade Biologics, Invitrogen, USA) are cultured in Endothelial Cell Media with Phenol Red free on collagen coated tissue culture flask. Cells were used between third and fifth passages. All endothelial cells are maintained in CO_2 incubator at 37°C in a 100% humidified atmosphere of air containing 5% CO_2. The media was changed after second days of culturing and leave to reach 80%-90% confluence before detachment using 10x Trypsin plus 0.2% ethylenediaminenetetraacetic acid (EDTA) (Sigma-Aldrich, USA).

Substance P (SP)

SP were purchased from Sigma-Aldrich, USA and Bachem (Peninsula Lab, USA). The concentration of SP used are in a range of 1 to 100 nM. The lyophilized neuropeptide SP were prepared with 0.1 N Acetic Acid producing 3.71 x 10^{-2} M stock solution. For sub-stock preparation, the methods as stated by Moskowitz *et al*., 1987 were followed. To prevent repeated freeze thaw process, it was aliquot to smaller volume and stored in -80°C. Required concentrations of SP were prepared in the media as working solution and were added to cultures.

Endothelial Permeability Assay

BD Falcon cell culture inserts with 1.0 µm symmetrical pore size transparent polyethylene membrane together with 24-well tissue culture plates were used. Tissue culture insert are coated with collagen for 1 h. Culture medium is place in the upper and lower compartments at 250 µL and 500 µL, respectively. Cell are cultured for 72 hours, or until a monolayer is form, in a 37 °C CO_2 tissue culture incubator. The upper and lower compartment were washed with Cell Basal Medium and subsequently induced with SP with doses ranging from 1 to 100 nM at shorter (10, 20 and 30 minutes) and longer time-point (6, 12, and 24 h). Then, a high molecular weight FITC-Dextran (150 µL) were added to the upper compartment and leaves for 5 minutes. The extravasation of FITC-Dextran will be assess by collecting 100 µL medium from lower compartment and transferred to 96-well black plate for fluorescence measurement using a microplate reader (Tecan M200 Infinite, Mannedorf, Switzerland) with 485nm and 530nm filter set. Fluorescent intensity determined were compare with fluorescence intensity of basal (non-treated cell). Blank inserts is prepared following Kazakoff *et al*. (1995) and every samples were done in triplicate.

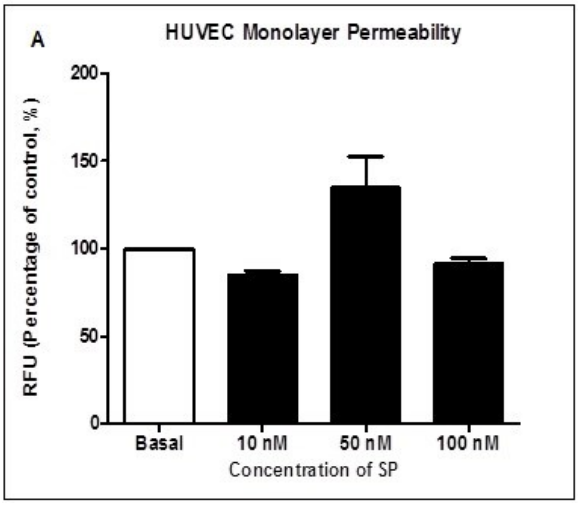
Statistical Analysis

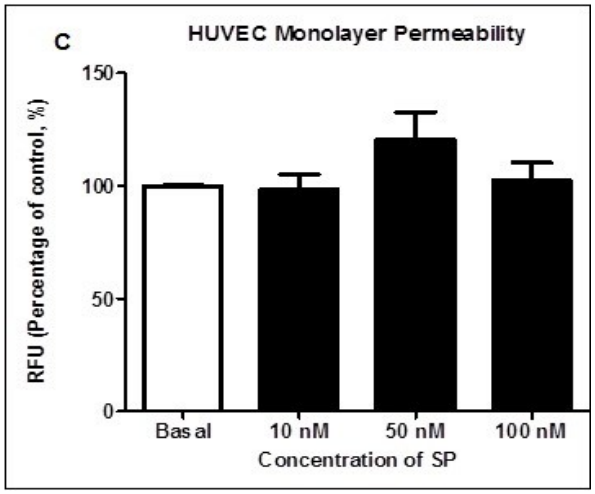
Data were expressed as means ± SEM (standard error of mean). Statistical analysis was performed with SPSS Statistics version 21 and Graphpad Prism 3.0 software. Data obtained were also analysed using one-way analysis of variance (ANOVA) and the differences between the groups with control group is determined using Dunnet post hoc test with $P<0.05$ as the limit of significance.

