Taurine Promotes Neuritic Growth of Dorsal Root Ganglion Cells Exposed to High Glucose in Vitro

Mengren Zhang, Inam-u-llah, Xiaoxia Shi, Pingan Wu, Kaixin Li, Raheel Suleman, Rana Muhammad Aadil, Muhammad Zubair Saleem, and Fengyuan Piao

Abstract Diabetic neuropathy (DN) is the most common chronic complication of DM and its major pathological changes show axonal dysfunction, atrophy and loss. However, there are few reports that taurine promotes neurite growth of dorsal root ganglion (DRG) cells. In current study, DRG neurons were exposed to high glucose (HG) with or without taurine. The neurite outgrowth of DRG neurons was observed by fluorescent immunohistochemistry method. Expression of Gap-43, Akt, phosphorylated Akt, mTOR and phosphorylated mTOR was determined by Western blot assay. Our results showed that HG significantly decreased the neurite outgrowth and expression of Gap-43 in DRG neurons. Moreover, phosphorylated levels of Akt and mTOR were downregulated in DRG neurons exposed to HG. On the contrary, taurine supplementation significantly reversed the decreased neurite outgrowth and Gap-43 expression, and the downregulated phosphorylated levels of Akt and mTOR. However, the protective effects of taurine were blocked in the presence of PI3K antagonists LY294002 or Akt antagonists Perifosine. These results indicate that taurine promotes neurite outgrowth of DRG neurons exposed to HG via activating Akt/mTOR signal pathway.

Mengren Zhang and Inam-u-llah as co-first author contributed equally to this work.

M. Zhang · Inam-u-llah · X. Shi · P. Wu · K. Li · F. Piao (✉)
Department of Occupational and Environmental Health, Dalian Medical University, Dalian, China

R. Suleman
Institute of Food Science and Technology, Graduate School of Chinese Academy of Agriculture Science, Beijing, China

R. M. Aadil
National Institute of Food Science and Technology, University of Agriculture Faisalabad, Faisalabad, Pakistan

M. Z. Saleem
Cancer Research Centre, Dalian Medical University, Dalian, China

Keywords High glucose · Diabetic neuropathy · Taurine · Dorsal root ganglion cells · Neurite outgrowth

Abbreviations

DRG   Dorsal root ganglion
HG   High glucose
T   Taurine

1 Introduction

Diabetes mellitus (DM) designates various metabolic disorders identified by elevated blood sugar level for an extended period of time as the pathology proceeds (American Diabetes Association 2014). DM is categorized into two major classes including Type 1 (insulin-dependent DM) and type 2 (non-insulin-dependent DM) (Sirdah 2015). The hyperglycemic condition of diabetes gives rise to increased risk of several complications that involve nephropathy, retinopathy, and neuropathy (Sarkar et al. 2017). Diabetic neuropathy (DN) is the most serious long-term complication of DM and its major pathological changes show axonal dysfunction, atrophy, and loss. (Inam-u-llah et al. 2018). DN causes diverse clinical manifestations such as sensory loss, pain and increase the risk of foot ulcers and amputation 5–7 (Kobayashi and Zochodne et al. 2018). About 30% of the diabetic patients have neuropathy while 50% of the patients are expected to develop neuropathy during disease (Asieh Hosseini and Abdollahi 2013), which is associated with considerable mortality and decline in life standard (Bril 2012). Although there are important symptomatic options in pain management, but the treatment that can unequivocally arrest or reverse progressive neuropathy is not still available beyond strict control of glucose levels. Therefore, promoting axonal regeneration and repair may be a good strategy for treating diabetic neuropathy.

