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Protective effect of HAMI 3379 against high glucose-induced PC12 cell injury

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Diabetic neuropathy is one of the most common diabetic complications, associated with long time exposure to high glucose. Hyperglycemia induces the production of reactive oxygen species (ROS) and reactive nitrogen species contributing to neuronal damage. We have previously reported that HAMI 3379, a selective antagonist of $CysLT_2$ receptor, is involved in neuron injury after ischemia. In this study, we investigated the protective effect of HAMI 3379 against high glucose-induced cell injury in PC12 neural cells. The PC12 cells were pretreated with various concentrations of HAMI 3379 (0.001 ~ 10 μM) for 1 h and then co-treated with HAMI 3379 and D-glucose (100 mM) for 48 h. HAMI 3379 (0.001 ~ 10 μM) protected PC12 cells against high glucose toxicity, as determined by cell viability and apoptosis after the evaluation by Hoechst 33342 staining assay. In addition, the increased nitric oxide production and nitric oxide synthase (NOS) activity in the media induced by high glucose was significantly inhibited by HAMI 3379. These results demonstrate that HAMI 3379 protected PC12 cells against high glucose-induced neurotoxicity by inhibition of apoptosis, nitric oxide production and nitric oxide synthase activity, suggesting that $CysLT_2$ receptor may be a potential target for diabetic neuropathy, and its antagonist may play a crucial role in the treatment of diabetic neuropathy.

Key words: Cysteinyl leukotriene receptor, glucose, nitric oxide synthase, nitric oxide.

INTRODUCTION

Diabetic neuropathy is one of the most common diabetic complications, characterized by neuropathic pain that occurs spontaneously (Arora and Singh, 2013), with approximately 50% of diabetic patients to suffer from pain and hyperalgesia (an increased level of glucose in the blood). Neuropathic pain is mostly associated with long-time exposure to high glucose, thus hyperglycemia is considered as a major factor of tissue damage (Afrazi et al., 2014). However, the mechanisms of diabetic neuropathy are not completely known. Recently, it has been reported that there is close relationship between diabetes and neurodegenerative disorders, such as Alzheimer's disease and Parkinson disease. Some evidence indicated that hyperglycemia induced atherosclerosis, contributing to insufficiency of blood flow to neuronal cells (Green et al., 2014). However, not much is known about the direct toxic effect of high glucose levels on neuronal cells. Here the effect of high glucose on cell viability was determined on high-differentiated PC12 cells as a model of neuronal cells.

It is well known that apoptosis has been regarded as possible mechanism for high glucose-induced neuronal

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dysfunction and cell death (Xu et al., 2012; Afrazi et al., 2014). Under hyperglycemic conditions, massive free radicals such as nitric oxide (NO) and reactive oxygen species (ROS) are produced, contributing to the oxidative stress and neuronal apoptosis increased (Renaud et al., 2014). Additional evidence indicated that eNOS, which synthesize nitric oxide (NO), is involved in neuron toxicity (Lipton et al., 1993; Koshimura et al., 1998). In the present study, possible involvement of NOS mediated NO production in high glucose-induced cell toxicity was investigated. HAMI 3379, a selective antagonist of cysteinyl leukotriene receptor 2, has been suggested as a potential protective substance in the central nervous system (Shi et al., 2012). In the present study, the possible protective effect of HAMI 3379 on high glucose-treated PC12 cells was investigated as an *in vitro* model of diabetic neuropathy.

MATERIALS AND METHODS

Drugs and chemicals

HAMI 3379 was purchased from Cayman Chemical (CAT.10580, USA). MTT [3-(4,5)-dimethylthiahiazo(-zy-l)-3,5-di-phenytetrazoliumromide] and Hoechst 33342 were purchased from Sigma-Aldrich (CAT.B2261, USA). RPMI 1640 was purchased from Hyclone (Thermo scientific, USA). Fetal bovine serum (FBS) was purchased from Sijiqing (Hangzhou, China). Nitric oxide metabolite detection kit and NOS detection assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (CAT. A012, A014-2, China).