III. RESULTS

Endothelial Permeability

HUVEC monolayers were seeded on collagen-coated CHEMICON® *In Vitro* Vascular Permeability Assay inserts. Basal (non-treated cell) serves as control while the rest are treated with various concentration of SP with various incubation time. After treatment, a high molecular weight FITC-dextran were added on top of the monolayers and leaves for 5 minutes to allow its permeation through HUVECs monolayer. Endothelial permeability were determined by fluorescence intensity reading; relative fluorescence units (RFUs) from the plate well solution. The reading are directly proportional with FITC-dextran released from upper compartment (inserts).





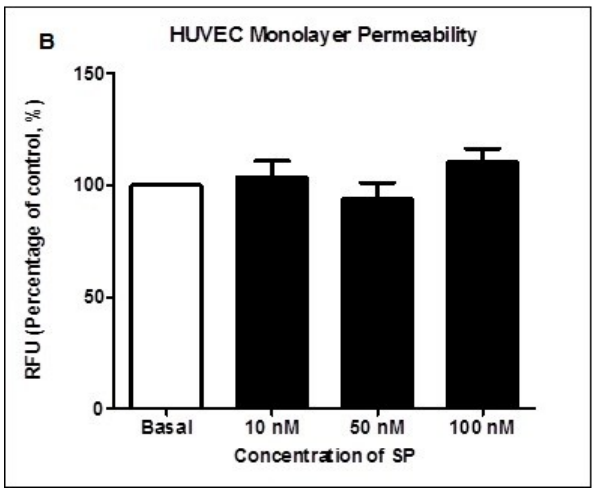
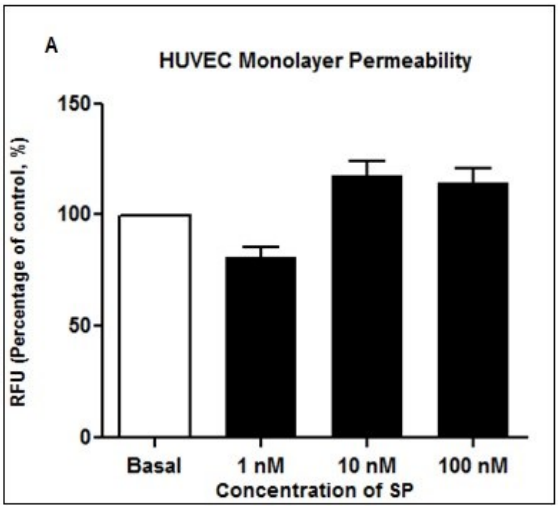
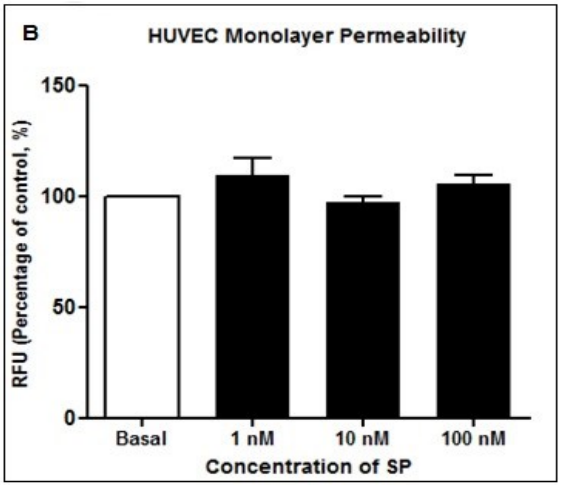


