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FFA-Fetuin-A regulates DPP-IV expression in pancreatic beta cells through TLR4-NFkB pathway

Snehasish Nag [a], Samanwita Mandal [a], Tanmay Majumdar [b], Satinath Mukhopadhyay [c], Rakesh Kundu [a, *]

[a] Cell Signaling Laboratory, Department of Zoology, Visva-Bharati University, Santiniketan, 731235, India
[b] National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, 110067, India
[c] Department of Endocrinology & Metabolism, Institute of Post-Graduate Medical Education & Research-Seth Sukhlal Karnani Memorial Hospital (IPGME&R-SSKM), Kolkata, 700020, India

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ABSTRACT

Dipeptidyl peptidase 4 (DPP-IV) is a ubiquitous proteolytic enzyme that cleaves incretin hormones, such as glucagon-like peptide 1 (GLP1) and gastric inhibitory protein (GIP), leading to reduced glucose stimulated insulin secretion from the pancreatic beta cells. The functionally active enzyme is present in a membrane bound form in several cell types as well as in a soluble form in the circulation. The present report deals with DPP-IV expression and its regulation in the pancreatic beta cells in presence of free fatty acids (FFAs) and Fetuin-A, a circulatory glycoprotein associated with insulin resistance in humans and animals. FFA and Fetuin-A individually or in combination trigger DPP-IV expression in MIN6 cells. Islets isolated from high fat diet fed (HFD) mice (16 weeks) showed higher levels of DPP-IV expression than standard diet (SD) fed mice. Fetuin-A increased DPP-IV expression in HFD mice (4 weeks). Inhibition of TLR4 or NFkB prevented palmitate-Fetuin-A mediated DPP-IV expression in MIN6. It has been seen that Fetuin-A alone also could trigger DPP-IV expression in MIN6 cells via NFkB. Additionally, palmitate treatment exhibited reduced level of soluble DPP-IV in the media of MIN6 culture, which corroborated with the expression pattern of its protease, KLK5 that cleaves and releases the membrane bound DPP-IV into the secretion. Our results demonstrate that FFA-Fetuin-A upregulates DPP-IV expression in the pancreatic beta cells through the TLR4-NFkB pathway.

1. Introduction

Dipeptidyl peptidase 4 (DPP-IV) is a 110 kDa glycoprotein, which acts as a proteolytic enzyme that cleaves incretin hormones glucagon-like peptide 1 (GLP1) and gastric inhibitory protein (GIP) [1,2]. It is a widely distributed enzyme found both in membrane bound form as well as soluble form [3]. Soluble DPP-IV is cleaved at the stalk region and shed from the membrane into the circulation and maintains its enzymatic activity [2]. The main role of incretin hormones (GLP1) is to stimulate secretion of insulin from pancreatic beta cells and limit secretion of glucagon from alpha cells [4]. By inhibiting incretin pathway, DPP-IV downregulates glucose stimulated insulin secretion leading to hyperglycemia [5,6]. DPP-IV also acts as a local mediator of inflammation and leads to insulin resistance in liver and adipocytes that aid in the pathogenesis of type 2 diabetes [6]. In rodents, DPP- IV was found to be expressed in pancreatic islets and also in rat insulinoma cell line INS-1832/13 [7,8], however its regulation in beta cell has not been worked out. A recent report has suggested that DPP-IV expression is partly influenced by TLR4 in human monocytes [9] and showed lipopolysachharide (LPS) stimulated TLR4 activation led to increase in the expression of DPP-IV. Our previous report demonstrated that Fetuin-A in combination with palmitate trigger TLR4 expression in beta cells [10]. Fetuin-A is a liver secretory glycoprotein which remains high in the circulation during hyperlipidemic condition [11,12]. We have also showed that palmitate-Fetuin-A promotes NFkB mediated secretion of pro-inflammatory cytokines that cause profuse inflammation in tissues [13,14]. All these findings raise question, whether FFA-Fetuin-A has role in DPP-IV expression as excess of which in tissues and circulation is associated with obesity and type 2 diabetes [1]. Therefore, in the present study, we have investigated the effect of FFAs and Fetuin- A individually or in

* Corresponding author.
E-mail address: rakesh.kundu@visva-bharati.ac.in (R. Kundu).

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combination on DPP-IV expression in pancreatic beta cell using in vitro MIN6 culture and in vivo high fat diet (HFD) fed mice model. The regulation of DPP-IV expression has been studied by inhibiting TLR4 or NFkB and its membrane shedding has also been demonstrated by studying the expression of its protease, KLK5. This study would definitely be helpful in understanding the regulation of DPP-IV in the pancreatic beta cells and its significance in maintaining beta cell activity.

