Nitric Oxide Production by Substance P Induced-Murine Raw 264.7 Macrophages

Nurfarahdilla, Z., Khairudin, N.N.H., Teh, S. S.,
Enoch, K. P., Zuraini, A., and Somchit, M.N.

Abstract **-- Substance P is a neuropeptide that release from small-diameter primary afferent neurons during neurogenic inflammation. During this process large amount of nitric oxide (NO) is generated. NO which are produced by activated macrophages are function as effectors molecules and involved in physiological and pathological responses in neural, vascular and immune system. Thus, measurement of NO levels has captured our attention for this study. This study was aimed to investigate the effects of Substance P on nitrite levels through Griess assay method. Quantification of NO, by measuring its oxidation product, nitrite was determined through the Griess reaction by a microplate assay method. Before Griess assay was performed, RAW 264.7 macrophages were introduced with 10-100nM of substance P for different incubation time (10 minutes to 4 hours). The results show that neither of short incubation time (10, 20 and 30 min) nor long incubation time (1, 2 and 4 h) of murine RAW 264.7 cell lines with substance P show any significant effect on NO release. Our current findings suggest that murine RAW 264.7 cell lines -induced substance P did not undergo inflammatory process since there is no nitrite levels in supernatant were detected as significant to control group.**

Keywords **-- Substance P, Neuropeptide, Neurogenic inflammation, nitric oxide, RAW 264.7 macrophages**

I. INTRODUCTION

Inflammation is a localized body protective response of living mammalian tissues to eliminate or limit the spread of harmful agents or stimuli brought out by injury or destruction. This reaction is characterized in the acute form by the classical signs of pain, heat, redness, swelling, and loss of function. When the harmful stimuli are eliminated, the inflammation process usually stops. However, inflammation sometimes if continue to exist can involve to some disease including rheumatoid arthritis, chronic asthma, multiples sclerosis, inflammatory bowel disease, and psoriasis.

Once nerve cells which contain inflammatory neuropeptides involves in that response, that process called neurogenic inflammation. Neurogenic inflammation is characterized by redness and warmth (secondary to vasodilation), swelling (secondary to plasma extravasation), and hypersensitivity (secondary to alteration of the excitability of certain sensory neurons) (Richardson & Vasko, 2002). This is triggered by the activation of primary sensory neurons and the subsequent release of inflammatory neuropeptides from sensory nerve terminals to transmit nociceptive messages to central neurons and also to be important in the inflammatory process in the periphery through axon-reflex mechanisms (Julius & Basbaum, 2001).

Inflammation leads to the upregulation of a series of enzymes and signaling proteins in affected cells and tissues (Liao, Deng, Lin, Lee, Lee, Hou, Huang, & Huang, 2012). During the inflammation, large amounts of the proinflammatory mediator, NO is generated by the inducible isoforms of NO synthase (iNOS) (Kim, Kim, Park, Ha, Choi, & Park, 2001). High levels of NO production mediate proinflammatory and destructive effects. On the other hand, low level of NO takes part in almost all kinds of physiological process, such as vascular functions, neurological functions and cytotoxic functions (Ouyang, Hong, Zhao, Shen, Shen, Zhang, & Zhang, 2008) and has a protective effect in some inflammatory responses as it is likely to contribute to the antimicrobial activity of macrophages against certain bacterial pathogens (Liao, Deng, Lin, Lee, Lee, Hou, Huang, & Huang, 2012). However, sometimes, lower level of NO of its normal level also contributes to inflammation.

NO production is mainly catalyzed by nitric oxide synthase (NOS) which exists in three isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). eNOS and nNOS are constitutively expressed and play an important role in normal physiological activities. iNOS is expressed in the response to proinflammatory stimuli, such as Substance P (SP). The iNOS-mediated NO production can promote pathological inflammation (Liao, Deng, Lin, Lee, Lee, Hou, Huang, & Huang, 2012; Nussler & Billiar,1996). iNOS production by activated macrophages was initially considered as a component of innate immunity, which contributes to the host immune defense against viruses and bacteria. However, high production has been associated with oxidative stress and with the pathophysiology of various diseases such as arthritis, diabetes, stroke, septic shock, autoimmune diseases, and chronic inflammation (Rimbach, Park, Guo, Moini, Qureshi, Saliou, Takayama, Virgili, & Packer, 2000).

