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Lipocalin-type prostaglandin D_2 synthase reduces glucagon secretion in alpha TC-1 clone 6 cells via the DP1 receptor

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ABSTRACT

Diabetes is associated with disturbances in the normal levels of both insulin and glucagon, both of which play critical roles in the regulation of glycemia. Recent studies have found lipocalin-type prostaglandin D_2 synthase (L-PGDS) to be an emerging target involved in the pathogenesis of *type*-2 diabetes. This study focused on the effect of L-PGDS on glucagon secretion from cultured pancreatic Alpha TC-1 Clone 6 cells. When cells were treated with various concentrations of L-PGDS (0, 10, 50, and 100 ug/ml) for 2 h in 1 mM glucose; glucagon secretion decreased to 670 ± 45, 838 ± 38, 479 ± 11, and 437 ± 45 pg/ml, respectively. In addition, pancreatic islets were isolated from C57BL/6 mice and stained for prostaglandin D_2 receptors, DP1 and DP2, using immunohistochemistry. Our results showed that these islets express only the DP1 receptor. Pancreatic islets were then stained for alpha and beta cells, as well as DP1, to find the primary location of the receptor within the islets using immunofluorescence. Interestingly, DP1 receptor density was found primarily in alpha cells rather than in beta cells. Our study is the first to report a correlation between L-PGDS and glucagon secretion in alpha cells. Based on our obtained results, it can be concluded that higher concentrations of L-PGDS significantly reduced the secretion of glucagon in alpha cells, which may contribute to the pathogenesis of diabetes as well as offer a novel therapeutic site for the treatment of diabetes.

1. Introduction

Lipocalin-type prostaglandin D_2 synthase (L-PGDS) is the enzyme which plays an important role in the metabolism of arachidonic acid by converting PGH2 into PGD2. PGD2 gets converted into numerous prostaglandins including and 15-deoxy-D12, 14-PGJ2 (15d-PGJ2) [1,2]. PGD2 is primarily produced in mast cells and in smooth muscle cells with a synthetic phenotype [3] which has also been reported for its accumulation during atherosclerotic lesion formation, suggesting their strong involvement in the pathogenesis of cardiovascular injuries [4]. Current literature has also shown a strong correlation between vascular injuries and diabetes. Briefly, diabetes mellitus is the result of imbalance of normal levels of both insulin and glucagon with their opposite effects on glycaemia as well as on the metabolism of nutrients. Insulin acts mainly on muscle, liver and adipose tissue with an anabolic effect, inducing the uptake of glucose into these tissues and its accumulation as glycogen and fat, while glucagon activates liver glycogenolysis and gluconeogenesis due to its catabolic effect which leads to increase blood glucose level [5]. Glucagon is secreted in response to hypoglycemia and is inhibited already when the glucose concentration reaches 4–6 mmol/l [6,7]. Abnormal function of alpha cells due to hyper secretion of glucagon may lead to dysregulation of glucose homeostasis and eventually diabetes mellitus [7]. Even though, it is a well known fact that hyperglycemia in diabetes is primarily due to insufficient or no secretion of insulin. However, there are ample evidences which support the phenomenon that hyper secretion of glucagon plays very important role in initiating and maintaining hyperglycemic conditions in diabetic animals and humans [8,9]. Therefore, the concept of glucagon signaling is becoming the emerging target for the treatment of hyperglycemia [9,10]. The condition of hyperglucagonemia in diabetes can be mitigated but not normalized completely by the insulin treatment [11]. The actual mechanism which causes hyperglucagonemia has not been fully studied yet.

Therefore, a novel concept for the inhibition of glucagon needs to be introduced. Interestingly, we found a novel enzyme, L-PGDS which can inhibit glucagon secretion. Increased plasma glucagon levels relative to insulin can be a determinant in the higher rate of hepatic glucose output, which seems to be critical in maintaining hyperglycemia in diabetic patients [12]. Recent studies have found

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Lipocalin-type prostaglandin D_2 synthase (L-PGDS) to be an emerging target involved in the pathogenesis of diabetes, especially *type*-II diabetes [13]. Therefore, we hypothesized that L-PGDS may have a role in the regulation of glucagon from alpha cells of pancreas and represent a future target for the development of antidiabetic drugs.