2. Materials and methods

2.1. Reagents and antibodies

Cell culture materials were purchased from Gibco-BRL/Life Technologies. Palmitic acid was purchased from SRL, India. Fetuin-A, DAPI, Percoll and others were purchased from Sigma-Aldrich (St. Louis, MO, USA). CLI-095 and PDTC were purchased from Invivogen and Sigma respectively. Anti- DPP-IV, anti-pNF-kBp65, anti-PDX-1, anti-KLK5 antibodies were purchased from ABclonal Technology. Anti-NFkB, anti-alpha-tubulin and anti-beta-actin antibodies were purchased from Santa- Cruz Biotechnology. RT^2-qPCR primers listed in Table 1 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of palmitate and Fetuin-A solution

Palmitate solution was prepared by conjugating palmitate with bovine serum albumin in the ratio of 1.35:2.0 [14]. The solution was checked with Pierce High Capacity Endotoxin Removal Spin Columns and found to be free of endotoxins. Fetuin-A solution was prepared by adding 1 mg of Fetuin-A per 1 ml of molecular biology grade water.

2.3. Cell line and cell culture

MIN6 cells (Mouse insulinoma cell line) was procured from the National Centre for Cell Science, Pune, India. The Cells were cultured in media containing DMEM with 15% (v/v) FBS (Fetal bovine serum), penicillin (100 units/ml) and streptomycin (100 mg/ml). The cells were grown in a humidified 5% CO2 atmosphere at 37°C. Before treatment, serum containing media was removed and cells were incubated in serum free media for 4 h. Cells were then incubated with Fetuin-A or palmitate at different doses and time periods ranging from 2 to 24 h. For inhibition studies, cells were pre-treated with TLR4 inhibitor, CLI-095 (3 μM) or NF-kB inhibitor, PDTC (pyrrolidine dithiocarbamate) (50 μM) for 1 h before treatment with palmitate and Fetuin-A.

2.4. Animal treatments and isolation of pancreatic islets

6–8 weeks old male C57BL/6J black mice weighing 18-24 gms were purchased and acclimatized for 7 days at 25 ± 2 °C in 12 h light/12 h dark cycle. Mice were segregated into 5 different groups containing 7 animals each. In first set of experiments, one group maintained under standard diet (SD) having 57.3% carbohydrate, 18.1% protein and 4.5% fat and the other group under high fat diet (HFD) containing 15% carbohydrate, 20% protein, 65% fat [14] for 16 weeks. In second set of experiments, one SD and two HFD groups received the diets for 4 weeks. One of the two HFD groups additionally received intraperitoneal injection (IP) of 0.35 mg/g body weight/day Fetuin-A solution for last 5 consecutive days before sacrifice. Fetuin-A solution for injection was prepared at 10 mg/ml in sterile PBS (0.05 M, pH 7.4) following our previous description [15]. On termination of the experiments, mice were sacrificed and pancreas were collected for further studies. Pancreatic islets were isolated from all the five groups using percoll gradient [16]. All animal experiments were approved by the Institutional Animal Ethics Committee, Visva-Bharati (IAEC/III-04/2020), following the guidelines prescribed by CPCSEA.

Table 1
Mouse (Mus musculus) primers for PCR amplification.

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
Dpp4	CACCTCTGATGGAAGCAGCTTC	GATAATCGCTGGTCAGAGCTTCG
Nf-kb	CTTCCTCAGCCATGGTACCTCT	CAAGTCTTCATCAGCATCAAACTG
Klk5	CGTGACTCTCACTCAGTGAAGC	TTCAGGCACTGGAGGACTTTCG
Gapdh	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT

2.5. Immunoblotting

Immunoblotting analysis was performed according to our earlier description [13]. 80 μg of total proteins collected from cell lysates or media were separated using 10% SDS-PAGE. The resolved proteins then transferred to a PVDF membrane (Millipore, Bedford, USA) using Wet/Tank Blotting System (Bio-Rad Laboratories Inc, Hercules, USA). Membranes were then incubated overnight at 4°C with different primary antibodies (1:500 dilutions). After proper washing, membranes were incubated for 2 h with respective alkaline phosphatase conjugated secondary antibodies following which the membranes were again washed and incubated with 5-bromo 4-chloro 3-indolyl phosphate/nitrobluetetrazolium (BCIP/NBT). BCIP/NBT acts as a substrate that develops protein bands [13]. The intensities of various protein bands were analyzed by Image Lab Software (Bio-Rad Gel DocTMXR+, USA).

2.6. Real-Time PCR

Total RNA from different cell samples were isolated using TRI Reagent (Sigma) following the manufacturer's protocol. cDNA synthesis was performed using Revert AidTM first-strand cDNA synthesis kit (Fermentas Life Sciences). Changes in expression of specific genes were analyzed using Real-Time PCR (Quant Studio 5, Applied Biosystems). Appropriate gene specific markers were selected and used to perform qPCR under following reaction conditions: activation step at 95 °C for 10 min, followed by 60 cycles of denaturation at 95 °C for 30 s, then annealing at 60 °C for 30 s and then extension at 72°C for 45 s. Ct values from each sample was corrected using the corresponding gapdh (glyceraldehyde-3-phosphate dehydrogenase) controls [13].