Fig. 1. HUVECs monolayer permeability at short incubation period, 10 minutes (A), 20 minutes (B) and 30 minutes (C)





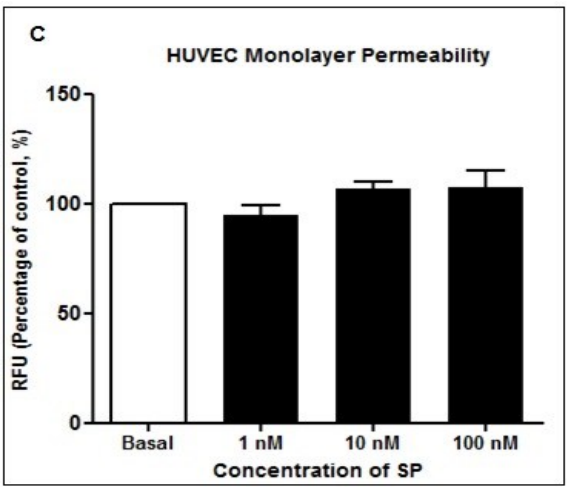


Fig. 2. HUVECs monolayer permeability at long incubation period, 6 hours (A), 12 hours (B), and 24 hours (C)

Based on the present study, we shall report that SP failed to enhanced endothelial permeability to HUVECs monolayer neither at short (minutes) time-point nor longer (hours) time-point. HUVECs monolayer showed there are no significant different ($p < 0.05$) as compare to basal (non-treated cell) at concentrations ranges from 1 nM to 100 nM.

IV. DISCUSSION

Endothelium is the layer of cell which lined up all blood vessels and lymphatics. It acts as barrier between blood and interstitial fluid (Payan, 1989). The wall of blood vessels are responsible to control the extent of vasodilatation and also the leakage of plasma fluid into surrounding tissue Chen, Tsai, Wang, Yu, & Chang, 2007). When there is increase in endothelial permeability, unregulated flow through these vessels to the surrounding tissue occurs. The tissue itself become inflated and swollen which leads to an events known as oedematous (Gallicchio, Rosa, Benetti, Collino, Dianzani, & Fantozzi, 2006; Bates, Hillman, Williams, Neal, & Pocock, 2002).

In vitro permeability assay is relatively objective and a high-throughput method (Khalil & Helme, 1989) to determine the extravasation process on endothelial cells. The effects of chemicals, drugs or cytokines on endothelial permeability can be studied with an *in vitro* vascular permeability assay. This assay provides an efficient system as it having the upper and lower compartment which enables us to measure the cell permeability. On the present study, we have employed this assay to mimic the endothelial hyper permeability induced by SP on HUVECs monolayer. Induction of SP on HUVECs monolayer were done in various incubation times with various concentrations and incubated at 37 ^{0}C in 5% CO_2 incubator. Then, FITC-dextran; a high molecular weight fluorescence molecule were added on top of the cells for about 5 minutes. This enables FITC-dextran to permeate through the endothelial gaps. Then, the extent of permeability can be determined by fluorescence produced from the plate well solution (Holzer, 1989).

In this study, we had conducted an *in vitro* endothelial permeability study on primary cultures of HUVECs induced by SP. We also demonstrated for the first time, endothelial permeability process on Human Umbilical Vein Endothelial Cells (HUVECs) were unaffected after being induced by a well-known neurogenic inflammatory mediator, SP. In contrast, a statement by Chen *et al*. (2007) stated that neurogenic release of SP from sensory C-fibres may leads to induction of vascular permeability in neurogenic inflammation. Disturbance of vascular permeability causes vascular leakage take place. Thus, the results that we had obtained have leaded us to further discuss the possible causes that may be involved in present findings.