2. Materials and methods

2.1. Cell culture

Alpha TC-1 Clone 6 cells (ATCC), an pancreatic alpha cell line isolated from C57BL/6 mice, at passage five were incubated at 37 °C and 5% CO_2 in low-glucose (5.5 mM) DMEM, supplemented with 15 mM HEPES, 0.1 mM non-essential amino acids, and 0.1 mM bovine serum albumin until glucagon extraction procedures.

2.2. Glucagon extraction

Overnight, incubating medium was replaced with a high glucose (20 mM) DMEM medium. The following day, high glucose DMEM was removed and cells were washed twice with glucose-free ringer's buffer. The cells were then supplemented with ringer's buffer containing various lower concentrations of glucose (0.5, 1, 5, and 16.5 mM) to induce secretion and incubated for 2 h. The cells used were disposed off. The buffer collected was then analyzed for glucagon concentration released using a multiplex kit (Millipore). After observation that lower concentrations of glucose induce secretion, 1 mM was chosen as a baseline glucose concentration for experimentation with L-PGDS. Addition of L-PGDS to the culture was used to shift the equilibrium of the PG H2 substrate pool towards PG D_2 and ensure a more physiological response. Using the same extraction procedure, a new set of alpha cells were supplemented with various concentrations (0, 10, 50, 100 μg/ml) of L-PGDS in 1 mM glucose. The buffer was then collected and analyzed for glucagon concentration.

2.3. Immunohistochemistry

Histology slides with 4 μm sections of pancreas from C57BL/6 mice were washed and deparaffinized. The sections were then blocked with hydrogen peroxide for 10 min and then washed in 1 × TBST+0.05% Tween. To perform antigen retrieval, slides were then submerged in 10 mM citric acid buffer (pH6+0.05% Tween) and heated to just below boiling for 20 min, then and washed in TBST and were left to cool down at room temperature. Slides were then given a protein block (Abcam) and incubated for 5 min at room temperature and then washed. The slides that were designated to receive antibody received a 1:100 concentration of DP1 polyclonal antibody from rabbit (Cayman) in TBST and normal goat serum and incubated at room temperature in a humidified chamber for 2 h and 15 min. From this point on, control slides were treated separately to avoid contamination. Sections were covered with biotinylated goat-anti-rabbit lgG(H+L) (Abcam) and incubated for 10 min at room temperature, then were washed and covered with drops of streptavidin peroxidase (Abcam) and incubated for 10 min at room temperature. All slides then received a solution of diaminobenzidine (DAB) chromagen (Abcam) and left for 10 min to stain. Slides were then washed and treated with hematoxolyn, rinsed with tap water, and mounted for observation.

2.4. Immunofluorescence

New slides with sections of pancreas were deparaffinized and given the same antigen retrieval protocol as in the immunohistochemistry procedure. Slides were then blocked in 5% donkey serum in PBS with 0.3% triton for 1 h at room temperature and then aspirated without rinsing. Primary antibodies were then combined into a cocktail into a 1:100 solution in PBS (0.3% triton). Primary antibodies included insulin (guinea pig) and glucagon (rabbit) on one slide, and DP1 (rabbit) on another. Sections were covered with primary antibody solution and incubated overnight at 4 °C. The following day, slides were rinsed three times for five minutes each in PBS (0.3% triton). A cocktail of secondary antibodies in a 1:250 was made in PBS (0.3% triton) and applied to sections and left to incubate in the dark at room temperature. The secondary antibodies included Anti-Guinea Pig Cy3 (red) and Anti-Rabbit FitC (green). The slides were then washed and mounted with DAPI (blue) mounting medium for observation.

2.5. Statistical analysis

All glucagon extractions were performed twice in triplicate. The results were analyzed using Sigma Stat for standard error and a Student *t*-test was performed to determine statistical significance at the [$p < 0.05$].