2.7. Immunofluorescence

The pancreatic beta cell line MIN6 was seeded onto sterile coverslips in 6 well plates. The cells were then either left as untreated or treated with palmitate (0.75 mM) or palmitate + Fetuin-A (100 μg/ml) for 24 h. In another set, MIN6 cells were pre-treated with 3 μM CLI-095 or 50 μM of PDTC for 1 h and then treated with palmitate + Fetuin-A (100 μg/ml) for 4 h. At the end of the incubation periods, the cells were immunostained with anti-DPP-IV antibody, followed by FITC-tagged secondary antibody. Nucleus was stained with DAPI (4′,6-diamidino-2-phenylindole).

2.8. ELISA

DPP-IV released in the cell culture medium were measured using mouse DPP-IV ELISA Kit (Bioassay Technology Laboratory, Shanghai, China) following the manufacturers' protocol.

2.9. Statistical analysis

Statistical analysis was performed using GRAPHPAD PRISM 8.0. Multiple groups of data were analyzed using one-way analysis of variance (ANOVA) while two groups of data were analyzed using Student's t-test. All values are expressed in means ± s.e.m. We considered P value < 0.05 as statistically significant.

3. Results

3.1. FFA mediates DPP-IV expression in pancreatic beta cells

Previous studies have shown that DPP-IV is expressed in pancreatic beta cells [7,17], however, its expressional pattern in different physiological conditions have not been studied. Here we have investigated the role of free fatty acids (FFAs) in DPP-IV expression. Thus, we have treated MIN6 cells with palmitate (0.75 mM) for 24 h (Fig. 1A), and the immunoblot analysis showed an increase in DPP-IV expression in response to palmitate. Palmitate also showed a dose (0.25 mM−1 mM) and time (2−8 h) dependent increase in the DPP-IV expression as evident from immunoblot analysis (Fig. 1B and C). As, NFkB is known to be a transcription factor of DPP-IV [1,18], we have checked NFkB expression and its phospho form, which also showed a time dependent increase (Fig. 1C). Real-Time PCR analysis depicted increase in DPP-IV gene expression on palmitate treatment by ~1.4 folds compared to the control (*P < 0.05) (Fig. 1D). Similar observation was found in the isolated islets from 16 weeks SD and HFD mice where DPP-IV and phosphoNFkBp65 levels showed an increase while PDX-1 (insulin gene transcriptor) [19] expression was found to be decreased in HFD islets compared to SD (Fig. 1E). Real-Time PCR analysis also showed increased DPP-IV expression by ~6 folds in HFD islets vs SD (**P < 0.05) (Fig. 1F) corroborating the immunoblot results. All these suggest that FFA mediates DPP-IV expression in pancreatic beta cells.

3.2. FFA-Fetuin-A combination promotes DPP-IV expression

To investigate the role of Fetuin-A in palmitate induced DPP-IV expression in beta cells, we treated MIN6 cells with palmitate (0.75 mM) or in combination with Fetuin-A (100 μg/ml) for 4 h. Immunoblot showed increased expression of DPP-IV (*P < 0.05), and phosphoNFkBp65 in pal + Fetuin-A treated cells compared to palmitate alone (Fig. 2A). Fetuin-A also showed increased DPP-IV expression in presence of palmitate in a dose dependent manner (Fig. 2B). Real-Time PCR analysis revealed an increased expression of DPP-IV (***P < 0.0001) and NFkB gene (*P < 0.01) in pal + Fetuin-A treated MIN6 cells as compared to palmitate alone (Fig. 2C). Immunostaining of DPP-IV in MIN6 cells similarly showed increased expression in pal + Fetuin-A treated cells as above (Fig. 2D). Next, to study the role of Fetuin-A in FFA induced DPP-IV expression in vivo, C57BL/6J male mice were fed with SD and HFD diets, followed by Fetuin-A injection (IP) in one batch of HFD mice for 5 consecutive days before termination of the experiment. Immunoblot and Real-Time PCR analysis from the islets isolated from these mice showed increased DPP-IV protein and gene expression in HFD + Fetuin-A compared to the HFD (*P < 0.05) (Fig. 2E and F). These findings strongly suggest that Fetuin-A promotes FFA induced DPP- IV expression in pancreatic beta cells.

