Laminaria Japonica polysaccharide promotes the expression of calcitonin receptor-like receptor in type 2 diabetes mellitus model mice

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ABSTRACT

Amylin, or islet amyloid polypeptide (IAPP), is a 37-residue peptide hormone[1-2]. It is cosecreted with insulin from the pancreatic β cells in the ratio of approximately 100:1[3-4]. Amylin receptor (AR) is composed of calcitonin receptor (CTR) and receptor activity-modifying proteins (RAMPs) heterodimer. CTR is a seven transmembrane domain. The aim of experiment was to investigate the effect of Laminaria Japonica polysaccharide (LJPS) on the expression of calcitonin receptor-like receptor (CrlR) and hypoglycemic activity in type 2 diabetes mellitus mice. Type 2 diabetes mellitus models were established by feeding high fatty forage and injecting alloxan in 40 healthy male mice. The LJPS was applied as additive in physiological saline to treat the mice by intragastric administration. The levels of fasting blood glucose (FBG) were detected by automatic blood glucose device. The tissue morphology of brainstem, liver and pancreas were analyzed by Hematoxylin-eosin assay. The expression of CrlR was determined by immunohistochemistry and Western blot, and the expression of CrlR mRNA was detected by RT-PCR. The results indicated after treated with LJPS, the serum level of FBG were significantly higher than that in model group (< 0.05). The morphology and structure of liver and pancreas tissue improved than those in model group. The expressions of CrlR mRNA and CrlR protein were significantly higher than those in model group (< 0.05). These results suggest that LJPS could play a hypoglycemic effect by promoting the expression of CrlR in liver and pancreatic tissue to lessen insulin resistance.
Introduction

Amylin, or islet amyloid polypeptide (IAPP), is a 37-residue peptide hormone [1-2]. It is cosecreted with insulin from the pancreatic β cells in the ratio of approximately 100:1[3-4]. Amylin receptor (AR) is composed of calcitonin receptor (CTR) and receptor activity-modifying proteins (RAMPs) heterodimer. CTR is a seven transmembrane domain, class B G protein-coupled receptor. RAMPs are single-transmembrane spanning proteins. There are three distinct subtypes of RAMPs, designated RAMP1, RAMP2 and RAMP3, which could be combined with calcitonin gene related peptide (CGRP), CTR and calcitonin receptor-like receptor (CrlR) to form the adrenal medullary receptor and islet amyloid peptide receptor (AMY1-3), and form stable heterologous dimers expressed on the cell membrane. Amylin receptor is the isomer of the calcitonin receptor. Under the action of RAMPs and G protein-coupled receptors (GPCRs), the structure of calcitonin receptor generate glycosylation modification to change into amylin receptor structure, which transferred to cell surface and combined with amylin to play its roles. Also, the state of amylin receptor glycosylation determines its ligand specificity [5]. Amylin receptor distributes in the nervous system, pancreas, skeletal muscle and kidney cortex [6]. For the amylin receptor independent gene encoding has not yet been determined [7] and amylin is the member of CGRP family, CrlR can be seen as the elements of amylin receptor [8]. CrlR is a G protein-coupled receptor with seven transmembrane domins (its amino terminal has 3 N-glycosylation sites). There is 55% homology in amino acid sequence and nearly 80% same area in the transmembrane with CTR [7]. Previous study found that only combined with the N-terminal of RAMP1 or PAMP3 to form dimers, CrlR could be transferred to the membrane, thus mediating amylin activities [9]. CrlR is expressed in the nervous system [10], liver [11] and blood vessels, etc. RAMP1 is highly expressed in skeletal muscle, pancreas and brain tissue, while RAMP3 is lowly expression in rat tissue [12]. Research shows that CRT, CrlR and RAMPs expressed in pancreatic β cells at same time, influencing glucose metabolism by generating amylin receptor and transferring them to the membrane of the cell [13-14]. Kelp, a traditional Chinese medicine, which main activating component is Laminaria japonica polysaccharides (LJPS) and has the efficacy of cold-natured, salty flavor, soft lump loosen knot, dissolving phlegm and diuresis [15]. LJPS mainly consisted of sodium alga acid and fucoidan, it played anti-oxidant [16], anticoagulation [17], hypolipidemic and hypoglycemic effects [18-19] etc. The previous researches shown that LJPS could promote islet cell secretion function to play hypoglycemic activity by enhanced anti-oxidation [20-23], but rarely reported [24-25] about amylin receptor expression and insulin resistance (IR). In this study, we aim to investigate the influence of LJPS on the expression of CrlR and to explore the hypoglycemic effect and it mechanism in type 2DM.
MATERIALS AND METHODS

