Cardamonin inhibits nitric oxide production modulated through NMDA receptor in LPS-Induced SH-SY5Y cell in vitro model

Nur Khalisah Kaswan1, Nurul Syazwani Mohd Suhaimi1, Noor Aishah Mohammed Izham1, Tengku Azam Shah Tengku Mohamad1, Mohd Roslan Sulaiman1 and Enoch Kumar Perimal*

1Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.

ABSTRACT

Background: Cardamonin is a naturally occurring chalcone from the Alpinia species. It is known to possess antioxidant and anti-inflammatory properties. Our previous studies have shown that cardamonin has antihyperalgesic and antiallodynic effects on CCI-induced neuropathic pain in mice. Although the evidence of the association between cardamonin and neuropathic pain has been reported in animal studies, specific targets using in vitro models are still lacking.

Objectives/Methods: This study aims to investigate the effect of cardamonin on nitric oxide production using the LPS-induced neuropathic pain-like SH-SY5Y in vitro model through NMDA receptor expression. Results: Cardamonin administration in differentiated SH-SY5Y cells significantly reduced nitric oxide production assessed using Griess reagent. Western blot analysis demonstrated a significant reduction in GluN2B receptor expression in the cardamonin treated SH-SY5Y cells compared to the vehicle treated group. Conclusions: These data suggest that cardamonin reduces nitric oxide production modulated through NMDA GluN2B receptor subunit. Our results provide preliminary data to support the in vivo studies using cardamonin and may contribute to further understanding the mechanisms of action of cardamonin.

Keywords: Cardamonin; NMDA receptor; SHSY-5Y cells; neuropathic pain

INTRODUCTION

Neuropathic pain is caused by a lesion or disease of the somatosensory system as defined by the International Association for the Study of Pain (IASP). Alteration of the somatosensory system disrupts the normal sensory transmission and subsequently give rise to neuropathic pain. The changes may come in several forms including injury of neuronal cells leading to the changes in growth-factor expression, hypersensitization of intact uninjured nociceptors and generation of spontaneous activities resulting in persistent pain (Campbell & Meyer, 2006). Neuropathic pain affects both central and peripheral nervous systems and are commonly linked to some of the common chronic diseases such as Parkinson’s, multiple sclerosis, diabetic neuropathy and postherpetic neuralgia.

Individuals with these diseases usually suffer from hyperalgesia and allodynia. Hyperalgesia is referred to an exaggerating pain to a painful stimulus while allodynia is pain arising from an innocuous stimulus (Gilron et al., 2015). IASP has reported that based on general population studies, 7-8% of adults are currently suffering from chronic pain with neuropathic characteristics (Van Hecke et al., 2014). The numerous adverse effects arising from the use of current medications have attracted neuropathic pain related researches. For example, ketamine and pregabalin, the two commonly used drugs to manage neuropathic pain symptoms are known to cause unwanted side effects such as hallucination (Niesters et al., 2014) and drowsiness (Bansal et al., 2009).
In neuropathic pain conditions, the N-Methyl-D-Aspartate (NMDA), a glutamate receptor has been extensively studied. The NMDA receptor is responsible for the binding of glutamate at the postsynaptic neuron (Li et al., 2011). NMDA is required in the normal physiology of the body, however, an increase of NMDA receptor activation lead to the potentiation of numerous signaling cascade leading to pain, at peripheral and central nervous system (Petruncio et al., 2014). Injuries and lesions to the nerve leads to the activation of glutamate receptor specifically NMDA and triggers the nitric oxide (NO) pathway through the conversion of L-arginine into L-citrulline mediated through the Ca²⁺ influx (Luo & Czokova, 2000). Production of NO will further catalyze soluble guanylyl cyclase (sGC) which in turn generates cyclic guanosine monophosphate (cGMP) a secondary messenger causing pain hypersensitization (Maruyama et al., 2012; Ashlaw et al., 2014).