3.3. Palmitate-Fetuin-A regulate DPP-IV expression through TLR4-NFkB pathway

To understand the regulation of DPP-IV expression in pancreatic beta cells, we treated MIN6 cells with TLR4 and NFkB inhibitors. Pretreatment of MIN6 cells with TLR4 inhibitor CLI-095 (3 μM) for 1 h, followed by pal + Fetuin-A treatment showed down-regulation of DPP-IV protein and gene expression compared to pal + Fetuin-A treatment (**P < 0.05) (Fig. 3A and C). Again, MIN6 cells pretreated with NFkB inhibitor PDTC (50 μM) for 1 h similarly showed reduced DPP-IV protein and gene expression (Fig. 3B and D). PDX-1 expression in contrast was restored in both the inhibitor treated

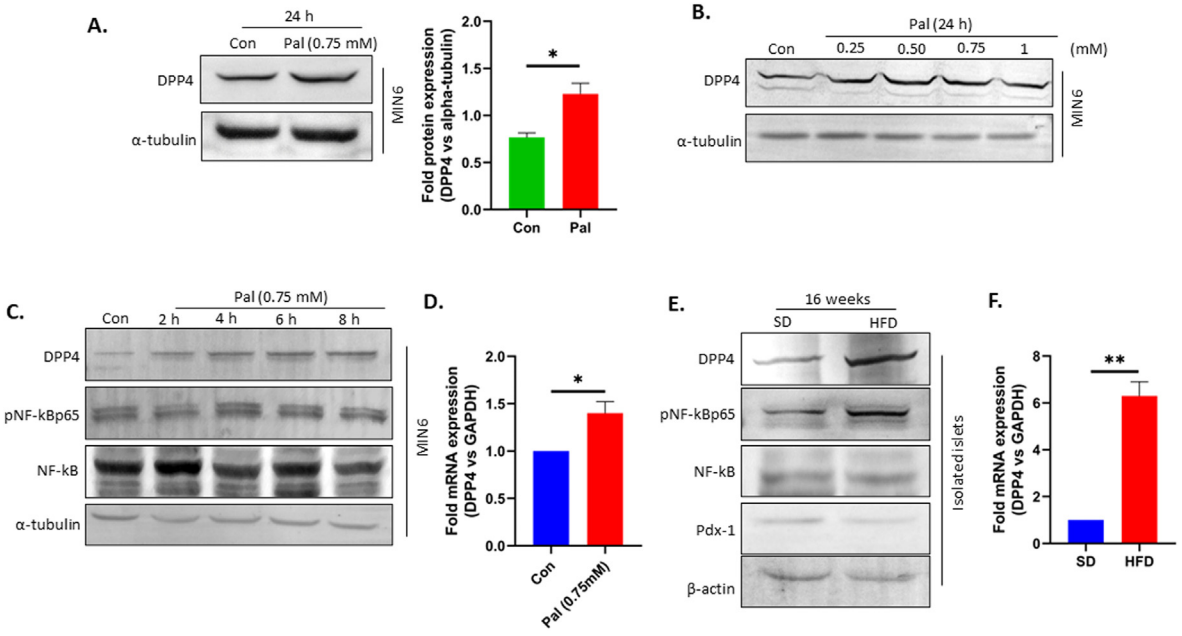

Fig. 1. (A, B, C) MIN6 cells were incubated with or without palmitate (Pal) at 24 h or at different dose and indicated time periods and DPP-IV expression was analyzed through immunoblot and densitometric plotting. α-tubulin served as loading control (*P < 0.05, compared with control). (D) Total RNA was extracted from pal treated MIN6 cells and subjected to qPCR analysis using DPP-IV specific primers taking gapdh as internal control. (*P < 0.05, compared with control). (E, F) Immunoblot and qPCR analysis of pancreatic islets isolated from SD and 16 weeks of HFD fed mice respectively (**P < 0.05, compared with control). Gapdh served as internal control.

