A curcumin derivative J147 ameliorates diabetic peripheral neuropathy in streptozotocin (STZ)-induced DPN rat models through negative regulation AMPK on TRPA1

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Abstract

Purpose: To investigate the specific molecular mechanisms and effects of curcumin derivative J147 on diabetic peripheral neuropathy (DPN).

Methods: We constructed streptozotocin (STZ)-induced DPN rat models to detected mechanical withdrawal threshold (MWT) in vivo using Von Frey filaments. In vitro, we measured cell viability and apoptosis, adenosine 5'-monophosphate-activated protein kinase (AMPK) and transient receptor potential A1 (TRPA1) expression using MTT, flow cytometry, qRT-PCR and western blot. Then, TRPA1 expression level and calcium reaction level were assessed in agonist AICAR treated RSC96 cells.

Results: The results showed that J147 reduced MWT in vivo, increased the mRNA and protein level of AMPK, reduced TRPA1 expression and calcium reaction level in AITCR treated RSC96 cells, and had no obvious effect on cell viability and apoptosis. Besides, AMPK negative regulated TRPA1 expression in RSC96 cells.

Conclusions: J147 could ameliorate DPN via negative regulation AMPK on TRPA1 in vivo and in vitro. A curcumin derivative J147 might be a new therapeutic potential for the treatment of DPN.

Key words: Curcumin. Peripheral Nervous System Diseases. TRPA1 Cation Channel. Protein Kinases. Rats.
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Lv J et al.

Acta Cir Bras. 2018;33(6):533-541

In 2011, Chen and Prior et al. discovered curcumin derivative J147 (CAS No.: 1146963-51-0) when screening compounds from plants with the ability to reverse brain aging cells and molecular marker. Several reports suggest that J147 can reverse memory deficits, enhance the production of new brain cells, and retard the progression of Alzheimer's disease in rats. Related the study of molecular mechanisms have found that J147 combines with the proteins mitochondrial proteins via ATP synthase, thereby promoting aging cells, mice and fruit flies to look younger. Besides, Lian et al. reports that J147 improves the depression-like behavior in mouse models caused by chronic stress, and its mechanism may involve the regulation of cAMP/pCREB/BDNF pathway.

Due to curcumin can relieve diabetic peripheral neuropathic pain, we aimed to investigate the specific molecular mechanisms and effects of J147 on DPN through the establishment of DPN rat models, cell viability and apoptosis assay in this study.

■ Methods

DPN rat models

Ethical Guidelines were approved by the Ethical Committee of Shanxi University of Traditional Chinese Medicine.

The 30 adult male SPF rats (weighing 200 ± 20g) were obtained from Institute of Laboratory Animal Science, Chinese Academy of Medical Science (Beijing, China). They were housed in stainless steel squirrel cages with food and water available ad libitum. The breeding room was kept at 20°C, 70% humidity and 12 h of light. SPF rats were weighed after fasting for 12 h, and streptozotocin(STZ) solution (Solarbio, Beijing, China) was injected intraperitoneally at 50 mg/kg weight. After 4 h of injection, the rats resumed their diet.
and were given water with glucose for 6 h, and then blood sugar was measured after 72 h. Subsequently, DPN rats were randomly divided into 3 groups: one group was fed with 10 μM of J147 (Myland Biological Technology Co., Ltd., Suzhou, China) at 10 mg/kg weight, the other group was fed with 100 μM of J147, while the control group was fed the same amount of normal saline. Then, to evaluate the hypersensitivity of mechanical pain after medication, mechanical withdrawal threshold (MWT) of algesia at 1, 2, 3, 4 and 5 days after feeding J147 were measured using Von Frey filaments (DanMic Global, California, USA).