3. Results

3.1. Effect of glucose on glucagon secretion

To determine the effect of glucose on glucagon secretion using various concentrations, we extracted the glucagon from the buffer and measured using milliplex kit. We measured the glucagon levels and compared with non glucose treated cells extraction. Glucagon level with no glucose was found to be 961 ± 14 pg/ml while glucagon concentrations decreased as glucose concentrations were increased and found to 912 ± 10, 916 ± 10, 890 ± 3 and 801 ± 15 pg/ml with glucose concentrations of 0.5, 1, 5, 16.5 mM respectively as shown in Fig. 1A. Based on the obtained results, as the glucose concentrations were increased, glucagon secretion was decreased.

3.2. Effect of L-PGDS on glucagon secretion

To determine the effect of L-PGDS on glucagon secretion, Alpha TC1 clone 6 cells were pretreated with 1 mM glucose concentration followed by the 2 h treatment with various concentrations of L-PGDS. The average concentrations of glucagon secretion were found to be 670 ± 45, 838 ± 38, 479 ± 11, and 437 ± 45 pg/ml from the respective concentrations of L-PGDS (0, 10, 50, and 100 μg/ml) as shown in Fig. 1B. Based on the obtained results, it can be stated that higher concentrations of L-PGDS reduce the glucagon secretion.

3.3. Immunofluorescent staining of pancreatic islets to determine the location of DP1 receptor in alpha and beta cells

Pancreatic islets were isolated from C57BL/6 mice and stained for prostaglandin D_2 receptors both DP1 and DP2 using immunohistochemistry to confirm that islets used in the study have alpha cells. As shown in Fig. 2, Nuclei were stained blue with DAPI mounting medium on the left panel. Islets were also stained for insulin (Cy-3, red) and glucagon (FIT-C, green) to distinguish between alpha and beta cells. Islets were also stained for DP1 and DP2 receptors (FIT-C, green) as shown on the right panel of Fig. 2. However, this observation confirmed the location of only DP1 receptor but not the DP2 receptor (*data not shown*) in these islets cells.

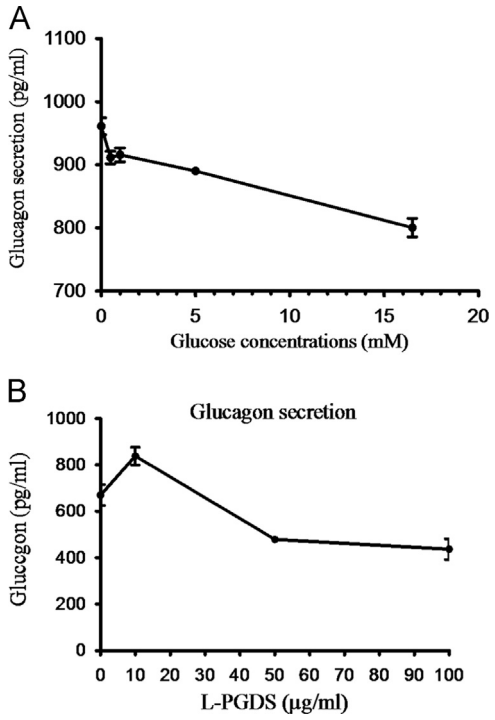

Fig. 1. (A) Treatment of Alpha TC1 Clone 6 cells with glucose concentration. Alpha cells treatment with supplementation of four different concentrations of glucose (0.5,1,5 and 16.5 mM) using Alpha TC1 Clone 6 cells over the period of 2 h and Glucagon secretion was measured. Data are expressed as Mean ± SEM and statistical analysis was performed using *t*-test with the significance of $p < 0.05$. As shown above, glucagon secretion decreases, glucose concentrations increases. (B)-Treatment of Alpha TC1 Clone 6 cells with L-PGDS. Alpha TC1 Clone 6 cells were treated with different concentrations of L-PGDS (0, 10, 50 and 100 µg/ml) over the period of 2 h and Glucagon secretion was measured. Data are expressed as Mean ± SEM and statistical analysis was performed using *t*-test with the significance of $p < 0.05$. As shown above, at lower concentrations, glucagon secretion increases; however, it decreases as the L-PGDS concentrations increases. Data are expressed as Mean ± SEM and statistical analysis was performed using *t*-test with the significance of $p < 0.05$.