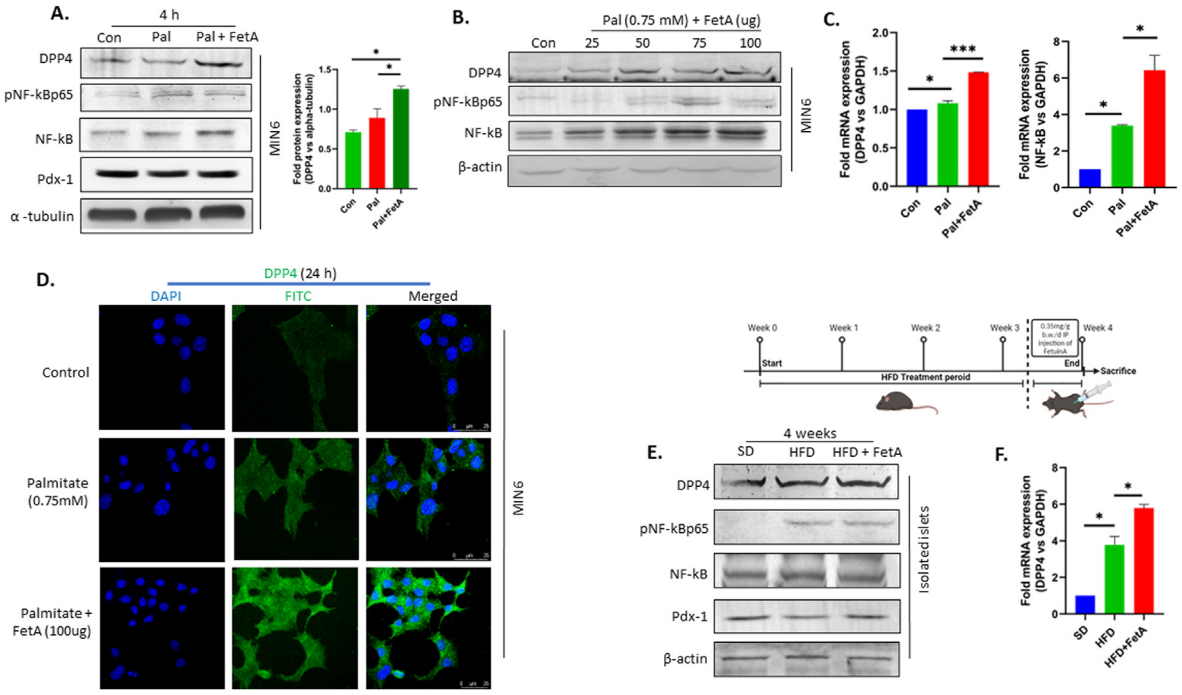

Fig. 2. (A) MIN6 cell were treated with palmitate (0.75 mM) alone or in combination with Fetuin-A (100 μg/ml) for 4 h and expression of DPP-IV, phosphoNF-kBp65, NF-kB, and PDX1 were analyzed using immunoblot. Densitometric plotting of DPP-IV was performed using α-tubulin as loading control. (*P < 0.05, compared with control). (B) MIN6 cells were treated with pal + Fetuin-A for 4 h at different doses of Fetuin-A for immunoblot analysis. (C) Total RNA was extracted from pal and pal + Fetuin-A treated cells and subjected to qPCR analysis using DPP-IV (***P < 0.0001, compared with palmitate) and NF-kB specific primers (*P < 0.01, compared with palmitate) taking gapdh as internal control. (D) Confocal images of DPP-IV immuno-stained MIN6 cells untreated or treated with palmitate or pal + Fetuin-A. DAPI was used as nuclear stain. Scale bar 25 μm. (E, F) Immunoblot and qPCR analysis of pancreatic islets isolated from SD, 4 weeks of HFD and 4 weeks HFD + IP injection of 0.35 mg/g body weight/day Fetuin-A for 5 days respectively (*P < 0.05, compared with HFD). Gapdh served as internal control.