SP is a neuropeptide that release from sensory nerve terminal serves to mediate the initiation process of neurogenic inflammation. SP is secreted by nerves and inflammatory cells such as macrophages, eosinophils,

Nurfarahdilla, Z., Khairudin, N.N.H., Enoch, K. P., Zurain,i A., & Somchit, M.N. are with Physiology Research Laboratory, Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

Teh, S. S. is with Chemistry Research Laboratory, Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

Somchit, M.N. is with Sports Academy, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia (Email Address: nazrulh@upm.edu.my).

lymphocytes and dendritic cells. . In macrophages, SP induces nitric oxide production and oxidative burst of macrophages, resulting in the production of reactive oxygen intermediates (Yaraee, Ebtekar, Ahmadiani, Sabahi, & Ghazanfari, 2007). SP also enhances antigen presentation and phagocytosis by macrophages during cellular immune responses. SP, at nanomolar (nM) concentrations caused selective chemokine response in murine macrophages which is release from macrophages infiltrating into local and distant damaged tissues (Sun, Ramnath, Zhi, Tamizhselvi, & Bhatia, 2008).

The current study is aimed to investigate the effects of Substance P on nitrite level in macrophages by using RAW 264.7 cells, a macrophage-like cell line as a model.

II. MATERIALS AND METHODS

Cells and Culture

A murine macrophage cell line RAW 264.7 was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37^{o}C. Confluent monolayers were passaged routinely by trypsinization. The cells were added in 1 x 10^6 to 24-well plates to form a monolayer then the supernatant were obtained for used in Griess assay.

Substance P (SP)

SP was purchased from Bachem (Peninsula Lab, USA). Cell culture medium (DMEM) was purchased from Gibco BRL, Life tech., USA. Stock solution of SP (37.1mM) was prepared by dissolving in 0.1M acetic acid and stored at -20^{o}C. Further dilutions were conducted in deionized water.

Measurement of Nitrite Level

Quantification of nitrite (NO2−) level on supernatant of cultured RAW 264.7 cells was determined through the Griess reaction by a microplate assay method. Nitrite is a stable breakdown product of NO (Rimbach, Park, Guo, Moini, Qureshi, Saliou, Takayama, Virgili, & Packer, 2000). Briefly, 50µl of macrophages supernatant were removed and incubated with an equal volume of 1%sulfnilamide/0/1%N-(1-naphtyl)-ethylene diamide dihydrochloride in 2.5% H3PO4] in 96-well plates at room temperature for 10 min in darkness. Nitrite concentration was determined by measuring the absorbance at 540 nm in Tecan M200 Infinite, Mannedorf, Switzerland. Concentrations were calculated from a standard sodium nitrite curve.

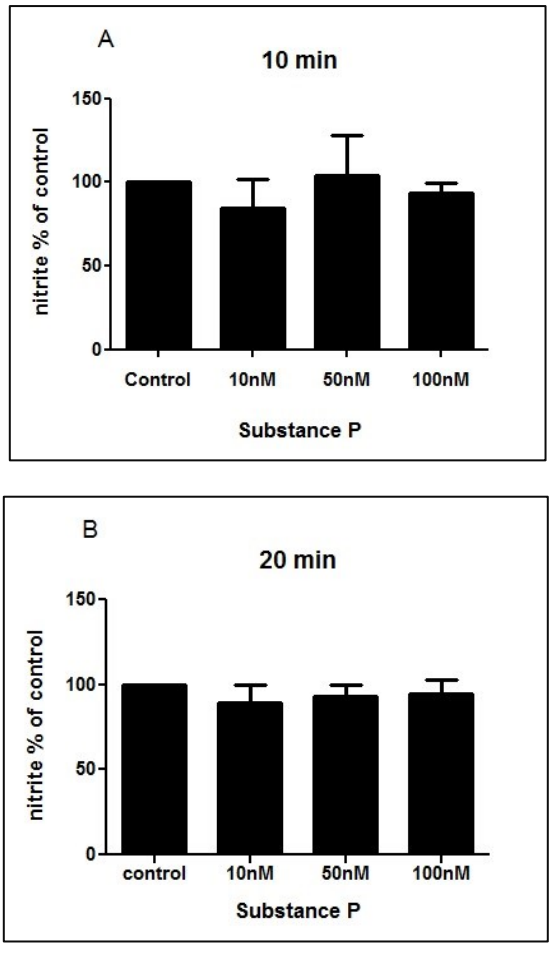
Statistical Analysis

Data were expressed as means ± SEM (standard error of mean). Statistical analysis were 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 are determined using Dunnet post hoc test. A *P* value <0.05was considered a statistically significant difference.