Cell culture and treatment

PC12 cells were obtained from the academia Sinica (Shanghai, China), maintained and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in a humidified environment of 5% CO_2 at 37°C. To produce hyperglycemia, the cells were treated with D-(+)-glucose at different concentrations (25, 50, 75, 100, 150, 300 mM) for 24 and 48 h. Later, the cells were pretreated with D-glucose (100 mM) for 1 h, then co-treated with HAMI 3379 and D-glucose for continuous 48 h. Control cells were cultured normally, not treated with HAMI 3379. HAMI 3379 was dissolved in mixed dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS). The final concentration of DMSO was less than 0.1% (v/v). Each independent experiment was carried out more than three times.

Cell viability analysis

The cells were seeded onto 96-well culture plates at 5000 cells/well and cell viability was assessed by a modified 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) reduction assay. MTT was dissolved in PBS and prepared a final concentration of 5 mg/ml. After glucose treatment in the absence or presence of HAMI 3379, 10 µl of MTT (5 mg/ml) solution was added to the cultured media (100 µl) at a final concentration of 0.5 mg/ml. The cells were incubated at 37°C for 4 h. Then the media were removed carefully and 100 µl of dimethyl sulfoxide (DMSO) was added to each well. The absorption was determined at 570 nm by an enzyme linked immunosorbent assay (ELISA) reader. Results were expressed as percentages of control group.

Hoechst 33342 staining

Hoechst 33342 staining followed by analysis with fluorescence microscopy assessed apoptosis. PC12 cells were seeded in the 24-well plates at 3×10^5/well and treated with Hoechst 33342 fluorochrome (10 µg/ml) for 10 min at 37°C. After repeated washing in PBS, the cells were fixed in cold methanol (-20°C). Then the stained cells were observed under a fluorescence microscope (Olympus BX51, Tokyo, Japan). The apoptotic cells were determined as condensed nuclei with strong bright Hoechst 33342 staining. A total of 200 cells from five random fields were counted and the percent of apoptotic cells were expressed as percentages of total cells.

Nitric oxide and NOS assay

After glucose (100 mM) and HAMI 3379 (0.001 ~ 10 µM) treatment for 48 h, the supernatant was collected. NO generated by cells was quantified by measuring nitrate and nitrites in the culture media with Nitric oxide detection assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, 100 µl of the supernatant was added to the mixed solution (1.8 ml), after incubated at 37°C for 60 min, the absorption was determined at 550 nm by an ELISA reader. NOS was measured using a NOS activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, 100 µl of the supernatant was added to the mixed solution (1.95 ml), after incubated at 37°C for 15 min, the absorption was determined at 530 nm by an ELISA reader. All the procedures were operated strictly according to manufacturer's protocols.

Statistical analysis

Data are expressed as means ± standard error of mean (SEM). The differences were analyzed by one-way ANOVA followed by the Newman-Keels test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of glucose on PC12 cell viability

The effects of different concentrations of glucose on PC12 cells viability were evaluated by using the MTT assay. After the initial grow period, the cells were exposed to glucose at the concentration of 25, 50, 75, 100, 150, 300 mM for 24 and 48 h. MTT assay showed that glucose could decrease the viability of PC12 cells in a concentration-dependent manner after 48 h (Figure 1A). The level of 100 mM glucose for 48 h had about 35% reducing effect on relative cell viability, while no effect was found for 24 h. This toxicity was time-dependently increased, so the concentration was selected to induce cell injury and evaluate the protective effect in the next sessions.