3.4. Presentation of DP1 receptor with or without primary antibody

Immunohistochemistry was performed to show the difference between with or without primary antibody for DP1 receptor. As shown in Fig. 3, two different pancreatic were isolated from the islets of the same C57BL/6 mouse. (A) Without primary antibody (B) Islet with primary antibody for DP1 receptor. In image B, there was a clear background staining throughout most of the islet, however, specific staining occurred in the periphery of the islet cells, shown in dark brown.

4. Discussion

Increased secretion of glucagon contributes to the dysregulation of glucose homeostasis in diabetes [7]. Even though, it is a well known fact that hyperglycemia in diabetes is mainly due to insufficient or no secretion of insulin. However, there are ample evidences which support the phenomenon that hyper secretion of glucagon plays very important role in initiating and maintaining hyperglycemic conditions in diabetic animals and humans [8,9]. Therefore the concept of glucagon signaling has been considered as an important target in the treatment of hyperglycemia [9,10]. Glucagon concentrations in diabetes can be reduced but not completely normalized by the insulin treatment [11] and moreover, the actual cause of underlying the hyperglucagonemia have not been fully elaborated yet. Therefore, a novel concept for the inhibition of glucagon needs to be introduced.

Our study is the first to report an interesting correlation between L-PGDS and glucagon secretion in alpha cells (Alpha TC1 Clone 6) derived from C57BL/6 mice. First of all, pancreatic islets were isolated from C57BL/6 mice and stained for DP1/DP2 receptor as well as alpha and beta cells using immunohistochemistry which confirmed the expression of only DP1 receptor not the DP2 receptor (*data not shown*) in alpha cells not in beta cells as shown in Fig. 2 where nuclei are stained blue with DAPI mounting medium on the left panel. Islets were also stained for insulin (Cy-3, red) and glucagon (FIT-C, green) to distinguish between alpha and beta cells. Islets were also stained for DP1 receptor (FIT-C, green) as shown on the right panel of Fig. 2. Islets were also stained for DP2 receptor but there was no expression observed (*data not shown*). This observation confirmed that isolated islets have alpha cells. Since it is a well know concept that prostaglandin D2 acts through DP1 and DP2 receptor [14]. As shown in Fig. 3, alpha cells were compared with and without primary antibody for DP1 receptor and interestingly these cells expressed only DP1 receptor which can be clearly distinguished between left (A) and right panel of Fig. 3B. After the confirmatory studies of alpha cells, alpha TC-1

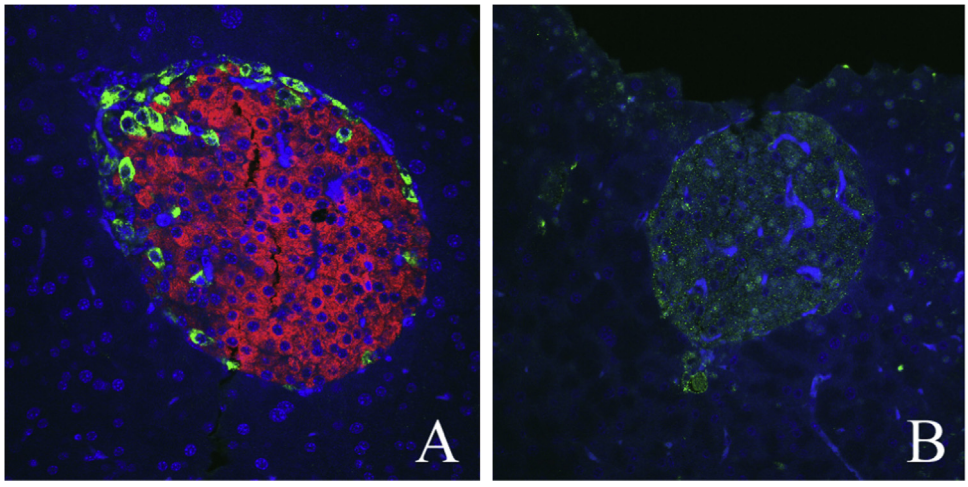

Fig. 2. Immunofluorescent staining of pancreatic islets for DP1 receptor as well as insulin and glucagon. Two different pancreatic islets were isolated from the same C57BL/6 mouse. Nuclei are stained blue with DAPI mounting medium. (A) Islet stained for insulin (Cy-3, red) and glucagon (FIT-C, green) to distinguish between alpha and beta cells. (B) Islet stained for DP1 receptor (FIT-C, green). Image 2B displays very punctate staining throughout the islet; however, the stain shows a tendency of cluster formation on the periphery of the islet. Islets were also stained for DP2 receptor but there was no expression observed (data not shown).