Taurine, 2-aminoethylsulphonic acid, is a non-protein amino acid which is present in approximately all animal tissues and most abundant as a free amino acid in human cells (Kim et al. 2012). Especially, Taurine is one of the highly abundant free amino acids in the nervous system and is vital for normal nervous system development and growth. Taurine is considered to exhibit multiple functions, including: osmoregulation, modulation of calcium fluxes, normalization of the electroretinogram, modifying protein phosphorylation, stabilization of cell membranes, antioxidant properties, favoring of migration of cell in brain and retina (Huxtable 1989; Lima 1999). Many studies have revealed the therapeutic effect of taurine and its products against diabetes mellitus (Sarkar et al. 2017). Taurine enhanced glucagon activity, favored glycemic stability, improved glucose levels, addressed hyperglycemia and improved insulin secretion, and insulin resistance. It has also a protective

effect against DN (Inam-u-llah et al. 2018). It was shown that taurine administration reverses deficits of hind limb and improves nerve conduction velocity sciatic motor, threshold in sensory nerves and blood flow in nerves Zucker diabetic fatty rats (Li et al. 2006). It is also known to have neurotrophic actions. It promotes repairing of nerve and regeneration (Oja and Kontro 1990). It was exhibited previously that taurine increased extensions from goldfish ganglions in retina after mild type of crush in optic nerve (Lima et al. 1988) and increased the number of extensions in separated cultured retinal cells (Matus et al. 1997). However, there is small number of reports about that discuss about axonal regeneration by taurine in DN.

In the present study, DRG or dorsal root ganglions cells were subjected to high glucose (HG) with or without taurine. The neurite extensions from DRG neuron was observed by immunofluorescent staining of SMI312. Expression of Gap-43, Akt, phosphorylated Akt, mTOR and phosphorylated mTOR were checked by Western blot assay. Moreover, these parameters were also examined with PI3K antagonists LY294002 or Akt antagonists Perifosine. The results show that taurine promotes neurite development from DRG neuron exposed to HG in vitro via activating Akt/mTOR signal pathway. These results may provide a new therapeutic approach for diabetic neuropathy and a novel insight for explaining protective mechanism of taurine.

2 Methods

2.1 Primary Cultures of Rat DRG Neurons

After 1–2 days of birth SD rats were sacrificed, the vertebrae were exposed under aseptic conditions. The neurons were removed under a microscope and moved to a centrifuge tube containing L15 (Gibco, USA) medium. After washing with PBS, type II collagenase (sigma, USA) was added. After incubation at 37 °C for 45 min, removed and 2 ml of PBS containing 20% FBS was added, and incubated at 37 °C for 10 min, washed with L15 medium containing 1% BSA, and inoculated at a density of 5×105 cells/ml. The transwell lower layer of Matrigel was plated. After 24 h, Ara-C was added at final concentration of 5 µg/ml. After 24 h, the normal medium was changed.

2.2 Experimental Grouping

The cells were classified into several groups, Con group: cells were cultured by normal culture medium, HG group: cells were exposed to 50 mM glucose within culture medium, three taurine treatment groups: cells were co-treated at 50 mM glucose with 10 mM, 20 mM and 40 mM taurine. HG + T group: 50 mM glucose

and 40 mM taurine were added in the medium. HG + T + LY294002 group: 20 µM PI3K antagonists LY294002 (Beyotime, China) added into the medium with 50 mM glucose and 40 mM taurine. HG + T + Perifosine group: 50 mM glucose and 40 mM taurine with added 5 µM Akt antagonists Perifosine (Beyotime, China) in the medium.

2.3   Immunofluorescence Staining

The cells were secured in 4% paraformaldehyde at 4 °C for 15 min. These were washed with PBS for 10 min three times. 10% goat serum (10% goat serum, 1% BSA and 0.3% triton 100 dissolved in PBS) closed for 1 h. These were incubated overnight at 4 °C with primary antibody SMI312 (1:400, Biolegend, USA). Three Washings with PBS for 10 min each, Incubation of secondary antibody Alexa Fluor 594 conjugated donkey anti-mouse IgG secondary antibody (1:500, Jackson, USA) at 37 °C while avoiding light, washed with PBS for 10 min three times. Sample was mounted sample by mounting medium, observed by fluorescence microscope (Olympus, Japan) in dark room.