III. RESULTS

Effect of Substance P on Nitrite Production

We investigated the effect of SP on NO production by RAW 264.7 by Griess assay method. Nitrite accumulated in the culture medium was estimated by the Griess reaction as an index for NO release from the cells. SP was added at final concentration of 10nM, 50nM and 100nM at 10min, 20min, 30min, 1h, 2h and 4h before griess assay. As shown in Fig. 1 NO formation was not significantly detected as nitrite in the culture medium after 10 min (A), 20 min (B) and 30 min (C) of incubation. The same findings were obtained for a longer incubation period with SP for 1h (A), 2h (B) and 4 h(C) (Fig 2).



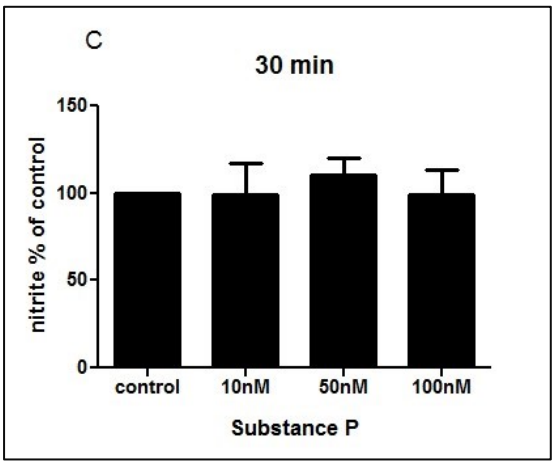
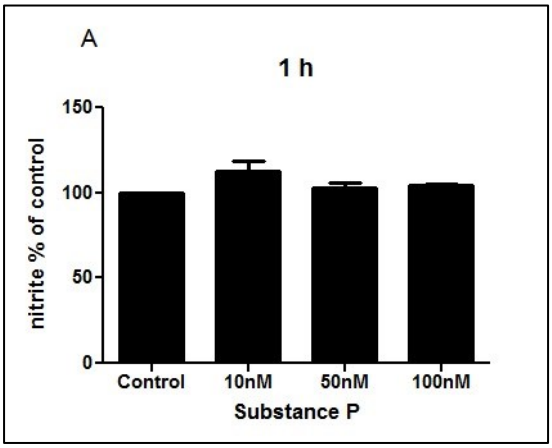
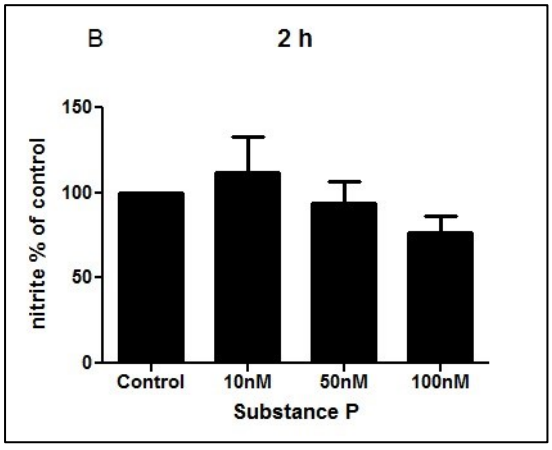


Fig. 1. Nitrite accumulation at 10 minutes (A), 20 minutes (B) and 30 minutes (C)





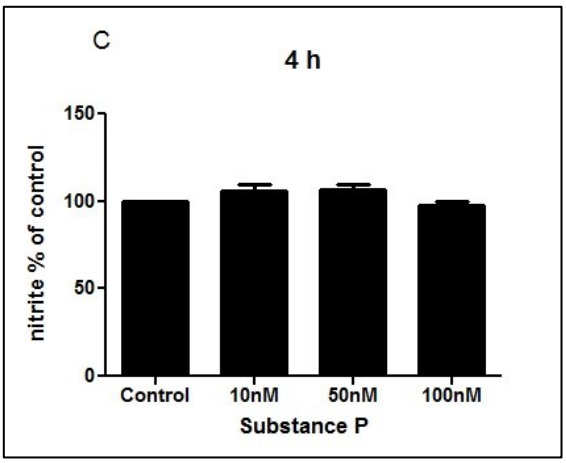


Fig. 2. Nitrite accumulation at 1 hour (A), 2 hours (B), and 4 hours (C)