Effect of HAMI 3379 on high glucose-induced cell toxicity

To elucidate the protective effect of HAMI 3379 *in vitro*,

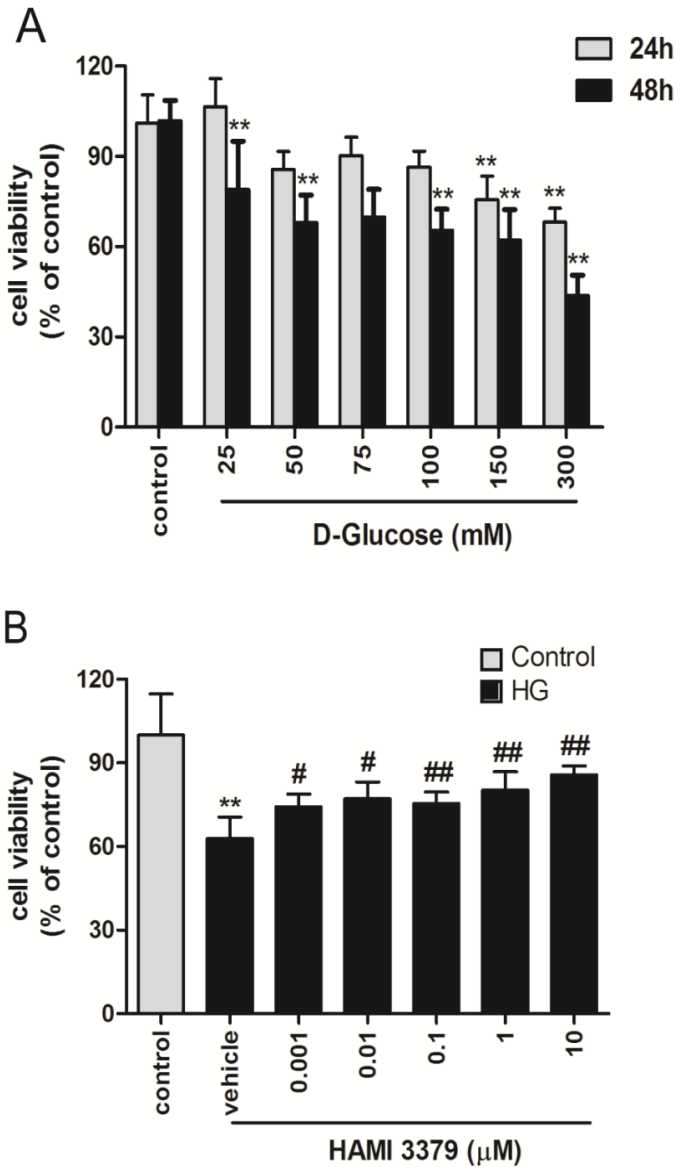

Figure 1. Effect of high glucose and HAMI 3379 on PC12 cell viability. A. PC12 cells were incubated in media containing varying concentrations of glucose (25, 50, 75, 100, 150, 300 mM) for 24 and 48 h. Cell viability was determined by MTT assay. B. The cells were treated with HAMI 3379 (0.001-10 μM) 1 h and then exposed to high glucose (100 mM) for 48 hours. The data are expressed as means ± S.E.M. n = 8 for each group. $^{**}P < 0.01$ versus control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus vehicle group.

we study the effect of HAMI 3379 on cultured PC12 cells with high glucose treatment. As shown in Figure 1B, D-glucose (100 mM) for 48 h reduced PC12 cells viability, however, the pretreatment with HAMI 3379 at 0.001 ~ 10 μM concentration-dependently prevented the cytotoxicity induced by high glucose ($P < 0.05$). No toxic effect was found on normal medium-incubated cells, treated with HAMI 3379 at concentrations of 0.001 ~ 10 μM.

HAMI 3379 inhibited high glucose-induced apoptosis

HG-induced apoptosis, we study the effect of HAMI 3379 on HG-induced cultured PC12 cells apoptosis stained by Hoechst 33342. As shown in Figure 2A, exposure to high glucose (100 mM) for 48 h increased the ratio of apoptotic cells with cell shrinkage, chromatin condensation, and strong bright fluorescent nuclei. The