2.4   Western Blot Analysis

Protein was extracted from DRG cell cultures. Equal amounts of protein were separated on 12% SDS – polyacrylamide gels and moved to PVDF membrane (Millipore, USA). The membrane was blocked with TBS containing 0.1% Tween-20 (TBST) and 5% nonfat dry milk, reacted with primary antibodies, include GAP-43 (1:1000, Sigma, USA), Akt (1:1000, Sigma, USA), p-Akt (1:1000, Sigma, USA), mTOR (1:1000, Abcam, USA), p-mTOR (1:1000, Abcam, USA), β-actin (1:1000, Abcam, USA), overnight at 4 °C, followed by incubation with horseradish peroxidase (HRP)-conjugated second antibodies about 2 h with wash between each step. The membrane was developed with enhanced chemiluminescence reagent (keyjen, China) and ChemiDoc-It Imaging System UVP Gel Imaging Analysis System Scans PVDF Films. Quantitative analysis was performed Using ImageJ1.4.3.67 software.

2.5   Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM) and analyzed by SPSS using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. Statistical significance was considered at $p < 0.05$.

3   Results

3.1   Effect of Taurine on the Axon Growth of DRG Neurons Exposed to HG

In order to study the consequences of taurine supplementation on axon growth of HG-exposed DRG neurons, the axon outgrowth and the expression of Gap-43 was measured. As shown in Fig. 1a, the axon development of DRG neurons was inhibited in the presence of HG, but taurine promoted axon outgrowth in HG-treated DRG neurons in a dose-dependent manner, as shown in Fig. 2b, the neurite length
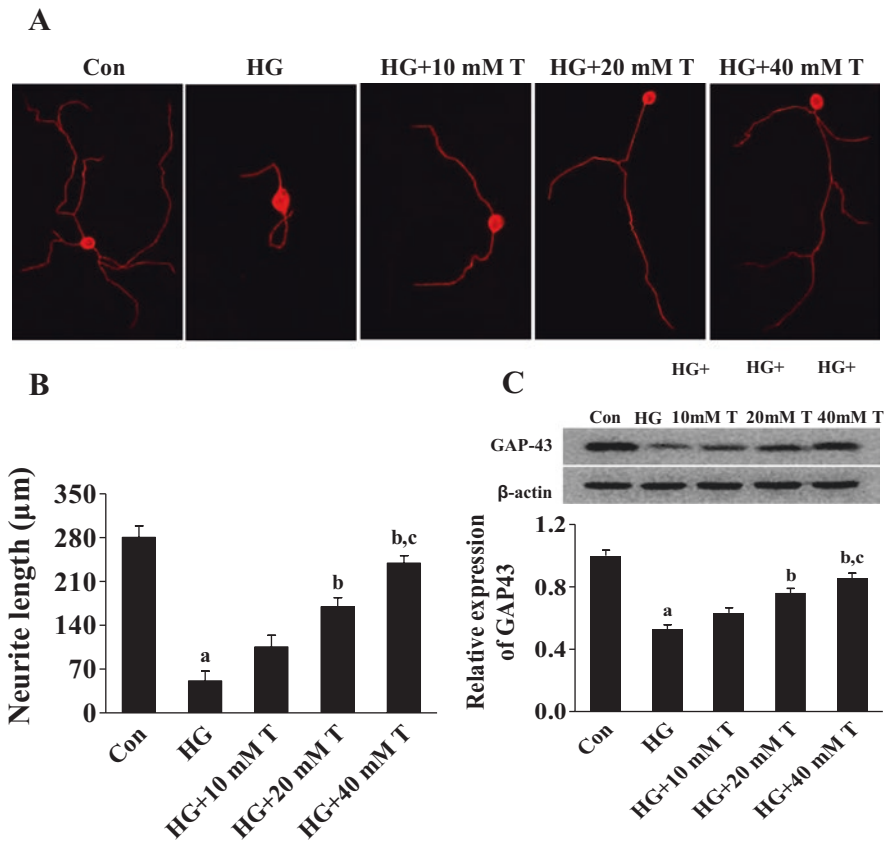