1. Mouse Model

Forty healthy male Kunming mice weighing 23-27g were purchased from the Experiment Animal Center of Qingdao Drug Inspection Institute (SCXK (LU) 20110010). Animals were acclimatized to feed with normal forage for 7 days. Ten mice were randomly selected as a control group and given general forage. The remaining 30 mice were fed with high fatty forage. After 4 weeks of dietary manipulation, alloxan (50mg/kg body weight) was injected intraperitoneally once every other day for 3 times to establish type 2 DM models [24-25]. Mice in the control group were administered with equivalent amounts of normal saline. Fasting blood glucose (FBG) was measured third day after the final injection. The type 2 DM animal model as the successful markers for establishing model was when FBG differed by more than two standard deviations from the control group. Ten experimental mice were excluded because they did not satisfy the standard. The remaining 20 type 2 DM model mice were divided randomly into model group (n=10) and treatment group (n=10).

2. Intervention

According to our previous research confirmed LJPS effective therapeutic dose 3.00g/kg body weight [24-25], LJPS was diluted with normal saline to desired concentrations (300mg/ml) with intragastric administration once a day for 2 weeks. In control group and model group, mice were orally administrated an equivalent amount of normal saline. Meanwhile, all of animal model mice were fed normal forage for 2 weeks.

3. Specimen collection

3.1. Blood sampling: Blood samples 1.0ml for each mouse were collected from heart and centrifuged for 10 min at 4000r/min to separate serum and then stored at -20℃. Using automatic blood glucose meter (Johnson & Johnson Medical Ltd) and Onetouch Ultra test strips to detect FBG (mmol/L).

3.2. Hematoxylin-eosin (HE) staining: Five mice form each group were perfused and fixed form heart with 0.9% saline 45ml and 4% paraformaldehyde 45ml. Then brainstem, liver and pancreas tissues were collected and fixed in 4% paraformaldehyde for 2h and distilled water for 4h. The sample tissues were subjected conventional ethanol dehydration, transparent of xylene, paraffin embedding, and cut into serial section with 5 mm thickness by microtome (Leica 2015, Shanghai). The paraffin section of brainstem, liver and pancreas tissues were general hydration of dewaxing with hematoxylin for 2 min. After 1% hydrochloric acid in ethanol differentiation, the nucleus becomes blue and the cytoplasm showed different degrees of red.

3.3. Immunohistochemistry staining: Rabbit anti-mouse CrIr antibody (Sc-30028, Santa Cruz). SABC immunohistochemistry kit (SP-90001, ZSGB-BIO). Firstly, paraffin sections were de-waxed and incubated 3%H2O2 10 min, soaked by PBS with 5min. Dropped reagent A on the section to incubate at room temperature for 10min, and washing with PBS for 5min×3times. Dropped primary CrIr antibody (1:500) to incubate at 37℃ for 1h and then PBS washing 5min×3times. Secondly, dropped reagent B to react at 37℃ for 15min and then PBS douching 5min×3times. Thirdly, dropped reagent C to react at 37℃ for 10min, and then PBS douching 5min×3times. At last, colored by DAB chromogenic reagent and re-stain by haematoxylin. Under microscope, which cytoplasm or membrane appeared brown granules was considered as positive cells, while negative control sections stained with 0.1mol/L PBS instead of without primary antibody and no color appeared. Under
optical microscope with magnified 400 times, five non-overlapping visual fields in each section were randomly selected to observe and analysis the absorbance value (A) of CrlR expression by Image-Pro Plus software. The CrlR expression intensity was presented by the positive cells A subtracting the background A.