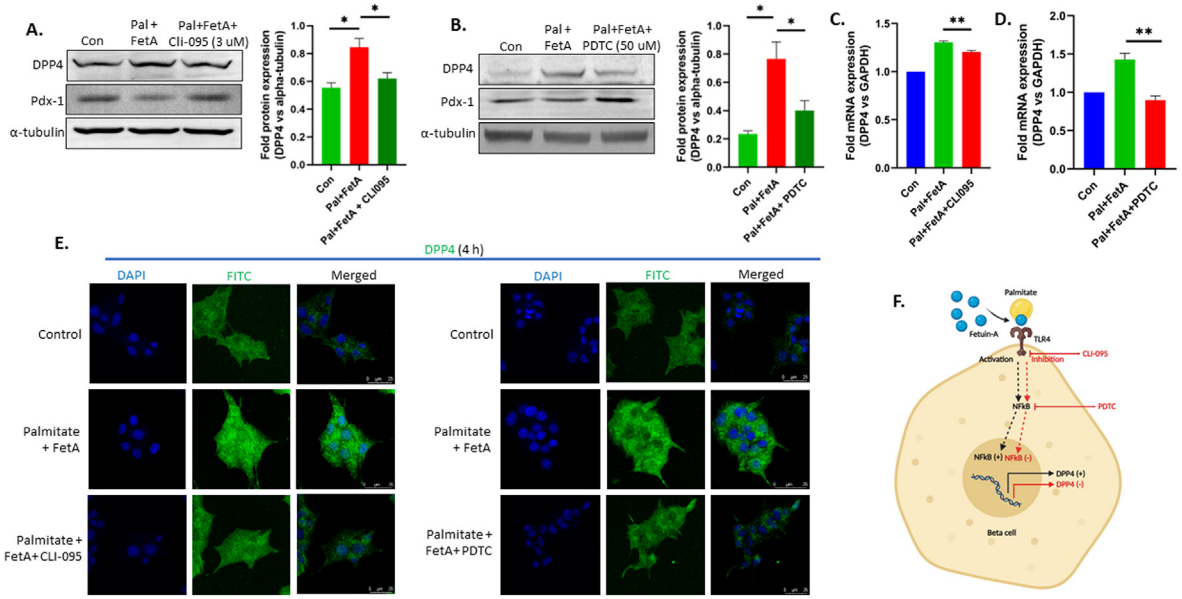

Fig. 3. MIN6 cells were pre-treated with TLR4 inhibitor CLI-095 for 1 h followed by 4 h of treatment with palmitate (0.75 mM) in combination with Fetuin-A (100 μg/ml) and analyzed with (A) Immunoblot and densitometric plotting, α-tubulin serving as loading control (*P < 0.05, compared with pal + Fetuin-A) and with (C) qPCR using DPP-IV specific primers, gapdh serving as internal control (**P < 0.05, compared with pal + Fetuin-A). MIN6 cells were also pre-treated with NF-kB inhibitor PDTC for 1 h followed by pal + Fetuin-A treatment for 4 h and performed (B) immunoblot and densitometric analysis. α-tubulin serving as loading control (*P < 0.05, compared with pal + Fetuin-A), and with (D) qPCR using DPP-IV specific primers, gapdh serving as internal control (**P < 0.05, compared with pal + Fetuin- A). (E) Confocal images of DPP-IV immuno-stained 1 h CLI-095 pretreated (3 μM) and 1 h PDTC pretreated (50 μM) MIN6 cells treated with pal + Fetuin-A. DAPI was used as nuclear stain. Scale bar 25 μm. (F) Model of the palmitate-Fetuin-A regulated DPP-IV expression in beta cells through TLR4- NFkB pathway.

cells (Fig. 3A and B). Immunostaining of DPP-IV in inhibitor treated MIN6 cells demonstrated reduced expression of DPP-IV in presence of pal + Fetuin-A (Fig. 3E). These findings suggest that palmitate-Fetuin-A induce DPP-IV expression in pancreatic beta cells through TLR4-NFkB pathway (Fig. 3F).

3.4. Fetuin-A alone mediates DPP-IV expression via NFkB

As we have previously demonstrated that Fetuin-A alone can promote NFkB activation in both human adipocytes and Raw 264.7 cells [14], we have checked the role played by Fetuin-A alone in DPP-IV expression. Treatment of MIN6 cells with Fetuin-A showed increased DPP-IV expression and phosphoNFkBp65 level as evident from immunoblot analysis (Fig. 4A). However, no apparent change in PDX1 expression was observed. Comparison of DPP-IV expression in MIN6 cells treated with Fetuin-A or palmitate or pal + Fetuin-A showed that Fetuin-A alone could induce DPP-IV expression, which further intensified with palmitate (**P < 0.005) (Fig. 4B). Increased gene expression of DPP-IV (**P < 0.005) as well as NFkB (**P < 0.005) has been observed in Real-Time PCR data in Fetuin-A treated MIN6 cells (Fig. 4C). Immunoblot with densitometric analysis also showed reduced expression of DPP-IV in PDTC treated MIN6 cells in presence of Fetuin-A (*P < 0.05) (Fig. 4D). Immunostaining of DPP-IV revealed an increased expression on treatment with Fetuin-A compared to the untreated cells (Fig. 4E). These results thus indicate that Fetuin-A increases DPP-IV expression through the activation of NFkB.

3.5. Palmitate impede DPP-IV secretion from MIN6 by reducing KLK5

As both membrane bound and soluble form of DPP-IV maintains its functional activity, next, we studied the effect of palmitate on the cleavage of membrane bound DPP-IV in pancreatic beta cells. We treated MIN6 cells with palmitate and pal + Fetuin-A for 4 h and immunoblot analysis found significant reduction in soluble DPP-IV level in the culture medium of treated cells compared to the control (*P < 0.05) (Fig. 5A). ELISA analysis of DPP-IV secreted in the medium also corroborated with the immunoblot data (**P < 0.05) (Fig. 5B). However, no significant change in the serum DPP-IV level was observed in both the SD and HFD mice (data not shown). To better understand this reduced secretion of DPP-IV on palmitate treatment, we looked for the expression of another protein kallikrein-related peptidase 5 or KLK5 in beta cells, which is supposed to be a sheddase that cleaves DPP-IV at the extra-cellular stalk region (Fig. 5E) [20,21]. Interestingly we found a reduced level of KLK5 protein and gene expression in both palmitate and pal + Fetuin-A treated MIN6 cells (Fig. 5C and D). These findings suggest that by reducing the expression of KLK5 protease, palmitate may abrogate DPP- IV secretion from MIN6 cells. A detailed diagram of KLK5 cleavage site of membrane bound DPP-IV has been shown in Fig. 5E as described by Nargis et al., 2017 [21].