Fig. 1 Neurite outgrowth and the expression of GAP-43 in DRG neurons exposed to high glucose in presence of taurine. (**a**) Immunofluorescent images showing SMI312 immunoreactive DRG neurons. (**b**) Neurite length of DRG neurons. (**c**) Relative expression of GAP-43 in DRG neurons. [a]$p < 0.05$, compared with Con group; [b]$p < 0.05$, compared with HG group; [c]$p < 0.05$, compared with HG + 10 mM T group; [d]$p < 0.05$, compared with HG + 20 mM T group. Data are expressed as mean ± SEM
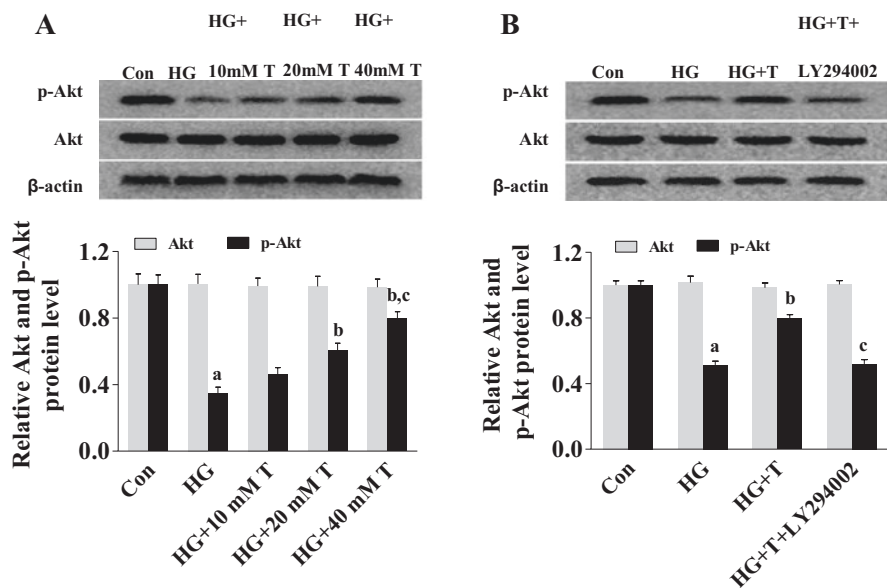
Fig. 2 Akt and p-Akt expression in the treated DRG neurons in presence of taurine. (**a**) Relative Akt and p-Akt protein levels in DRG neurons. (**b**) Relative Akt and p-Akt protein levels in DRG neurons within or without PI3K antagonists LY294002. [a]$p < 0.05$, compared with Con group; [b]$p < 0.05$, compared with HG group; [c]$p < 0.05$, compared with HG + 10 mM T group or HG + T group; [d]$p < 0.05$, compared with HG + 20 mM T group. Data are expressed as mean ± SEM

of DRG cells was significantly decreased in HG group compared with control group ($p < 0.05$). However, the neurite length of DRG neurons were significantly higher in taurine treatment groups than that in HG group ($p < 0.05$), increasing dose-dependently. Axon outgrowth relative protein Gap-43 of DRG neurons was studied by western blot analysis. Then, as shown in Fig. 1c, the Gap-43 expression was evidently decreased in HG group compared with control ($p < 0.05$). However, the expression of Gap-43 were significantly higher in taurine treatment groups than that in HG group ($p < 0.05$), increasing dose-dependently. The results presented that taurine promoted the axon outgrowth in HG-induced DRG neurons.

3.2 Effect of Taurine on Akt (p-Akt) in DRG Cells Exposed to High Glucose

The expression of Akt and p-Akt was studied by Western blot. The results showed as Fig. 2a, it was shown that the expression of Akt was not different among groups ($p > 0.05$). Nevertheless, the p-Akt expression was significantly decreased ($p < 0.05$) in HG group, but increased ($p < 0.05$) after use of taurine in a dose-dependent manner. The expression of Akt and p-Akt in culture DRG neurons were detected at the presence of PI3K antagonists LY294002. As shown in Fig. 2b, the expression of Akt

was not different in different groups (p > 0.05). However, It was found that the increase in expression of p-Akt after taurine in HG treated DRG neurons was significantly decreased in the presence of LY294002 (p < 0.05). It was implied that taurine increased the phosphorylation of Akt in HG-induced DRG neurons via activation of PI3K.