3.4. Western blotting: Five mice from each group were perfused from heart with 0.9% saline 45ml, and then collected brainstem tissue 50mg, liver tissue 100mg and pancreas tissue 100mg. Add RIPA lysis buffer (P0013B, Beyotime Co. Ltd.) to grind tissues into homogenizer on the ice, then centrifuged (Eppendorf 5801, Germany) at 12000r/min for 15 min to separate the supernatant, and determine the concentration of protein by enhanced BCA protein assay kit (P0010, Beyotime Co. Ltd.). According to total protein 50μg, CrlR protein was separated by 8%SDS-PAGE electrophoresis, transferred to a PVDF membrane and sealed for 1h by 5% evaporated skimmed milk. Then added rabbit anti-mouse primary antibody (CrlR 1:500; GAPDH, TA-08, 1:10000), 4℃concentrating table to stay overnight, TBST washing 10min×3times. Added peroxidase labeling goat anti-rabbit secondary antibodies (Abcam, Ab6721, 1:5000) and horseradish enzyme marker goat anti-mouse IgG (ZB-2305, 1:10000) to incubate at 37℃ for 1h. Took out the membrane and washed with TBST for 10min×3times, TBS for 5min×2times. Then plus plus A, B liquid developer (1:1) and imaged by Vilber Fusion FX7 imaging system. Using Quantity One software to analyse the absorbance value (A) of the internal reference (36kD), and calculate the relative content of CrlR (CrlR A/GAPDH A value). The experiment was repeated 3 times and result was presented as mean ± standard deviation (x ± s).

3.5. RT-PCR: Trizol extraction kit was purchased from Invitrogen Co. Ltd. Firstly, added 1ml Trizol solution into brainstem tissue 50mg, liver tissue 100mg and pancreas tissue 100mg, ground 5min 4℃, and centrifuged at 12000r/min for 15min to separate the supernatant into sterile EP tube. Secondly, added 0.2ml chloroform, mixed 15s at room temperature, and centrifuged at 12000r/min for 15 min to separate the colorless aqueous phase into another sterile EP tube. Thirdly, added 0.5ml isopropanol, mix gently and centrifuged at 12000r/min for 15 min to discard the supernatant carefully, and added 75% precooling ethanol 1ml to wash RNA precipitation to , centrifuge (4℃) at 7500r/min for 5min. Then discard the supernatant carefully to dry about 20min in cupboard (RNA precipitate becomes transparent). At last, added 0.1% DEPC-H2O 100μl to dissolve RNA for 10min at 57℃water bath. Micro-spectrophotometer (K5500, Beijing Kaiao Tech. Co., Ltd) was used to detect RNA abundance. Primers were synthesized by Shanghai Yingjieka & Co. Ltd. CrlR forward primer: 5′-GGT ACC ACT ACT TGG CAT TG -3′, reverse primer: 5′-GTC ACT GAT TGT TGA CAC TG -3′, product length 262bp; GAPDH forward primer: 5′-ACC ACA GTC CAT GCC ATC AC -3′, reverse primer: 5′-TCC ACC ACC CTG TTG CTG TA-3′, product length 452bp. RT-PCR: semi-quantitative PCR was conducted according to Takara DRR014A PrimeScript™ RT-PCR kit. The reactions were pre-degeneraed at 95℃ for 5min, then degenerated at 94℃ for 30s, amplified at 56℃ (for CrlR) or 60℃ (for GAPDH) for 30s and extension at 72℃ for 40s, 35 cycles, and then followed by 72℃ for 10min. Electrophoresis: 50μl PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The absorbance (A) of interest gene CrlR and internal reference GAPDH was imaged by Vilber Lourmat gel imaging system and analysed by Quantity One software. The CrlR mRNA expression level was presented as a ratio of CrlR A/GAPDH A. The experiment was repeated 3 times and result was presented as mean ± standard deviation (x ± s).
4. Statistical analysis

