Biomedicine & Pharmacotherapy 100 (2018) 221–225

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Swertisin ameliorates diabetes by triggering pancreatic progenitors for islet neogenesis in Streptozotocin treated BALB/c mice

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ARTICLE INFO

Keywords:
Islet neogenesis
Pancreatic progenitors
Diabetes mellitus
Swertisin

ABSTRACT

In the present study, Swertisin's role in triggering resident pancreatic progenitors for islet neogenesis in Streptozotocin (STZ) diabetic mice was explored. STZ diabetic mice when treated with Swertisin demonstrated reversion to normoglycemia and significant elevation of fasting serum insulin levels. On screening the pancreatic tissue post Swertisin treatment in the STZ diabetic mice, we observed significant up-regulation of key transcription factors viz. Pdx1, Neurog3, MafA and Nkx6.1 required for islet neogenesis and beta cell homeostasis. We further observed increase in expression of Nestin and Neurog3 positive population; Nestin and Glut2 positive population and increase in c-peptide and Glucagon positive population within the Islets of Langerhans indicating increased pancreatic progenitor activity and their differentiation into Insulin producing beta cells in Swertisin treated STZ diabetic mice. Thus, this short study highlights pancreatic innate capability to regenerate and recover using its own resident progenitors upon appropriate stimulus, which could culminate into an effective diabetic therapy.

1. Introduction

The hallmark of diabetes is loss of insulin producing beta cells leading to hyperglycaemia due to insufficient insulin production [1]. Various therapies have evolved till date for the amelioration of diabetic condition including pancreatic regenerative therapy [2].

The origin of the newly created islets or the pancreatic progenitor source or their exact characteristics has been a highly controversial topic in the field of islet biology. Hence, existing reports suggest that pancreatic progenitors can have either acinar, ductal and/or islet source within the pancreatic tissue [3]. Popular convection although still controversial, suggests that there exists pancreatic progenitors that migrate and differentiate into islets when required [4]. The advantage of pancreatic progenitors is to differentiate very efficiently and very quickly into islets or insulin producing beta cells [5].

In recent years various stem cell sources have been identified to produce insulin producing beta cells and different stem cells require different induction along with culture media conditions to be differentiated into functional islet clusters. There are many molecules that have been previously reported for islet neogenesis e.g. Activin-A, Betacellulin, KGF, Exendin-4, Nicotinamide etc. [6]. In the present study, we have used Swertisin, which was isolated from an Indian herb *Enicostemma littorale* (EL). EL is an anti-diabetic plant used in traditional Indian medicine and swertisin has been characterised as a potent islet differentiating agent. Our group have previously reported Swertisin's islet neogenic potential on NIH3T3 and PANC1 cell lines in vitro and in pancreatectomised mice model in vivo. Swertisin in these studies successfully induced differentiation into insulin producing islets which responded to glucose challenge. Further, when these islets were transplanted in the type 1 diabetic mice model, they were able to ameliorate the diabetic condition effectively [7,8]. Here, we have tried to emphasize that a small molecule Swertisin can trigger pancreatic progenitors by up-regulating key transcription factors essential for replenishment of lost beta cells and recovery of pancreatic endocrine function, thus providing an incredible therapeutic intervention in the treatment for diabetes mellitus.

2. Material and methods

2.1. Chemicals

All chemicals, culture media and molecular biology reagent used in this study were purchased from Sigma Aldrich and Invitrogen, ThermoFisher Scientific. The details of the antibodies used are given in

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https://doi.org/10.1016/j.biopha.2018.01.096
Received 29 November 2017; Received in revised form 16 January 2018; Accepted 24 January 2018

Table 1
List of Antibodies.

Sr. No.	Antibody	Company & Catalog No.	Isotype IgG	Mono/ Polyclonal Ab	Mol. Weight (kDa)	Application	Dilution
1	Pdx-1	BD#554655	Mouse	Mono	40	Immunoblotting/ Immunofluorescence	1:1000/ 1:200
2	Neurogenin-3	Sigma #SAB1306585	Rabbit	Poly	23	Immunoblotting/ Immunofluorescence	1:1000/ 1:100
3	MAFA	Sigma #SAB2105099	Rabbit	Mono	40	Immunoblotting	1:1000
4	Nkx 6.1	DSHB #F64A6B4	Mouse	Poly	40	Immunoblotting/ Immunofluorescence	1:40/1: 20
5	GLUT2	Sigma #SAB1303865	Rabbit	Mono	61	Immunoblotting/ Immunofluorescence	1:1000/ 1:100
6	β-Actin	BD#612657	Mouse	Mono	42	Immunoblotting	1:10000
7	Nestin-PE	BD#561230	Mouse	Mono	177	Immunofluorescence	1:100
8	Glucagon	Sigma#G 2654	Mouse	Mono	3.48	Immunofluorescence	1:100
9	C-Peptide	CST#4593	Rabbit	Mono	5	Immunofluorescence	1:100
10	Anti-Mouse-IgG-	Jackson ImmunoResearch #115-035-003	Goat	Poly		Immunoblotting	1:5000
11	Anti-Rabbit-IgG-HRP	Jackson Immuno Research #111-035-003	Goat	Poly		Immunoblotting	1:5000
12	Anti-Rabbit-IgG-FITC	Sigma#F9887	Goat	Poly		Immunofluorescence	1:200
13	Anti-Mouse-IgG-CF555	Sigma#SAB4600299	Goat	Poly		Immunofluorescence	1:100

Table 1.