3.3 Effect of Taurine on mTOR (p-mTOR) in DRG Cells Exposed to High Glucose

The expressions of mTOR and p-mTOR were studied by Western blot. As shown in Fig. 3a, it was found that the expression of mTOR was not different among groups (p > 0.05). However, p-mTOR was down-regulated (p < 0.05) in HG group, but up-regulated after taurine treatment in a dose-dependent manner (p < 0.05). The expressions of mTOR and p-mTOR in culture DRG neuron cells were noticed in the presence of Akt antagonists Perifosine. It was found that the expression of mTOR was not different among groups (p > 0.05). However, the increasing expression of p-mTOR after taurine in HG treated DRG neurons was significantly decreased in the presence of Perifosine (p < 0.05) (Fig. 3b). The results indicated that taurine promoted the posphorylation of mTOR in DRG neurons via activation of Akt.
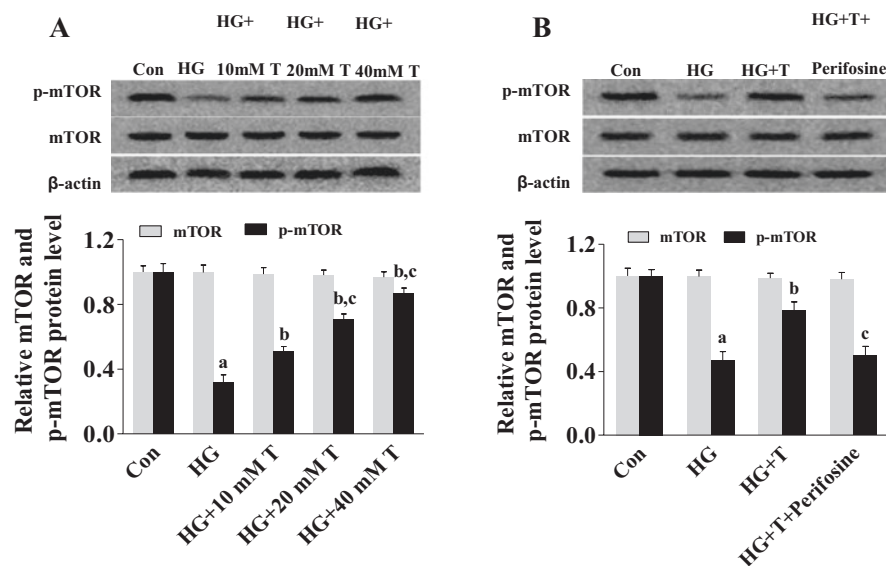

Fig. 3 mTOR and p-mTOR expression in the DRG neurons in presence of taurine. (**a**) Relative mTOR and p-mTOR protein levels in DRG neurons. (**b**) Relative mTOR and p-mTOR protein levels in DRG neurons with added Akt antagonists Perifosine. [a]$p < 0.05$, compared with Con group; [b]$p < 0.05$, compared with HG group; [c]$p < 0.05$, compared with HG + 10 mM T group or HG + T group; [d]$p < 0.05$, compared with HG + 20 mM T group. Data are expressed as mean ± SEM

3.4 Promotive Effect of Taurine on Neurite Outgrowth of DRG Neurons Exposed to High Glucose via Akt/mTOR Signalling Pathway

Immunofluorescence was used to detect axon growth of DRG neurons, it was detected by and the expression of Gap-43 by western blotting. It showed that the axonal extensions of DRG neurons were inhibited in the presence of HG, but taurine promoted axonal outgrowth in HG-treated DRG neurons and this effect was reversed in the presence of Perifosine (Fig. 4a). As showed in Fig. 4b, the neurite length of DRG neurons weas significantly higher in taurine treatment group than that in HG cells ($p < 0.05$). However, the increasing neurite length of DRG neurons after taurine in HG treated DRG neurons were significantly decreased in the presence of Perifosine ($p < 0.05$). Figure 4c showed that the increasing expression of GAP-43 after taurine in HG treated DRG neurons was significantly decreased in the presence of Perifosine ($p < 0.05$). It was implied that taurine favored the neurite outgrowth in HG-induced DRG neurons via activation of Akt/mTOR signalling pathway.