According to SPSS 17.0 software analysis, Data were expressed as mean±standard deviation (x±s) that multi-groups were compared using analysis of variance (ANOVA), two groups were compared by LSD-t test.

RESULTS

1. FBG levels

Before modeling, there was no significant difference of animal FBG level among two groups and control group (P >0.05). After modeling, these groups of animals FBG level had significant difference by analysis of variance (F=14.32, q=0.01-1.57, P<0.05), There was significantly higher compared model group or control group(t=2.64, P <0.05). each treatment group animals FBG levels were significantly lower than the model group (F=4.02, q=0.01-2.94, P <0.05). Table 1.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before modeling</th>
<th>After modeling</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>7.40±1.23</td>
<td>6.70±1.05</td>
<td>6.19±1.27</td>
</tr>
<tr>
<td>Model group</td>
<td>7.40±1.23</td>
<td>10.18±0.97 a b</td>
<td>8.47±0.91 b</td>
</tr>
<tr>
<td>Treatment group</td>
<td>7.40±1.23</td>
<td>9.95±1.03 a</td>
<td>6.86±1.46 c</td>
</tr>
</tbody>
</table>

a Compared with before modeling, P<0.05 ; b Compared with control group, P<0.05 ; c Compared with model group, P<0.05

2. Tissue pathology

There was no significant difference among these groups of brainstem nerve cells with edge sharpness and normal structures. In control groups, hepatic lobule arranged in order, but in model groups appeared focal hepatocytes vacuolar degeneration. In control group, islets was demarcation clear and more cells, while reduced significantly in model groups of islet cells with vacuoles degeneration. After treated with LJPS, the morphology and structure of hepatocytes and islets improved significantly than those in model group.

Figure 1.
3. Immunohistochemical staining

There was no significant differences of CrIR expression level in brainstem tissue between control, model and treatment groups ($P > 0.05$). In liver and pancreas tissue, CrIR expression levels in model group decreased significantly than those in control groups ($P < 0.05$), while in treatment group increased significantly than those in model groups ($P < 0.05$). Figure 2 and Table 2.
A-C: Brainstem (control, model and treatment groups); D-F: Liver (control, model and treatment groups); G-I: Pancreas (control, model and treatment groups)

Table 2 The expressions of CrlR in different tissues of mice (\(\bar{x} \pm s, n=5\))

<table>
<thead>
<tr>
<th>Groups</th>
<th>CrlR-B</th>
<th>CrlR-L</th>
<th>CrlR-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0.285±0.091</td>
<td>0.193±0.026</td>
<td>0.149±0.051</td>
</tr>
<tr>
<td>Model group</td>
<td>0.274±0.010</td>
<td>0.131±0.048(^a)</td>
<td>0.103±0.011(^{a,b})</td>
</tr>
<tr>
<td>Treatment group</td>
<td>0.281±0.007</td>
<td>0.175±0.004(^{a,b})</td>
<td>0.132±0.003(^{a,b})</td>
</tr>
</tbody>
</table>

\(^a\)Compared to control group, \(^{a,b}\)t=25.39, \(^{a,b}\)t =8.53, P<0.05 ; \(^\wedge\)Compared to model group, \(^{a,b}\)t=-15.63, \(^{a,b}\)t =-5.82, P<0.05

5. Western blotting

No significant differences of CrlR protein expression level in brainstem tissue existed between control, model and treatment groups (P>0.05). In liver and pancreas tissue, CrlR protein expression levels in model group were significantly lower than those in control group (P<0.05), but in treatment group higher than those in model groups (P<0.05) . Figure 3 and Table 3.