Neuropathic pain is also mediated through neuroinflammation at peripheral and central nervous systems leading to the initiation and maintenance of persistent pain (Ellis & Bennett, 2013). Neuroinflammation triggers the pro-inflammatory mediators to be released during neuronal injury including interleukin 1β and 6, tumor necrosis factor α (TNF α) and NO and thereby induce neuronal hypersensitization (Boje et al., 2003; Üçeyler et al., 2007; Miyamoto et al., 2009; Lurie, 2018).

SY-SYST is a human neuroblastoma cell line that possess a neuronal phenotype that has been widely used to study neurodegenerative diseases (Zhao, 2009; Kovalevich & Langford, 2013). The differentiated SH-SY5Y cells have been shown to be almost similar in morphology, biochemical and pathological properties to the living neurons in the human brain (Xie et al., 2010). Besides, upon the differentiation of the cells, SH-SY5Y was shown to express similar receptor activation and signaling pathways as in neuropathic pain (Inada et al., 1998; Renaud & Spengler, 2002; Andaloussi-Lija et al., 2009). On the other hand, lipopolysaccharide (LPS), a gram negative bacteria induced neuroinflammation is an established model to induced neuropathic pain condition in numerous in vivo and in vitro study (Clark et al., 2010; Yoon et al., 2012; K. Sharma et al., 2018). Induction of LPS to SH-SY5Y was shown to increase the release of pro-inflammatory mediators and increase glutamate receptor channel expression which is important in maintenance of neuropathic pain (Mengke et al., 2016; Chia et al., 2020). Therefore, LPS-induced SH-SY5Y cell model is a reliable model to be used in this study to mimic the neuropathic pain-like condition in vitro.

In this study, cardamomin, a type of naturally occurring chalkone that is isolated primarily from fruits or seeds of Alpinia species (Rao et al., 1976) was used to evaluate its potential to treat neuropathic pain in LPS-induced neuropathic pain-like condition in vitro model of SH-SY5Y human neuroblastoma cells. A previous study by (Lee et al., 2012) reported that cardamomin exhibits anti-inflammatory and other numerous pharmacological properties such as anti-cancer (Park et al., 2013) and anti-oxidant (Baijai et al., 2011). Our previous study also demonstrated that cardamomin was able to alleviate hyperalgesia and alldynia in CCI-induced neuropathic pain mice (Sambasevam et al., 2017). However, a study on how cardamomin affects defined molecules or proteins in alleviating neuropathic pain is still lacking. This in vitro study was conducted to further support our in vivo findings. Therefore, this study aims to investigate the effect of cardamomin in LPS-induced neuropathic pain-like condition in vitro model of SH-SY5Y cells modulated through the NMDA receptor and NO pathway.

MATERIALS AND METHODS

Materials

Cardamomin or 2,4-dihydroxy-6-methoxychalcone was purchased from Calbiochem (US), RIPA buffer, Protease inhibitor and Chemiluminescent reagent kit. Dimethyl sulfoxide and Griess reagent were purchased from Sigma Aldrich (St. Louis, US), Dulbecco’s modified essential medium/Ham’s Nutrient Mixture (DMEM:F12), 1% Penicillin-Streptomycin solution, Trypsin, and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide (MTT) from Nacalai Tesque (Tokyo, Japan). Inactivated fetal bovine albumin (FBS) and Non-Essential Amino Acid (NEAA) were purchase from Gibco-BRL (Grand Island, NY). Lipopolysaccharide (LPS) and BCA reagent kit were purchased from Merck (Darmstadt, Germany) and Thermo Fisher (Massachusetts, US) respectively.

Compound preparation

100 μg/ml of cardamomin stock was freshly prepared in 100% DMSO and further diluted with media containing DMEM/Nutrient Mixture F-12 (DMEM:F12), 1% Penicillin-Streptomycin solution and 15% heat-inactivated fetal bovine albumin (FBS) to a concentration of interest; 2.5 μg/mL, 5.0 μg/mL and 10.0 μg/mL (Hatizcieria et al., 2006; Kim et al., 2010; El-Naga, 2014) The final concentration of DMSO was kept constant at 0.1% and it does not affect the cell viability (Pascoal et al., 2014).