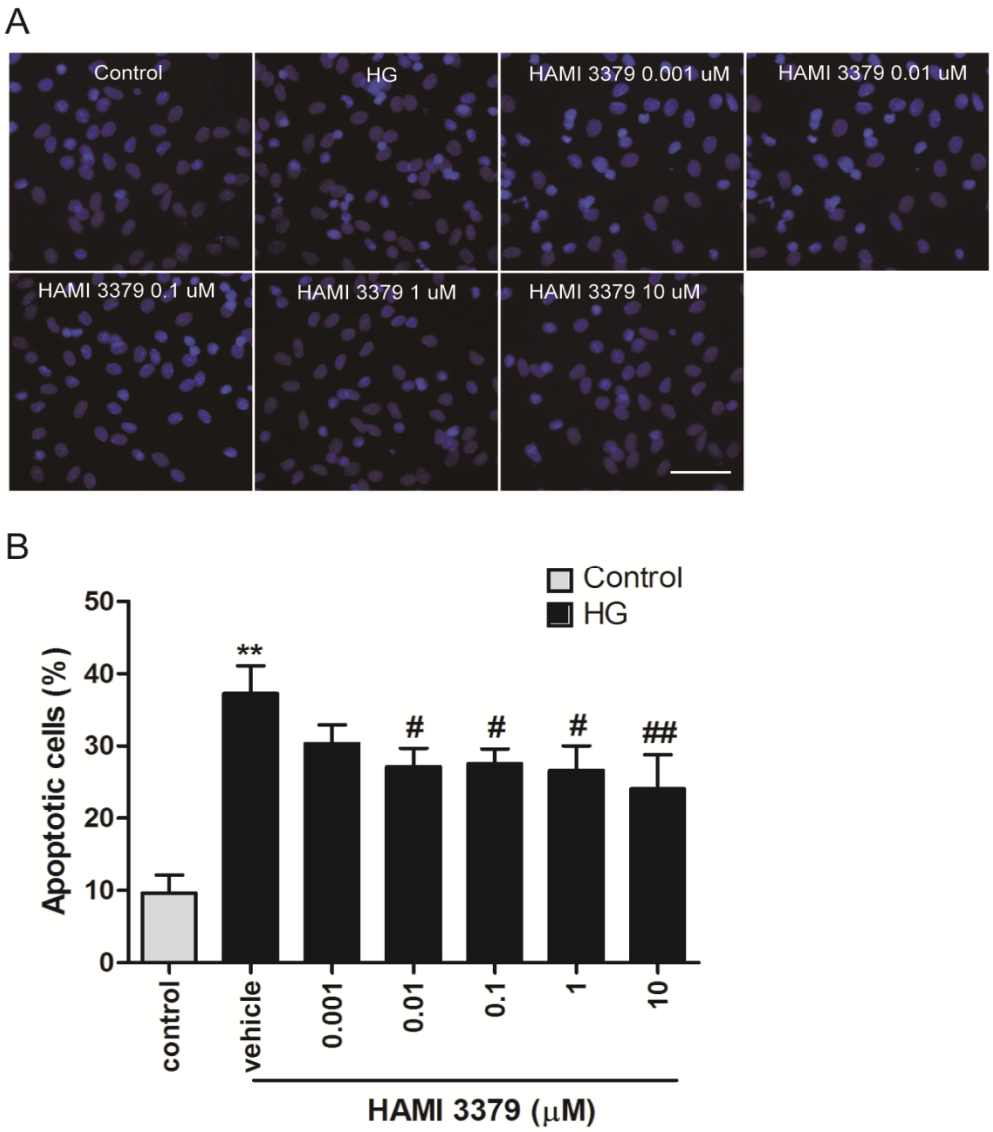

Figure 2. HAMI 3379 reduced high glucose-induced apoptosis in PC12 cells. The cells were treated with HAMI 3379 (0.001-10 μM) 1 h and then exposed to high glucose (100 mM) for 48 h. After 48 h, cells were collected and stained with Hoechst 33342, and apoptotic cells were numbered. A. Representative photographs of PC12 cells stained with Hoechst 33342. B. Summarized data of apoptotic cells. Data are expressed as means ± S.E.M. n = 8 for each group. $^{**}P < 0.01$ versus control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus vehicle group.

ratio of apoptotic cells was significantly reduced by HAMI To examine whether HAMI 3379 protect PC12 cells from 3379 increased concentrations of 0.01 ~ 10 μM (Figure 2B, P < 0.05). Vehicle group (DMSO 0.1%) or HAMI 3379 (0.001 μM) had no effect on the number of PC12 apoptotic cells under high glucose conditions.