Table 3 The expressions of CrlR protein in different tissues of mice (\(\bar{x} \pm s, n=5\))

<table>
<thead>
<tr>
<th>Groups</th>
<th>CrlR-B</th>
<th>CrlR-L</th>
<th>CrlR-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0.463±0.015</td>
<td>0.437±0.010</td>
<td>0.389±0.015</td>
</tr>
<tr>
<td>Model group</td>
<td>0.455±0.009</td>
<td>0.313±0.006(^a)</td>
<td>0.256±0.006(^{a,b})</td>
</tr>
<tr>
<td>Treatment group</td>
<td>0.460±0.009</td>
<td>0.383±0.009(^{a})</td>
<td>0.331±0.004(^{a,b})</td>
</tr>
</tbody>
</table>

\(^a\)Compared to control group, \(^a\)t=22.92, \(^{a,b}\)t=18.01, P<0.05 ; \(^\wedge\)Compared to model group, \(^{a,b}\)t=-13.70, \(^{a,b}\)t=-20.56, P<0.05

6. RT-PCR
In liver and pancreas tissue, CrlR mRNA expression in model groups were significantly decreased than those in control groups \((P < 0.05)\), while increased significantly than those in model groups \((P < 0.05)\). There was no significant differences of CrlR mRNA expression in rainstem tissue between the control, model and treatment groups \((P > 0.05)\). Figure 4 and Table 4.

**DISCUSSION**

Type 2 diabetes mellitus is a metabolic disease that its key features as insulin resistance (IR), relative insulin deficiency and hyperglycosemia. Amylin is produced in the pancreatic β-cells, released into the bloodstream where they travel to body tissue, the Combination of membrane of target tissue and amylin receptor, and generate physiological effect. Amylin has been reported\[7\] to regulate gastric emptying and suppress glucagon secretion and food intake, influence bone forming and absorbing\[26\]. Amylin could inhibit secretion of the digestive enzymes\[27-28\]. Whether dramatic increasing through injection or slowly through feeding, the high blood concentration of amylin reduce significantly the frequency and the amount of the feeding, making the postprandial blood glucose well controlled\[29-30\]. Grunberger\[31\] found that treatment of T2DM’s new drug pramlintide is naturally amylin analogues both in the pharmacokinetic and pharmacodynamic characteristics. It treats mainly DM by inhibiting gastric emptying and glucagon secreting, which is the theoretical basis for therapy pramlintide with insulin in type 1 diabetes and type 2 diabetes clinically \[32\]. Although the physiological function of amylin and receptor is not fully clear in the biological organism, but their hypoglycemic effect and mechanism broaden the cognition of diabetes, which also provides more choices for the treatment for diabetes, becoming the current focus of attention. Believing that with research going on, there will be more meaningful findings.

The experiment confirmed that FBG of animal had significantly higher level, focal cell vacuolar degeneration in the liver...
and pancreatic tissues in model groups, pancreatic β cell be damaged and insulin secretion function synchronized declined on type 2 diabetic mouse. Immunohistochemistry, Western blot and RT-PCR experiments indicated that no significant difference of CrlR expression in brainstem tissue between groups of mouse. CrlR has no affected by blood glucose and maintain its stability, importance to maintaining stability of the physiological functions of the brain. In liver and pancreatic tissues, CrlR mRNA and CrlR protein expression levels decreased significantly in model groups, so induced IR due to CrlR number reducing and sensibilities descend in type 2 DM. After treated with LJPS, CrlR expression levels increased significantly, while tissues morphology has improved and FBG levels were decline in liver and pancreatic. The further confirmation of LJPS could improve CrlR expression levels, and reduce IR and glucose metabolism processes in type 2 diabetes, to play hypoglycemic activity in liver and pancreatic tissues.

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REFERENCES