4. Discussion

DPP-IV is a ubiquitous proteolytic enzyme that exists as a membrane bound form and a soluble form in the circulation, both the forms retaining their enzymatic activities [2,3]. Our interest in this current investigation is to study DPP-IV expression in the pancreatic beta cells which we thought might play a role in its function and health. Studies suggest that FFAs can stimulate the expression of DPP-IV in HepG2 cell line [22]. Researchers have also shown that oxidized LDL (low-density lipoprotein) significantly increases the expression of DPP-IV in bone marrow derived macrophages (BMMs) from C57BL/6 black mice. This increased expression of DPP-IV appears to occur partly through the TLR4

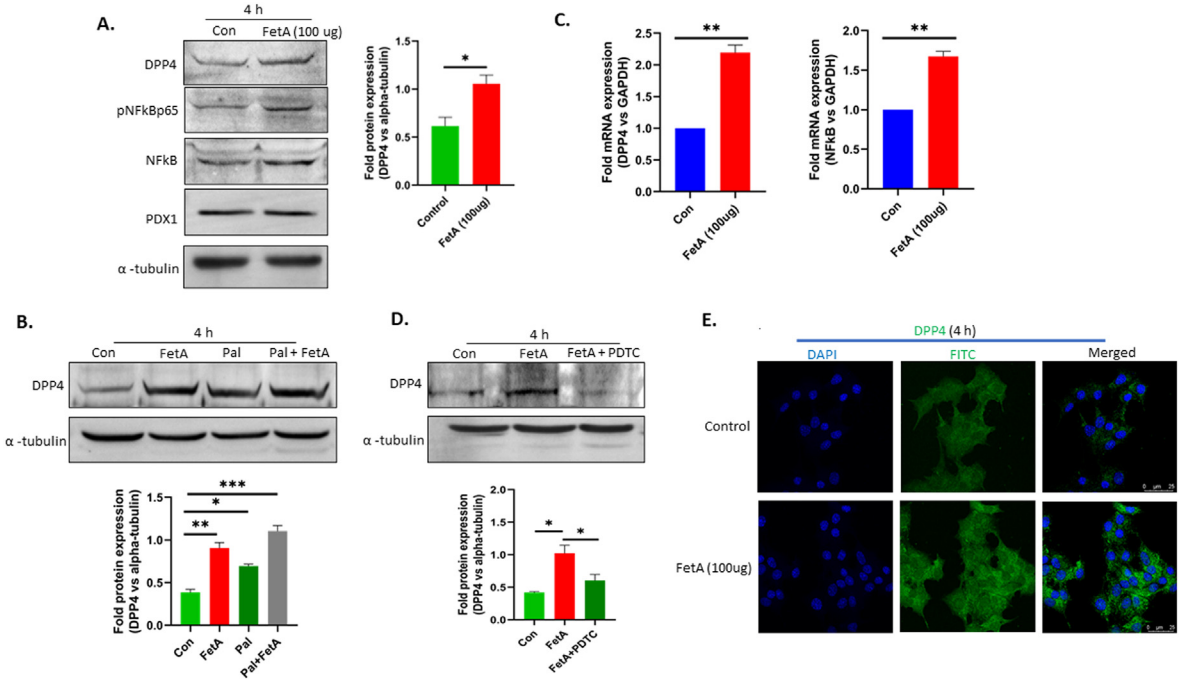

Fig. 4. (A) Immunoblot analysis of DPP-IV, phosphoNF-kBp65, NF-kB, and PDX1 expression in 4 h of Fetuin-A (100 μg/ml) treated MIN6 cells. Densitometric plotting of DPP-IV was performed using α-tubulin as loading control (*P < 0.05, compared with control). (B) MIN6 cells treated with Fetuin-A (100 μg/ml), palmitate (0.75 mM) and palmitate + Fetuin-A for 4 h and analyzed through immunoblotting and densitometry (**P < 0.005, compared with control). (C) Total RNA was extracted from 4 h of Fetuin- A (100 μg/ml) treated cells and subjected to qPCR analysis using DPP-IV (**P < 0.005, compared with control) and NF-kB (**P < 0.005, compared with control) specific primers. Gapdh is used as internal control. (D) Immunoblot with densitometric analysis of DPP-IV in 1 h of 50 μM PDTC pretreated MIN6 cells treated with Fetuin-A (100 μg/ml) for 4 h (*P < 0.05, compared with Fetuin-A). (E) Confocal imaging of DPP-IV immuno-stained MIN6 cells treated with Fetuin-A (100 μg/ml) for 4 h. DAPI was used to stain the nucleus. Scale bar 25 μm.