Several studies on endothelial permeability had been conducted both *in vivo* and *in vitro* involving SP. Walsh and his colleagues (Walsh, Weg, Williams, & Nourshargh, 1995) had shown that SP induce inflammatory reaction in guinea pig skin by producing oedema after injection with SP as lowest as 10^{-13} M and reached maximum level during first 1 H. In this study, we had chosen the range of 10^{-10} M to 10^{-8} M of SP to induce endothelial permeability in HUVECs. We had subjected HUVECs monolayer to SP at 10 nM, 50 nM and 100 nM concentration and incubated for 10 minutes, 20 minutes and 30 minutes. Conversely, our observation had revealed that SP was unable to promote endothelial permeability neither with the lowest (10 nM) nor with the highest concentration (100 nM) tested within less than 1 hour time.

An *in vivo* study conducted by Bakhle *et al*. (1996) demonstrated that in response to either exogenous or endogenous SP, upper airways of GH (genetically hypertensive strain) rats exhibit a marked reduced capacity to increase microvascular permeability regardless to the hypertensive conditions of the strain. However, in the presence of another pro-inflammatory mediator such as 5-Hydroxytryptamine, 5-HT; GH airways exhibit a normal capacity for microvascular permeability. These finding suggested that SP may increase permeability in the presence of other pro-inflammatory mediators. However, these findings contradict to a study conducted by Khalil and Helme (1989). They revealed that other mediators are not involved in early SP-mediated response concerning plasma extravasation process, yet late responds may involves interaction between SP, mast cell mediators and prostaglandins.

Another possibility involving the unresponsive of endothelial cells towards SP would be the cells was selectively insensitive to SP. The used of primary cultures endothelial cells to study endothelial physiology often show large degrees of heterogeneity between preparations. These variations somehow due to the differences between individuals as well as in culture conditions (Gifford, Grummer, Pierre, Austin, Zheng, & Bird, 2004). The endothelium might irresponsive and reduced its ability to change their structures hence permeability. This was also shown by Bakhle *et al*. (1999), where the microvasculature of GH animals were selectively insensitive to SP thus its permeability responses is reduced. This might be due to reaction of several group of mediators such as vasoactive cytokines, growth factor and signals from mediators with endothelial cell substructure which control the permeability process. They also mention that the microvasculature of GH strain were unresponsive to SP is not due to the altered circulatory dynamics in the GH.

SP elicits the inflammatory reaction by binding to NK-1 (O'Connor TM, O'Connell, O'Brien, Goode, Bredin, & Shanahan, 2004), NK-2 and NK-3 receptor (Gallicchio, Rosa, Benetti, Collino, Dianzani, & Fantozzi, 2006). The interaction between SP and NK-1 receptor generates neurogenic inflammation and is heavily depend on the production, release and binding of SP as well as NK-1 activation (Chen, Tsai, Wang, Yu, & Chang, 2007). Other mechanism such as phosphorylation of NK-1 receptors may also responsible for desensitization of cell to SP. Likewise,

internalization of NK receptors on cell surface may also contribute to desensitization of cells to SP. The responsiveness of endothelial cells to SP was limited after rapid removal of NK-1 receptors from cell surface by internalization. Thus, internalization and desensitization of NK-1 receptor towards SP can also be one of the causes that limit the amount of plasma leakage on site of inflammation (Bowden, Garland, Baluk, Lefevre, Grady, Vigna, & Bunnett, 1994), which may also explain the flow of FITC-dextran in the present study were non-significant to control (non-treated) group at any concentrations and times tested.

Another possibility that might involves is the present of Neutral Endopeptidase (NEP). NEP or also known as membrane metallo-endopeptidase is an enzyme that inactivates and cleaves several peptides hormones (e.g.: Substance P, neurotensin, bradykinin, glucagon) at the amino side hydrophobic residues (Pereira, Aksoy, Moon, Peng, Redfield, Burnett, Wieben, Yee, & Weinshilboum, 2010). Expression of this enzyme on cell surface will accelerate degradation of SP at extracellular fluid thus terminating its pro-inflammatory effects (Okamoto, Lovett, Payan, Bunnett, 1994). This phenomenon may also explain why endothelial permeability was not affected after induced with SP during present study.