Cell culture

SH-SY5Y human neuroblastoma cell lines were obtained from ATCC, thawed and cultured using growth medium mixture; DMEM:F12 which contains 4.5g/l glucose with 2mL of L-glutamate and sodium pyruvate supplemented with 15% heat-inactivated FBS, 1% of Penicillin-Streptomycin mixed solution and 1% NEAA maintained at 37°C humidified incubator containing 5% CO₂ were seeded in a 96-well plate with cell density of 1X10⁴ cells/well. The cells were induced with 10μM of all-trans retinoic acid for 5 days to induce differentiation in differentiation media (DMEM:F12, supplemented with 2.5% fetal bovine serum (FBS) and 1% of Penicillin-Streptomycin mixed solution) (Chia et al., 2020; Mohammed Isahm et al., 2018).

Cell viability

MTT assay was used measure the viability of SH-SY5Y cells. The media on the cell were discarded. 50 μL of serum-free media and 50 μL of MTT solution were added in each group and incubated for 3 hours at 37°C. After incubation, 150 μL of MTT solvent was added into each well. The plate was wrapped in foil and shaken for 15 minutes. Then, the absorbance was read at 590nm. The percentage of cell viability was compared to the non-treated group, and the percentage of viable cells were calculated.

LPS induction and treatment groups

LPS was used to induce neuronal sensitization to mimic neuropathic pain like condition in vitro. Following differentiation, the cells were induced with 1μg/mL of freshly prepared LPS (except normal control group) for 12 hours maintained in 37°C and 5% CO₂ incubator (Chia et al., 2020). 12 hours post LPS induction, three different concentrations of cardamomin (2.5 μg/mL, 5.0 μg/mL and 10.0 μg/mL), 16 μg/mL of L-NAME as positive control and vehicle (0.1% DMSO) were added to the LPS-induce cell culture for 24 hours.

NO measurements

NO concentration was assessed using Griess’ reagent (Bryan & Grisham, 2007). After 24 hours treatment, 100 μL of cell culture media with an equal volume of Griess’ reagent were added to each group and incubated for 15 minutes at room temperature. The incubation was done in a light-protected environment and absorbance was read at 540 nm using a microplate reader. The amount of nitrite in the media was calculated from sodium nitrite (NaNO₂) standard curve.

Western blot

Further evaluation on NMDA protein expression was done using western blot. 24 hours post treatment, the media was removed and the cells were washed with pre-chilled PBS, 200μg of RIPA and protease inhibitor mixture were added before scraping the cells in the plate. The
samples were then centrifuged at 10,000 rpm for 10 minutes and the supernatants were used to measure the protein using bicinchoninic acid assay (BCA). Equal amount of 10 μg of proteins were separated in 4-20% of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred into polyvinyldene di-fluoride (PVDF) membranes. The membranes were then blocked using 5% skimmed milk in TBS-T for 1 hour and washed for 3 times with 5 minutes interval. Then the membranes were incubated with primary antibody (GluN2B, 1:1000; Cell Signaling Technology, US; β-actin, 1:5000; Abcam Group, UK) overnight followed by secondary antibody (anti-goat IgG, 1:5000; Abcam Group, UK) for one hour with continuous agitation. The membranes were visualized using ECL solution (Advansta, USA) and detected by ChemiDoc™ imaging system. The band were measured and analysed using NIH ImageJ software.

Statistical analysis

All results were presented as mean ± S.E.M. Data was statistically analysed using one-way analysis variance (ANOVA) followed by post-hoc Tukey test using GraphPad Prism software, v8.0 (GraphPad San Diego, CA). Results were considered significant when p<0.05.

RESULTS

Cell viability

The effect of cardamonin on SH-SY5Y cell viability was measured using MTT assay. 12 hours post-treatment of three different cardamonin doses produced an increase in cell viability compared to the non-treated cells. Figure 1 shows a significant increase in SH-SY5Y cell viability when treated with 5 μg/mL and 10 μg/mL of cardamonin with significant level of p<0.05 and p<0.001 respectively. On the other hand, the lowest dose of cardamonin 2.5 μg/mL did not significantly increase or decrease the SH-SY5Y cell viability. Thus, we suggest that all the cardamonin doses do not cause toxicity to the cells.