HAMI 3379 reduced high glucose-induced NO release and NOS activity

To explore whether NOS/NO is involved in the protective effect of HAMI 3379 on HG-induced PC12 cell injury, NO production and NOS activity in the media were determined. We found that increased nitric oxide (NO) level induced by 100 mM glucose was reduced by pretreatment with HAMI 3379 (0.01 ~ 10 μM) in a concentration-dependent manner (Figure 3A.). As expected, nitric oxide syntheses (NOS) activity was significantly increased in the high glucose-treated PC12 cells as compared to those exposed to control medium. HAMI 3379 (0.01 ~ 10 μM) significantly inhibited NOS activity induced by high glucose compared to vehicle group (Figure 3B). HAMI 3379 (0.001 μM) or Vehicle

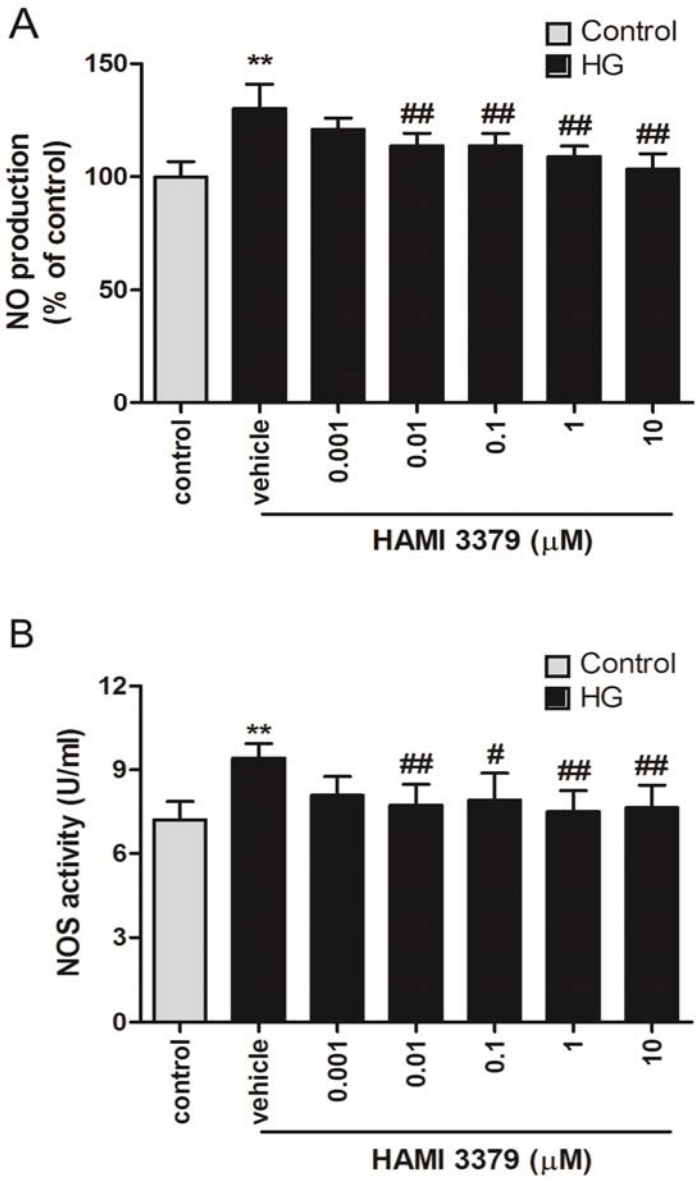

Figure 3. Effect of HAMI 3379 on NO level and NOS activity in the medium after HG exposure in PC12 cells. The cells were treated with HAMI 3379 (0.001 to 10 μM) 1 h and then exposed to high glucose (100 mM) for 48 h. After 48 h, the media were collected and NO level (A) and NOS activity (B) were detected by ELISA. HAMI 3379 significantly decreased NO release and NOS activity in a concentration-dependent manner. Data are expressed as means ± SEM. n = 6 for each group. $^{**}P < 0.01$ versus control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus vehicle group.

group (DMSO 0.1%) had no effect on NOS activity of PC12 cells under the condition of high glucose.