After induction of SP to endothelial cell, the leakage of Evans blue in plasma extravasation assay are peaked within 2 minutes and then declined rapidly with half-life of about 1 minute. Moreover, the second dose produced 56% less Evans blue leakage than did a single dose when another two sequential injections of SP are given in three minutes apart (Bowden, Garland, Baluk, Lefevre, Grady, Vigna, Bunnett, & McDonald, 1994). Likewise, metabolic clearance rate of SP after intravenously injected to normal subject (man) was 0.361 kg^{-1}/min and the half-life of exogenous SP was 1.6 minute (De Muckadell, Aggestrup, & Stentoft, 1986) . Another study by Watson (1983), one of the data shown that it was almost 12% SP (2×10^{-9} M) degradation occurs within 10 minutes of time and up to 45% degradation within 20 minutes of time after induce on guinea pig ileum bath fluid. Walsh and colleagues had injected SP (10^{-9} mol) intradermally into guinea-pig and leave for 1 h and 2 h. The results showed that oedema formations reached maximum level within the first hour (Walsh, Weg, Williams, & Nourshargh, 1995). This had indicated that SP may produce endothelial permeability associated with oedema formations maximally within 60 minutes. However, based on the results that we obtained, SP unable to shows the permeability effects even at shortest time incubation, which are 10, 20 and 30 minutes. Thus, extensive and rapid degradation of SP in *in vitro* bioassay can also be the major cause of discrepancy that happened in our assay.

V. CONCLUSIONS

In present study, we had conducted a study involving permeability of endothelial cells using SP as inducer. Based on literature, endothelial permeability was enhanced by induction of SP and was tested by assessing the extravasation of high molecular weight molecule, FITC-dextran. However, due to certain circumstances, we had failed to shows that SP enhance endothelial permeability after following certain incubation time on HUVECs. Nevertheless, we believe that our discovery has uncovered some important constraints that need to be considering in utilizing SP as inducer for occurrence of neurogenic inflammation involving endothelial cells. It is highly sensitive neuropeptide with short half live and proper storage is highly advised.

List of Abbreviations

HUVEC	Human Umbilical Vein Endothelial Cell
SP	Substance P
RFU	Relative Fluorescence Unit
FITC-Dextran	Fluorescein isothiocyanate–dextran
GH	Genetically Hypertensive Strain
5-HT	5-Hydroxytryptamine
NK-1, -2, -3	Neurokinin-1, -2, -3
NEP	Neutral Endopeptidase
EDTA	Ethylenediaminetetraacetic acid
SEM	Standard Error of Mean
ANOVA	Analysis of Variance

Figures

Fig. 1. - HUVECs Monolayer Permeability at Short Incubation Period (minutes)

SP were subjected to HUVECs monolayer at various concentrations and incubate for 10 minutes (A), 20 minutes (B) and 30 minutes (C). HUVECs at passage 3 were seeded at $1x10^6$ cells per inserts and cultured for 72 H in endothelial cell medium until monolayer is formed. At the following days, the monolayer were subjected to SP at 10 nM, 50 nM and 100 nM concentration and incubate for 10 minutes, 20 minutes and 30 minutes except for Basal group (non-treated cell). FITC-dextran permeability testing were performed after respected incubation time were ended. Triplicate samples shown that SP unable to increase endothelial permeability in dose-dependent manner of short incubation time and none of these are found to be significant. Bars are represent mean + SEM and values of $p< 0.05$ are considered significant.

Figure 2 - HUVECs Monolayer Permeability at Long Incubation Period (hours)

SP were subjected to HUVECs monolayer at various concentrations within 6 hours (A), 12 hours (B) and 24 hours (C) incubation time. HUVECs at passage 3 were seeded at $1x10^6$ cells per inserts and cultured for 72 H in endothelial cell medium until monolayer is form. At the

following days, the monolayer were subjected to SP at 1 nM, 10 nM aand 100 nM concentration and incubate for 6 hours, 12 hours and 24 hours except for Basal group (non-treated cell). FITC-dextran permeability testing were performed after respected incubation time were completed. Triplicate samples shown that SP unable to increase endothelial permeability in dose-dependent manner and none of these are found to be significant. Bars are represent mean + SEM and values of $p< 0.05$ are considered significant.

COMPETING INTERESTS

We declare that there is no competing interest.

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