**Cell culture**

RSC96 cells were purchased from ATCC (Manassas, USA) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Thermo Fisher Scientific, Waltham, USA) with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, USA), dimethylsulfoxide (DMSO, Thermo Fisher Scientific, Waltham, USA) and 150 mM of high-glucose (Thermo Fisher Scientific, Waltham, USA). The cells were divided into 3 groups, and respectively treated with J147 (10 and 100 μM) and PBS at 37°C. In addition, each group of partial cells was divided into two parts for follow-up experiments. One part was added 1 mM AMPK activator AICAR (AICAR+, Sigma-Aldrich, St. Louis, USA), while the control group was not added AICAR (AICAR-).

**Cell viability assay**

In order to detect the growth of cells, MTT Cell Proliferation and Cytotoxicity Assay Kit (Sangon Biotech, Shanghai, China) was used. Cells were seeded in 96-well plate (2000 cells/well) for 24 h. After adding 5 mg/ml of MTT solution to each well, cells were incubated with 5% CO₂ at 37°C for 4 h, and were added to 100 μl of formazan solubilization solution. Then, the absorbance was measured at 450 nm (OD450) by microplate reader (DeTie, Nanjing, China).

**Apoptosis assay**

Cell apoptosis was measured using Annexin V-FITC/PI Apoptosis Assay Kit (Sangon Biotech, Shanghai, China). Cells (2 × 10⁵ cells/ml) were re-suspended in 195 μl of 1 × Binding Buffer. Then, cell suspension were added to 5 μl of Annexin V-FITC and incubated for 10-15 min at the room temperature. Cells were washed in 1 × Binding Buffer twice, re-suspended again and added to 10 μl of PI. Finally, flow cytometry (Thermo Fisher Scientific, Waltham, USA) was used to observe the fluorescence within 4 h, and the percentage of apoptosis was calculated.

**Quantitative real-time polymerase chain reaction (qRT-PCR) assay**

RSC96 cells were cleaved by Trizol solution (Thermo Fisher Scientific, Waltham, USA) and incubated for 5 min at the room temperature. The RNA was precipitated with chloroform and isopropanol (Thermo Fisher Scientific, Waltham, USA), washed three times in 75% ethanol (Thermo Fisher Scientific, Waltham, USA), and was transcribed into cDNA using All-in-One™ First Strand cDNA Synthesis Kit (GeneCopoeia, Rockville, USA). Then, 4 μl of cDNA was combined with primers (β-actin as internal reference) to perform PCR under conditions of 95°C for 5 min, 94°C for 30 s, 55°C for 40 s and 72°C for 1 min. The 10 μl of product was electrophoresed and photographed using 1% agarose gel (Thermo Fisher Scientific, Waltham, USA). The grayscale was measured by ImageTool (IT) 3.0 (University of Texas Health Science Center, San Antonio, USA), and the relative mRNA expression level was calculated.
Western blot assay

RSC96 cells were lysed using RIPA Lysis Buffer (Beyotime, Shanghai, China). Lysed cell fluid was detected the protein concentration using Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China). Then, the protein samples were added 2 × SAS-PAGE Sample Loading Buffer (Beyotime, Shanghai, China), heated for 3-5 min at 100°C, and transferred to Polyvinylidene Difluoride (PVDF) membrane for 30 min using Bio-rad Trans-Blot SD (Bio-Rad, Hercules, USA). Subsequently, the protein membrane was washed in Western Wash Buffer (Beyotime, Shanghai, China) for 1 min and added Western Blocking Buffer (Beyotime, Shanghai, China) for 1 h at the room temperature. The primary antibodies of Adenosine 5′-monophosphate-activated protein kinase (AMPK), transient receptor potential A1 (TRPA1) and GADPH were purchased from Abcam (Shanghai, China), incubated at 4°C overnight and washed in Western Wash Buffer for 5 min three times. The secondary antibody marked by horseradish peroxidase was incubated for 1 h at 4°C and washed in Western Wash Buffer for 5 min three times. Finally, the proteins were detected and observed using BeyoECL Plus Kit (Beyotime, Shanghai, China) and X-OMAT BT film (Carestream, USA).