DISCUSSION

Neuropathy is one of the most common and devastating complications of diabetes mellitus and causes pain, decreased motility till amputation. Increasing evidence indicated that hyperglycemia played a key role in the development and progression of diabetic neuropathy (Singh et al., 2014). Hyperglycemia induces oxidative stress to generate reactive oxygen species and reactive nitrogen species in diabetic neurons resulting in neuronal damage and dysfunction (Vincent et al., 2005). It has been shown that high glucose induces apoptosis in

primary neurons by increasing the production of ROS (Liu et al., 2013). In the present study, we also found that high glucose levels reduced cell viability and increased apoptosis in PC12 neuronal cells. We previously reported that HAMI 3379 exerts powerful neuroprotective activity in neuronal injury against ischemia. Since neuronal injuries are often associated with inflammatory mediators such as cysteinyl leukotrienes (CysLTs). So, we examined whether HAMI 3379 have the potential to safely modulate neuronal injury under hyperglycaemia conditions.

CysLTs are potent inflammatory mediators as metabolites of 5-lipoxygenase, playing important roles in the pathogenesis of neurodegenerative diseases such as stroke. In streptozotocin-induced diabetic rats, previous researchers reported activation of the 5-lipoxygenase (5-LO) pathway in aorta associated with increased 5-LO expression and CysLTs production (Hardy et al., 2005). Abundant 5-LO expression was found in human atherosclerotic carotid lesions in diabetic plaque more than in non-diabetic plaque (Zhou et al., 2007). It is reported that hyperglycemia was considered to induce atherosclerosis. The present study indicated that besides atherogenic effect, high glucose levels had direct toxic effect on neural cells, suggesting a possible role of 5-LO pathway in mediating high-glucose induced neuron injury.

CysLTs acts through their receptors, and two receptors ($CysLT_1$ and $CysLT_2$) have been cloned. $CysLT_2$ receptor mediated ischemia-induced neural injury through microglia activation and cytokine production such as TNF-α and IL-1β. Our data showed that HAMI 3379 possibly contributing in the selective blocking of the $CysLT_2$ receptor leading to the reduction of the high glucose induced PC12 cell toxicity. It seems that $CysLT_2$ receptor also play an important role in the pathogenesis of neural cell injury under hyperglycemia conditions. However, further study is necessary to be done to investigate how 5-LO/CysLTs pathway mediates hyperglycemia-induced neuron injury.

In allergic rhinitis rats, LTD_4 induced production of NO in nasal cavity, and LTD_4-induced vesodilation was significantly inhibited by L-NMMA (an inhibitor of NOS) (Sakai et al., 2010). In the present study, nitric oxide and NOS activity in the medium was increased after the incubation with 100 nM of glucose. When the cells were cultured in the presence of HAMI 3379, the glucose-induced NO production and NOS activity in the media was decreased. These results reveal a protective effect of HAMI 3379 on glucose toxicity in PC12 cells. It has been already reported that high glucose-induced toxicity in PC12 cells is mediated through NOS activation to product nitric oxide (Koshimura et al., 2002). In rats with type 2 diabetes, iNOS expression was increased in aorta and retina after injection of streptozotocin, indicating an important role in the pathogenesis of diabetic retinopathy and atherosclerosis. Excess amount of NO produced via NOS activation was deeply involved in cardiovascular dysfunction during endotoxemia (Carmo et al., 2000; Bournival et al., 2012).

In summary, the current study demonstrated that the protective effect of HAMI 3379 on high glucose-induced cell injury and apoptosis in PC12 neural cells is related with the inhibition of NOS activation and NO production. Our results may provide a new clue for hyperglycemia induced diabetic neuropathy. However, more cellular and molecular studies will be needed to cast a light on the powerful roles of these compounds as preventive and complementary strategies for diabetes therapy.

Conflict of interest

The authors declare that there are no conflicts of interest.

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