Nitric oxide production

The effect of cardamonin on nitric oxide production in LPS-induced SH-SY5Y human neuroblastoma cells was evaluated by measuring total nitrate concentration as presented in Figure 2. The cells (except normal, N group) were administered with 1 μg/mL LPS for 24 hours. Following that, the cells were treated with respective doses of cardamonin for 24 hours before the total nitrite concentration was measured using Griess reagent. LPS and vehicle treated groups showed the highest total nitrate concentration compared to the normal SH-SY5Y cell. Different concentrations of cardamonin at 2.5 μg/mL, 5 μg/mL and 10 μg/mL significantly reduced the total nitrate concentrations at p<0.001 compared to LPS only and vehicle treated groups. The highest dose of cardamonin 10 μg/mL showed highest total nitrate concentration reduction. Based on this study and previously used dose of cardamonin, 10 μg/mL will be used for further evaluation on protein expression.

NMBA GluN2B protein expression

NMBA protein expression specifically the N2B subtype in cells was evaluated using western blotting. Figure 3 shows a significant reduction with p<0.05 on NMBA GluN2B expression in cardamonin treated cells compared to LPS only group. Cardamonin treated cells also managed to further reduce the NMBA GluN2B protein expression compared to the vehicle treated group however no significant difference was found between the two groups.

DISCUSSION

Our previous studies have demonstrated that cardamonin possesses antihyperalgesic and antiallodynic effects in CCI-induced neuropathic pain mice (Sambeavam et al., 2017). Even though numerous in vivo studies have been conducted to understand the mechanisms of neuropathic pain, the exact pathway remains unclear. One of the main reason is due to the multiple factors involved in the progression of neuropathic pain, types of immune cells activated, site of injury and type of injury involved (Colburn et al., 1999; Obata et al., 2006; Calvo et al., 2012; Inoue & Tsuda, 2018).

Figure 1: Cell viability of SH-SY5Y human neuroblastoma cells were determined using MTT assay. Data were presented in mean ± SEM with n=4. Significant difference was measured through one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. P level was set at *p<0.05/ ***p<0.001 compared to non-treated group. CDN (Cardamonin).

Figure 2: Total nitrate concentration in SH-SY5Y human neuroblastoma cells was determined using Griess assay. Data were presented in mean ± SEM with n=3. Significant difference was measured through one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. P level was set at *p<0.05, **p<0.01 compared to LPS group and ***p<0.001 compared to normal group. N (Normal); VEH (Vehicle, 0.1% DMSO); CDN (Cardamonin).

Figure 3: NMBA GluN2B receptor expression in LPS-induce SH-SY5Y cells samples of normal, vehicle, LPS only and cardamonin-treated groups were evaluated using western blotting. Data were presented in mean ± SEM with n=4; p<0.05/***p<0.001 as compared to LPS only group and #p<0.05 as compared to normal group. N (Normal); VEH (Vehicle, 0.1% DMSO); CDN (Cardamonin, 10 μg/mL).
Studies using specific in-vitro models may be beneficial in finding specific pathways and may help to provide more effective treatment in the future for neuropathic pain. This study aimed to investigate the effect of cardamomin in LPS-induced SH-SY5Y cells a neuropathic pain-like condition in-vitro model.

As described earlier, neuropathic pain arises due to a lesion or injury at the somatosensory system (Campbell & Meyer, 2006) Several mechanisms have been postulated to involve in neuropathic pain disease, progression including inflammation (Scholz & Woolf, 2007; Baron et al., 2010). Nerve injury or lesion of the neuron leads to the infiltration of pro-inflammatory mediators to cause pain sensitization (Moalem & Tracey, 2006; Thacker et al., 2007). Excessive inflammation that occurs in peripheral and central nervous systems promotes the neurotransmitter activation and sensitizes the primary afferent neurons that contribute to the initiation and maintenance of pain (Moalem & Tracey, 2006; Ellis & Bennett, 2013).