Calcium imaging assay

RSC96 cells were treated with AITCR, seeded in 24-well plate, added Fura-2AM (Tocris bioscience, Bristol, UK) solution for 30 min at the room temperature, and washed in Ringer solution for 30 min in the dark. The Fura-2 loaded cells were exposed for 30s at the excitation wavelength distribution of 340nm and 380nm using a fluorescence microscope (Leica Microsystems, Weitzlar, Germany). The calculated F340/F380 ratio represented the relative calcium level.

Statistical analysis

All data were represented by the mean ± standard deviation (SD). SPSS13.0 analysis software (SPSS Inc., Chicago, USA) was used to perform t-test between groups. The difference was statistically significant at p<0.05.

Results

J147 reduced MWT of STZ-induced DPN rats in vivo

In order to study the effect of J147 on DPN, we detected MWT of rat models in vivo using Von Frey filaments at 10 μM and 100 μM of J147. As shown in Figure 1, we found that the thresholds of rats fed J147 were decreased as time went on. Besides, the threshold was significantly reduced in the 10 μM J147 group compared to the control group, while it after feeding 100 μM of J147 also clearly lower than that 10 μM of J147.

Figure 1 - J147 reduced MWT of STZ-induced DPN rats in vivo. The MWT was measured by Von Frey filaments at 1, 2, 3, 4 and 5 days after feeding J147.

J147 had no obvious effect on cell viability and apoptosis

Subsequently, we examined the effect of J147 on RSC96 cells in vitro using MTT and flow cytometry. In Figure 2A, cell viability did not change in cells treated with J147. Similarly, there was no significant difference in apoptosis between the 3 groups (Figure 2 B and C).
A curcumin derivative J147 ameliorates diabetic peripheral neuropathy in streptozotocin (STZ)-induced DPN rat models through negative regulation AMPK on TRPA1

Lv J et al.

Acta Cir Bras. 2018;33(6):533-541

Figure 2 - J147 had no obvious effect on cell viability and apoptosis. Cell viability and apoptosis were measured by MTT and flow cytometry assay.

J147 increased AMPK expression and reduced TRPA1 expression

To study the molecular mechanism of the influence of J147 on DPN, we detected the expression levels of AMPK and TRPA1 using qRT-PCR and western blot assay. As show in Figure 3A, AMPK expression was clearly up-regulated in 10 μM and 100 μM of J147 treated cells, and the increase in the 100 μM J147 group was more obvious. On the contrary, the expression of TRPA1 was down-regulated in the 10 μM and 100 μM group compared with the control group (Figure 3B). Subsequently, Figure 3C also suggested that J147 increased the protein expression level of AMPK, and decreased TRPA1 expression level.

Figure 3 - J147 increased AMPK expression and reduced TRPA1 expression. The mRNA and protein expression levels of AMPK and TRPA1 were assessed by qRT-PCR and western blot.
AMPK negative regulated TRPA1 expression in RSC96 cells

In this study, we used agonist AICAR to treated RSC96 cells, and detected TRPA1 expression. In Figure 4A, qRT-PCR results showed that TRPA1 expression level in the agonist AICAR group was significantly lower than that in the non-agonist group. Analogously, western blot analysis results also suggested that agonist AICAR remarkably decreased the protein expression level of TRPA1 compared with the AICAR- group (Figure 4B).

Figure 4 - AMPK negative regulated TRPA1 in RSC96 cells. Cells were treated with AICAR. The mRNA and protein expression level of TRPA1 was assessed by qRT-PCR and western blot.

J147 reduced calcium reaction level in AITCR treated RSC96 cells

Finally, in order to investigate the function of TRPA1, RSC96 cells were treated with agonist AICAR and detected using calcium imaging assay. We found that calcium reaction level was significantly decreased in the 10 μM and 100 μM of J147 group than the control group, and the lowest level of calcium declined in the 100 μM of J147 group (Figure 5).