4   Discussion

DPN is an extremely lively process with interrelating mechanisms causing progressive abnormalities, the main features of which are impairment of axonal fuctioning, atrophy, and loss. Henceforth, DPN is described as an axonopathy of dying back type (Sima et al. 1988). Jia et al. also reported that HG locally inhibited neurite growth in DRG neurons (Hsu et al. 2013). In the present study, the results indicated a decline in neurite growth of DRG neurons meaningfully in HG group compared with control group. Moreover, the Gap-43 expression also significantly decreased in HG group compared with control group, being in accordance with the neurite's result. However, taurine supplementation significantly reversed the decreased neurite outgrowth and the down-regulated Gap-43 expression in HG-treated DRG neurons in a dose-dependent manner. Taurine is known to have a trophic impact on the nervous system. Moreover, it was shown that taurine stimulated neuritic outgrowth from retinal explants and ganglions (Lima et al. 1989; Matus et al. 1997), being supportive to our results. These results propose that taurine promotes the neurite growth of extensions in DRG neurons exposed to HG. However, the molecular mechanisms behind this are still unclear.

The serine/threonine kinase AKT, recognized as protein kinase B, is the major effector of the phosphati-dylinositol 3-kinase (PI3K) signaling pathway. Overexpression and persistent activation of AKT are caused by oncogenes, growth factors, and cytokines. The activated AKT controls many cellular functions including cell growth and survival (Alessi et al. 1996; Andjelkovic et al. 1997). Moreover, Akt has been shown as an important mediator of various features of neurite outgrowth, including elongation, branching and caliber (Read and Gorman 1997). To
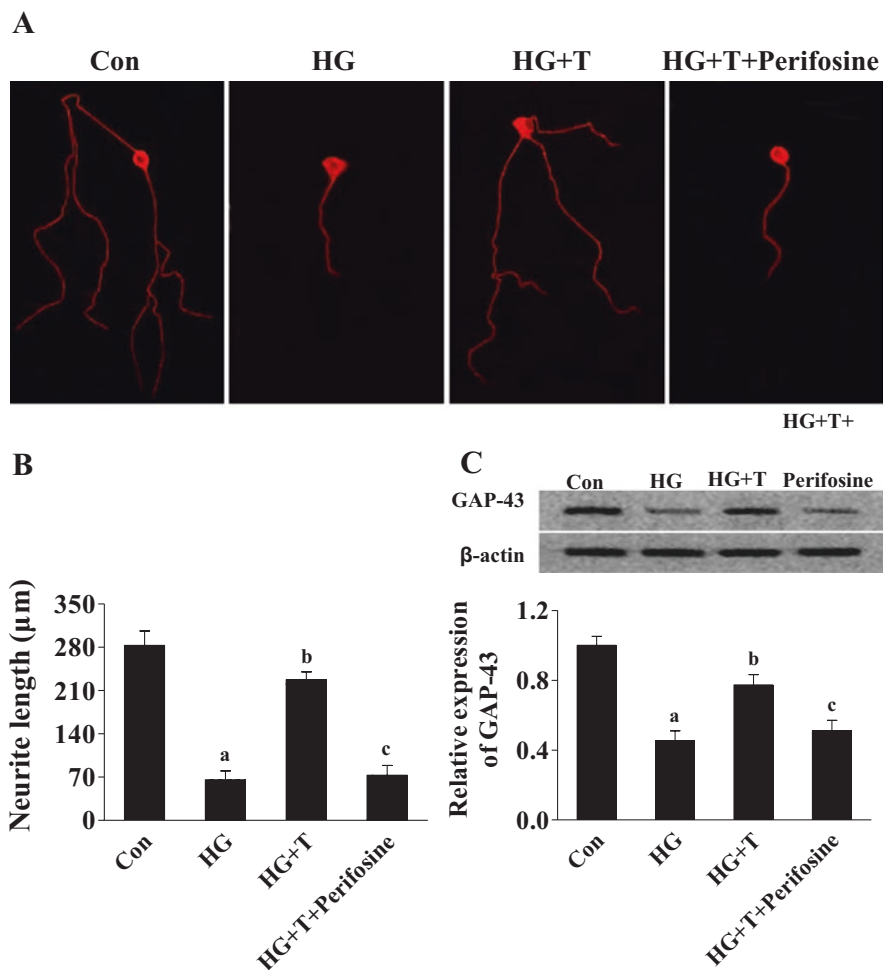
A