Several types of pro-inflammatory mediators are released during inflammation that mediates neuropathic pain sensitization including IL1β, IL6, TNF-α and NO (Milligan et al., 2003; J. Sharma et al., 2007; Leung & Cahill, 2010; Clark et al., 2013). Compared to the other pro-inflammatory mediators, nitric oxide has been studied widely in the pathophysiology of neuropathic pain (Luo & Cizkova, 2000) This is due to the dual function of NO that is able to promote and inhibit nociception depending on the availability and downstream pathway activation in peripheral and central nervous system (Sousa & Prado, 2001; Curry et al., 2011). Nitric oxide production is known to be enhanced in neuropathic pain models through the increase of nitric oxide synthase activity at the injured nerve (Levy & Zochodne, 2004; Naik et al., 2006). Therefore, inducing nitric oxide production in cells can be used to mimic the neuropathic pain-like condition in in-vitro studies.

In this experiment, lipopolysaccharide (LPS) has been used to induce nitric oxide synthesis in human neuroblastoma cells (SH-SY5Y) to mimic the in-vitro model of neuropathic pain-like condition. LPS is a structure of gram-negative bacteria that is important in immunomodulatory effects (Thomson & Moland, 2000). High concentration of LPS in the cells is able to enhance the release preinflammatory mediators to trigger the inflammatory response causing the development of neuropathic pain (Caroff & Karibian, 2003; Tanga et al., 2005; Lin et al., 2008; Xu et al., 2013). According to the data reported by (Chen et al., 2001) LPS treatment in raw cells were able to enhance the production of nitric oxide meditated through nitric oxide synthase activity. In line with this, exposure of LPS in SH-SY5Y cells also enhance total nitrate concentration when compared to non-treated LPS cells. In the present study, our data shows that cardamomin reduced nitric oxide levels in the LPS-induced SH-SY5Y cells model indicating an anti-inflammatory effects on the neural nitric oxide synthase synthase.

Unlike the other inflammatory mediators, NO is produced through the activation of nitric oxide synthase (NOS) enzyme consisting of three different isoforms including neuronal NOS, inducible NOS and endothelial NOS. All nitric oxide synthases are Ca2+-calmodulin-dependent enzymes constitutively produce in the body except for inducible NOS which is depend on Ca2+ influx (Ortiz-Ortiz et al., 2009; Förstermann & Sessa, 2011; Joca et al., 2019). Transmission of Ca2+ into the postsynaptic neuron is facilitated through the glutamate channel, N-Methyl-D-Aspartate (NMDA) receptor. Nerve injury to the neurons enhances glutamate release at the presynaptic membrane and subsequently activate NMDA receptors, thereby increasing the Ca2+ influx which then triggers the nitric oxide cascade in the neuron (Curry et al., 2011; Liescher & Malenka, 2012; Mukherjee et al., 2014).

NMDA receptor is further subdivided into different subunit including GluN1, GluN2 and GluN3 (Cull-Candy et al., 2001). Among the NMDA subunits, NR2B subtypes are known to be responsible for pain transmission through the activation of the downstream pathway that leads to pain sensitization (Qiu et al., 2017). Our study demonstrated that GluN2B expression increased in SH-SY5Y cells upon LPS exposure. In addition, our result also reveals that cardamomin treatment in LPS induced group reduces NMDA GluN2B receptor subunit expression in SH-SY5Y cells which in turn reduces the nitric oxide production. Therefore, the current data suggests pretreatment of LPS and co-administration of cardamomin after LPS exposure is able to reduce neuropathic pain-like neuroinflammation condition in the in-vitro model used.

CONCLUSION

In summary, the present study shows that LPS successfully induced neuroinflammation in the SH-SY5Y used and is able to mimic neuropathic pain-like condition in an in-vitro model. This process is modulated through nitric oxide and NMDA GluN2B receptor. Cardamomin was shown to reduce the GluN2B expression and eventually reduce nitric oxide production in SH-SY5Y cells. Thus, this study provides an insight into the role of cardamomin in SH-SY5Y cells for the development of a more specific treatment for neuropathic pain in future.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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