To confirm that SP by itself cannot affect the basal level of nitrite production; we followed the study with the addition of LPS. RAW 264.7 cells were incubated with combination of SP and LPS for 24h for a final concentration of SP 10nM and 100nM and LPS (100ng/ml). As shown in Fig. 3 incubation with SP and LPS for 24h was significantly increased nitrite production when compared to control.

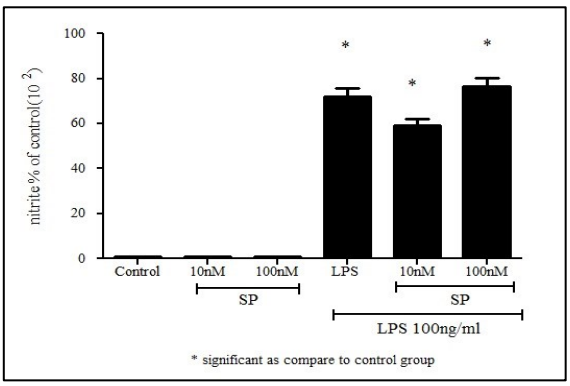


Fig. 3. Nitrite accumulation when RAW 264.7 cells were incubated with 10nM and 100nM of substance P in the presence or absence of LPS (100ng/ml) for 24h

Based on the present study, we shall report that SP failed to significantly increase of nitrite production in RAW 264.7 macrophages cells up to 4 hours. But, there was significantly increased of nitrite production in LPS and LPS-SP groups when compared to control. Thus, we suggest that RAW 264.7 cell lines -induced substance P did not undergo inflammatory process.

IV. DISCUSSION

Activated macrophages produce proinflammatory mediator, NO which are generated by inducible isoforms of nitric oxide synthase (iNOS) (Posadas, Terencio, Guillén, Ferrándiz, Coloma, Payá, & Alcaraz¸ 2000). A variety of studies have shown that this mediator was produced in

excessive amounts in activated inflammatory cells, which play key roles in the regulation of inflammation (Chen, Yang, & Lee, 2000; Korhonen, Lahti, Kankaanranta, & Moilanen, 2005). According to Qiao Wen and colleagues, macrophages, hepatocytes, smooth muscle cells, fibroblasts, mesangial cells, and some tumor cells begin to produce NO several hours after exposure to cytokines and microbial products (Xie, Cho, Calaycay, Mumford, Swiderek, Lee, Ding, Troso, & Nathan, 1992).

Griess assay is an assay that specialized to measure nitrite accumulation in the supernatant of cultured cells. After introducing SP into cultured macrophages, Griess reagent is added to each sample to quantify the nitrite level.

In this study, we demonstrated exposure of RAW 264.7 macrophages to SP was not associated with an accumulation of nitrite in the medium, implying a declined NO production. We found that when RAW 264.7 macrophages induced with 10nM to 100nM of SP, there are no effects on the NO production. In contrast, Yaraee have reported SP enhances NO production both in HSV- and mock-infected macrophages (Yaraee, Ebtekar, Ahmadiani, Sabahi, & Ghazanfari, 2007).

Study that conducted by Andoh and Kuraish showed that SP at 1 to 10 μM elicited NO production in cultured human keratinocytes after only 5 minutes of SP administration (Andoh & Kuraishi, 2003). Surprisingly, during our study, after up to 4 hours of incubation with SP, there are no sign of elevated of NO production by that activated macrophages.

When we get that negative result, we further the study by introducing LPS into cultured cells. This is supported by several studies that reported that SP works as priming factor rather that a direct stimulator. Which is SP should sensitize macrophages making them more responsive to LPS.