Figure 5 - J147 reduced calcium reaction level in AITCR treated RSC96 cells. Cells were treated with AICAR. The calcium reaction level was detected by calcium imaging assay.

Discussion

Due to curcumin can relieve diabetic peripheral neuropathic pain, and J147 as a derivative of curcumin, we concluded that it has a certain effect on DPN. Hence, in this study, we aimed to investigate the specific molecular mechanisms and effects of J147 on DPN through the establishment of DPN rat models, cell viability, apoptosis and calcium imaging assay. We found that J147 reduced...
MWT in vivo, increased the mRNA and protein level of AMPK, reduced TRPA1 expression and calcium reaction level in AITCR treated RSC96 cells, and had no obvious effect on cell viability and apoptosis. Besides, AMPK negative regulated TRPA1 expression in RSC96 cells.

It is reported that J147, as a positive neurotrophic factor, could promote animal memory, and maintain the normal presence of synaptic proteins and the basic level of cognitive ability in transgenic mice. In addition, J147 inhibits β-amyloid (Aβ) oligomerization and alleviates Aβ-induced cytotoxicity in neuronal cells. The underlying mechanism of mechanical hyperalgesia is the phenotypic transformation of Aβ fibers, thereby secreting neuropeptides expressed by small fibers. Because of DPN can produce corresponding sensory dysfunction and cause nerve damage, leading to loss of pain sensitivity in some areas. Therefore, in this study, we constructed STZ-induced DPN rat models to simulate DPN patients, and detected their MWT to facilitate the understanding of the effects of J147 on DPN in vivo. The results showed that J147 significantly reduced MWT of rat, and 100μM of J147 had a better effect.

AMPK is an enzyme that controls the energy balance in cells, consisting of 3 subunits of α, β, and γ. It can achieve energy balance via adjusting blood glucose levels, fatty acid oxidation and glycogen metabolism. The activity of AMPK is mainly regulated by intracellular AMP/ATP ratio. In the stress state with increased ratio, such as lack of nutrition, blood glucose deficiency, exercise, ischemia and hypoxia, AMPK activation can be induced. In our study, the results of qRT-PCR and western blot suggested that AMPK expression level was up-regulated in J147 treated cells, and could laterally reflect that J147 alleviated DPN in vitro.

TRPA1 is an instantaneous receptor-potential ion channel that was first discovered in the DRG and trigeminal ganglion neurons, and was cloned from the temperature sensitive neurons of the rats. It has been reported that blocking TRPA1 in the STZ-induced rodent acute diabetes model can reduce the high sensitivity of mechanical stimulation. A large number of oxidative stress reactions produced during the metabolic process of diabetes can emerge pain, hyperalgesia and neurogenic inflammation through activating TRPA1. Our results showed that J147 down-regulated the mRNA and protein expression level of TRPA1.

Subsequently, Wang et al. found that there is a functional link between AMPK and TRPA1 in dorsal root ganglion (DRG) neurons. AMPK activation leads to the down-regulation of TRPA1 and its channel activity within a few minutes. High-glucose treatment reduces AMPK activity levels and increases agonist-induced TRPA1 currents in cultured DRG neurons. Therefore, we studied the relationship between AMPK and TRPA1 by adding AICAR, and found that AMPK negatively regulated TRPA1 expression level in DPN cells model. In addition, activation of TRPA1 can cause calcium ions to permeate into cells. Then, we also detected calcium reaction level of AICAR-treated cells using calcium imaging assay. The results suggested that J147 reduced calcium reaction level in DPN cells, and were similar to those of previous studies.

Conclusions

Curcumin derivative J147 could ameliorate DPN through negative regulation AMPK on TRPA1 in vivo and in vitro. These studies provide a more sufficient experimental basis for clinical treatment of DPN.

References

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Conflict of interest: none
Financial source: none

1Research performed at Department of Prescription, Basic Medical College, Shanxi University, Traditional Chinese Medicine, China.