2.2. Isolation and characterization of Swertisin

Swertisin was isolated and purified from the whole dried plant of *Enicostemma littorale* as previously described [7].

2.3. Animal selection and induction of diabetes and in vivo experimental design

Adult virgin female BALB/c mice weighing 20–25 g aged 6–8 weeks were kept at animal house with 12 h light and dark cycle with water and pellet diet *ad libitum*. Gender was selected as per the availability of mice at the animal house at the time of the study. Diabetes was induced with STZ injection (65 mg/kg body weight) intraperitoneally for 5 days with overnight fasting. Diabetic status of animals was confirmed by monitoring Fasting Blood Glucose (FBG) using Accu-check Performa glucometer (Accu-check, Roche, USA) at regular intervals as shown in Fig. 1A. Eight animals were distributed per group.

Swertisin treatment Design: The STZ diabetic mice were treated with Swertisin (2.5 mg/kg body wt.) from 14th day of experiment till 30th day after which the mice were sacrificed. Swertisin was administered with saline intravenously through tail vein. Fasting serum insulin was estimated and compared within the groups on the day of sacrifice.

These studies were carried out in strict accordance as per the guidelines and approval of institutional Committee for the Purpose of Control and Supervision on Experiments on Animals, India (CPCSEA). Post experiment animals were euthanatized using xylazine (10 mg/kg) and ketamine (150 mg/kg) injection followed by cervical dislocation ensuring death.

2.4. Insulin ELISA

Fasting serum Insulin was analyzed using mouse-insulin ELISA (Mercodia Inc., USA) as per manufacturer's protocol.

2.5. Protein extraction and western blotting

Tissue were harvested and kept on ice. Tissues were minced with the help of a mortar and pestle in liquid nitrogen on ice. The mashed tissue powder was resuspended in Laemmli buffer with 4 M urea and sonicated on ice for 7 cycles of 20 s with 2 s on and 0.2 s off sequence with 2 min on ice between every cycle at 45% amplitude. 20 µg of total protein as estimated by Bradford's method was resolved on SDS-PAGE Tris-glycine gels and transferred to nitrocellulose membrane. Non-specific binding was blocked by incubating the membranes in 5% fat free skimmed milk with 0.1% Tween-20 in PBS/TBS for 1 h at RT. The blots were subsequently incubated overnight with primary antibodies against the following proteins: PDX-1, NEUROG-3, NKX6.1, MAFA, GLUT2 and β-ACTIN at 4 °C, with gentle agitation. Blots were washed with TBS/PBS containing 0.1% Tween (TBS/PBS-T) (4 × 15 min) and then incubated with respective secondary antibodies conjugated with HRP for 1 h at RT with gentle agitation (Table 1). After four washes with PBS/TBS-T and two washes with PBS/TBS; specific bands of immune-reactive proteins were visualized using Ultrasensitive enhanced chemiluminiscence reagent (Millipore, USA) and images were captured on chemigenious gel documentation system (Uvitech, Cambridge).

2.6. Cryosectioning & immunohistochemistry

After treatment period when mice were sacrificed, splenic pancreas were dissected out and fixed by immersion in 4% paraformaldehyde overnight at 4 °C and then cryo-protected in 15% and 30% sucrose solutions in 0.1 M sodium phosphate buffer (pH 7.4). These tissues were then embedded in tissue freezing medium (OCT, Leica), and frozen. Pancreatic tissue sections were mounted on Poly-L -lysine coated slides. Cryosectioning was performed on a cryostat (Leica CM1520) at 20 µm intervals. Sections were incubated in blocking buffer [2% fetal bovine serum, 2% bovine serum albumin, 0.1% Triton X-100 in Phosphate Buffer Saline (PBS) with pH 7.4] followed by incubation in primary antibody overnight at 4 °C (Table 1). After incubation, sections were rinsed in washing buffer (ten times diluted blocking buffer in PBS) and then incubated respective secondary antibody (Table 1). Further, sections were counterstained with the DNA stain DAPI, washed with PBS, mounted with coverslips and finally sealed with transparent nail paint. Immunostained sections were viewed under confocal microscope (Zeiss LSM 710) and the fluorescence above the negative slides (only secondary antibody treated) was captured.

2.7. Statistical analysis

The data is presented as mean ± SEM. The significance of difference was evaluated by the paired Student's *t*-test. When more than one group was compared with one control, significance was evaluated