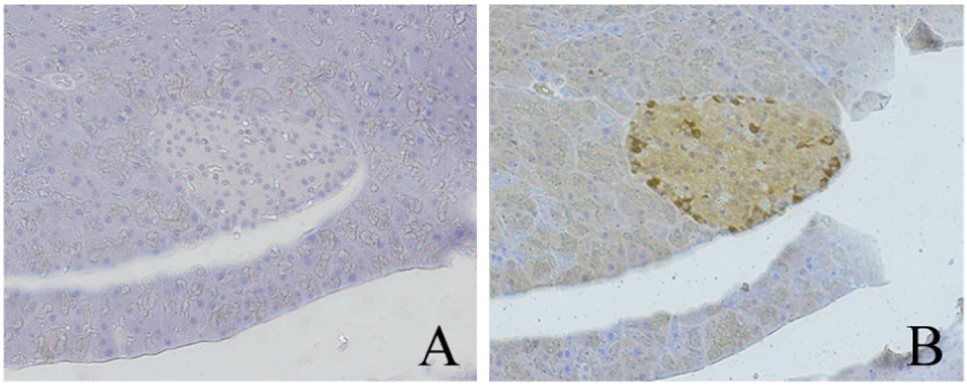

Fig. 3. Immunohistochemistry: Unstained and stained pancreatic islets for DP1 receptor. Two different pancreatic islets isolated from the same C57BL/6 mouse. (A) without primary antibody treated islets. (B) Primary antibody treated Islets for DP1 receptor. In image B, there is clear background staining throughout most of the islet, however, specific staining occurs in the cells in the periphery of the islet, shown in dark brown.

Clone 6, a cell line isolated from a carcinoma of C57BL/6 mice, were cultured in high glucose (20 mM) DMEM medium overnight. The following day, the media was replaced with various lower concentrations of glucose (0.5, 1, 5, and 16.5 mM) for 2 h to induce secretion. Glucagon concentrations measured were found to be 912 ± 10, 916 ± 11, 890 ± 3 and 801 ± 15 with treatment of 0.5, 1, 5, and 16.5 mM glucose respectively. Our results confirmed that lower concentrations of glucose cause the cells to secrete more glucagon as shown in Fig. 1A which is consistent with the existing literature [6,15] and 1 mM glucose concentration was chosen for further experiments. Cells were then treated with various concentrations (0, 10, 50, and 100 μg/ml) of L-PGDS for 2 h in 1 mM glucose; glucagon secretion was then measured using a Millipore Milliplex kit. High concentrations of L-PGDS were found to reduce the amount of glucagon secretion into the media. The average concentrations of glucagon secretion were found to be 670 ± 45, 838 ± 38, 479 ± 11, and 437 ± 45 pg/ml from the respective concentrations of L-PGDS (0, 10, 50, and 100 μg/ml). This showed an increase in basal glucagon secretion with a supplementation of a low concentration of L-PGDS (10 pg/ml) and an immediate drop below basal at high concentrations as shown in Fig. 1B.

Based on the obtained results, it can be concluded that islets isolated from the mice had alpha cells confirmed by immunofluorescence and immunohistochemistry. Moreover, alpha cells expressed only DP1 receptor which can be clearly seen in Fig. 2 and also been confirmed using with and without primary antibody for DP1 and found clear presence of DP1 receptor on the right panel as shown in Fig. 3B. Collectively it can be stated that higher concentrations of L-PGDS reduced the glucagon secretion from alpha cells which was possibly mediated through DP1 receptor. It could be more interesting to measure the PGD_2 production in the alpha cells but due to immediate non-enzymatic conversion of PGD2 into PGJ2 was found to be more challenging. Further studies need to be performed to elucidate the exact mechanism of action using diabetic animal model which is under progress.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.09.019.

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