| Con | HG | HG+T | HG+T+Perifosine |

HG+T+

B

C

| | Con | HG | HG+T | Perifosine |

GAP-43

β-actin

Fig. 4 Neurite outgrowth and the expression of GAP-43 in treated DRG neurons with added Akt antagonists Perifosine. (**a**) Immunofluorescent images showing SMI312 immunoreactive DRG neurons. (**b**) Neurite length of DRG neurons. (**c**) Relative GAP-43 protein levels in DRG cells of four groups with added Akt antagonists Perifosine. [a]$p < 0.05$, compared with Con group; [b]$p < 0.05$, compared with HG group; [c]$p < 0.05$, compared with HG + T group; [d]$p < 0.05$. Data are expressed as mean ± SEM

investigate whether the promotion of taurine is associated with an activation of Akt, the expressions of Akt and p-Akt in DRG neurons were measured by using Western blotting assay in present study. The results indicated that HG evidently decreased the expression of p-Akt in the DRG neurons.

On the contrary, taurine supplementation significantly reversed the downregulated phosphorylated level of Akt. However, the shielding effects of taurine were hindered in the presence of PI3K antagonists LY294002. Takatani et al. reported

that treatment of taurine significantly reversed the decline in phosphorylation level of Akt in cardiac myocyte cells taken from ischemic rats. It was also shown that acrylamide-induced reduction in the p-Akt expression in the rats was attenuated by taurine administration, being consistent with our findings. These results propose that taurine activates Akt in the DRG neurons exposed to HG (Sun et al. 2018).

Downstream of Akt, various substrates have been recognized which possibly play key roles in Akt-mediated outgrowth of neurites. mTOR is a direct target of Akt. In recent reports, mTOR encouraged axonal renewal in the adult central nervous system and improved the axonal growth in injured peripheral nerves (Park et al. 2008; Abe et al. 2010). In the present research, the results indicated that HG considerably lowered the expression of p-mTOR in the DRG neurons. On the other hand, taurine supplementation significantly reversed the reduction in phosphorylated levels of mTOR. Moreover, the protective effect of taurine was hindered with presence of Akt antagonists Perifosine. Li et al. reported that treatment of taurine significantly reversed decrease in phosphorylation level of mTOR in PC12 cells exposed to Methamphetamine (Li et al. 2012), being in accordance with our findings. Our results show that taurine activates p-mTOR in the DRG neurons HG condition. The PI3K/Akt pathway is known to normalize the mTOR pathway and PI3K/Akt/mTOR signaling promotes growth and branches in hippocampal neurons (Chen et al. 2012). Therefore, it is suggested that taurine may promote neuritic growth in DRG neurons under HG through modulating PI3K/Akt/mTOR signaling pathway. It is necessary to confirm further promotive effect of taurine on damaged axon regeneration in diabetic neuropathy in vivo via the PI3K/Akt/mTOR signaling pathway.

5 Conclusion

In the current study, our results showed that HG significantly reduced the neurite outgrowth and downregulated expression of Gap-43, phosphorylation levels of Akt and mTOR in DRG neurons. On the contrary, taurine supplementation significantly reversed these abnormal changes in DRG neurons under HG condition. However, the presence of PI3K antagonists or Akt antagonists hindered protective effects of taurine. These results suggest that taurine promotes neurite outgrowth in DRG neurons exposed to HG via activating Akt/mTOR signalling pathway. Hence, taurine can be helpful in treating axonal damage in diabetic neuropathy. These findings may provide a new therapeutic approach for diabetic neuropathy and a novel insight for explaining the protective mechanism of taurine.

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