Finding by Jeon et al., revealed that SP by itself failed to affect the basal level of nitrite production but on SP-LPS stimulated cells, after 24 hours of incubation, nitrite production were significantly increased in a dose dependent manner (1 to 1000nM) (Jeon, Jung, Choi, Oh, Shin, & Gwag, 1999). Thus, we had decided to choose SP concentration between 10nM to 100nM throughout the study and incubation time from 10 minutes up to 24 hours.

Again, we found that SP stimulation of NO production in LPS-activated macrophages also failed to increase the NO production. Different with finding by Jeon and colleagues and Guha and Mackman: stated that SP markedly augment LPS-induced NO production in time and concentration dependent manner (Jeon, Jung, Choi, Oh, Shin, & Gwag, 1999; Guha & Mackman, 2001). Thus, these findings ask us to seek the possibilities that might be occurred.

Binding of SP to NK-1R elicit the inflammation signaling. Sometimes, this binding causes rapid endocytosis and internalization of the receptor (Li, Leeman, Slack, Hauser, Saltsman, Krause, Blusztajn, & Boyd, 1997). The internalization and recycling of these receptors is of considerable functional importance because it contributes to desensitization, resensitization, and down-regulation of cellular responses to ligands (Grady, Garland, Gamp, Lovett, Payan, & Bunnett, 1995). Desensitization of cells to SP is believed to result from the limited amount of receptors toward SP. Moreover, macrophages are also capable for the production of SP which could increase the amount of SP in those cells, thus, competing with the external SP to the NK-1R.

Garland and colleagues reported that the NK-1R is rapidly desensitized to repeated exposure to SP (Grady, Garland, Gamp, Lovett, Payan, & Bunnett, 1995; Garland, Grady, Payan, Vigna, & Bunnett, 1994). They also demonstrated that within minutes of binding, both SP and its receptor are internalized into early endosomes and internalized SP is degraded, and internalization may be sufficiently rapid to contribute to desensitization. These events might be helpful to explain why nitrite secretion assay was not able to significantly increase the production of NO in RAW 264.7 cells.

V. CONCLUSIONS

We had conducted a study involving accumulation of NO (nitrite) in the culture medium using SP as inducer. Based on literature, SP play a role in the productions of NO and involved in inflammation processes. NO level was accessed by the specialised griess assay method. However, we had failed to shows that SP enhance NO production after following certain incubation time and dosage on RAW 264.7 macrophages. Nevertheless, we believe that our finding has uncovered some important constraints that need to be consider which involving highly sensitive neuropeptide.

List of Abbreviations

SP	Substance P
LPS	Lipopolysaccharide
NO	Nitric Oxide
NK-1R	Neurokinin-1 Receptor
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
SEM	Standard Error of Mean
ANOVA	Analysis of Variance

Figures

Fig. 1. - Effect of SP on Nitrite Production

The effect of SP on NO production in RAW 264.7 macrophages was measured by the Griess reaction. Cells were introduced with SP for 30 min (A), 20 min (B) and 30 min (C). SP at concentration 0 nM is in control group (basal). Cells were seeded at $5x10^5$ cells per well in 96 well plates and cultured for 24 hours in DMEM. The next day, cells 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). The data represents

the mean for triplicate and the bar represents the standard deviation. No significant different was observed.

Fig. 2. - Effect of SP on Nitrite Production

The effect of SP on NO production in RAW 264.7 macrophages was measured by the Griess reaction. Cells were introduced with SP for 1 h (A), 2 h (B) and 4 h (C). SP at concentration 0 nM is in control group (basal). Cells were seeded at $5x10^5$ cells per well in 96 well plates and cultured for 24 hours in DMEM. The next day, cells were subjected to SP at 10 nM, 50 nM and 100 nM concentration and incubate for 1 hour, 2 hours and 4 hour except for Basal group (non-treated cell). The data represents the mean for triplicate and the bar represents the standard deviation. Triplicate samples shown that SP unable to increase the NO level and no significant levels are found.

Fig. 3. - Effect of SP and LPS on Nitrite Production

RAW 264.7 cells were incubated with 10nM and 100nM of substance P in the presence or absence of LPS (100ng/ml) for 24h. Triplicate samples shown there were significantly increased of nitrite production in LPS and LPS-SP groups when compared to control. For SP group there were no significant level of NO are detected. The data represents the mean for triplicate and the bar represents the standard deviation. $*P < 0.05$ compared with control samples.

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