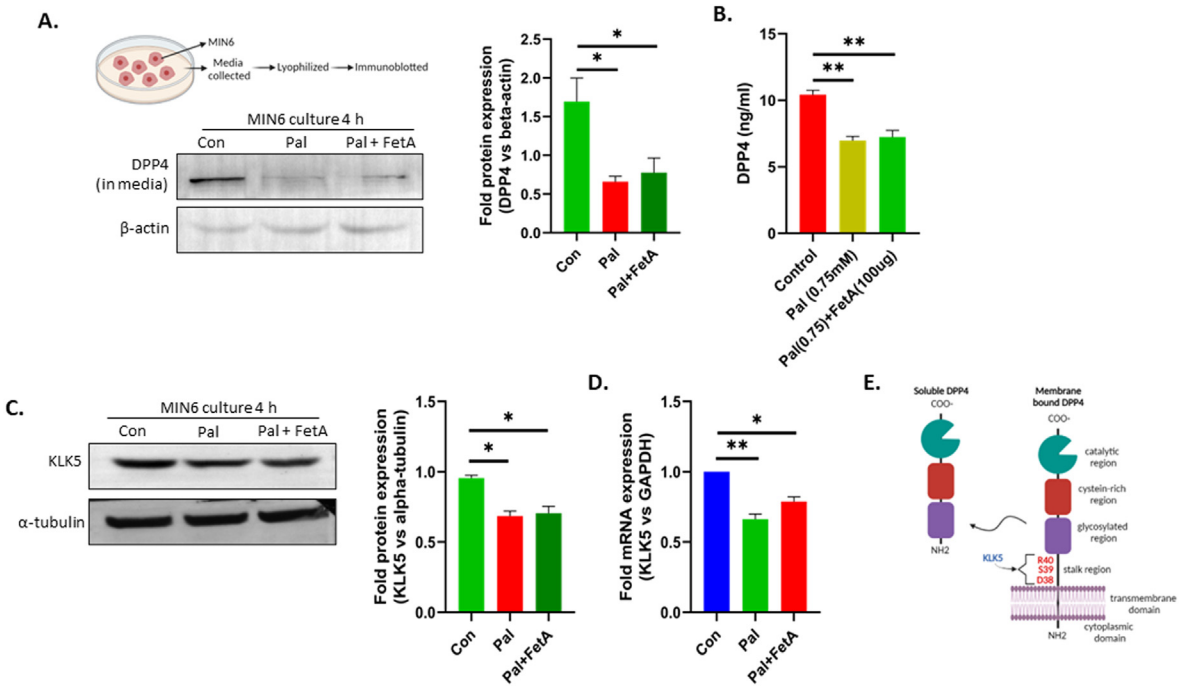

Fig. 5. (A) Immunoblot with densitometric plotting of DPP-IV secreted in the media of control, 4 h of palmitate (0.75 mM) and palmitate + Fetuin-A (100 μg/ml) treated MIN6 cells (*P < 0.05, compared with control). (B) ELISA analysis of DPP-IV secreted in similar treatment conditions (**P < 0.05, compared with control). (C) Lysates of MIN6 cells with identical treatments were also studied for KLK5 expression using immunoblot and densitometric analysis. α-tubulin served as loading control (*P < 0.05, compared with control). (D) qPCR analysis of palmitate and palmitate + Fetuin-A treated cells using KLK5 specific primers (**P < 0.05, compared with control). Gapdh is used as internal control. (E) Schematic representation of KLK5 interaction with the residues involved in DPP-IV cleavage.

pathway, as evident from LPS mediated increase in DPP-IV expression in human monocytes which was substantially reduced by TLR4 siRNA [9]. We previously reported increased TLR4 expression in pancreatic beta cells following FFA - Fetuin-A treatment [10], which incite question whether FFAs and Fetuin-A have a role in DPP-IV expression.

In rat insulinoma cell line RINm5F, palmitate upregulates the expression of DPP-IV [17]. As FFAs remain high during diabetic conditions, we checked DPP-IV expression on palmitate treatment in MIN6 cells as well as in pancreatic islets isolated from HFD mice. Treatment of MIN6 with palmitate increased the expression of DPP-IV (Fig. 1A and D). Similar results were also seen in the pancreatic islets isolated from HFD mice (Fig. 1E and F). These findings clearly show that FFAs stimulate DPP-IV expression in the mouse pancreatic beta cells, as reported earlier by Huang et al. in rat insulinoma cell line [17] and Lee et al. in HepG2 cells [22].

High circulating Fetuin-A levels have been consistently seen in people with diabesity [11,12]. Our previous reports showed that palmitate-Fetuin-A combination enhanced TLR4 expression in pancreatic beta cells [10]. A recent report showing increased TLR4-mediated DPP-IV expression in human monocytes [9], prompted us to look for DPP-IV expression in FFA and Fetuin-A treated MIN6 cells. Palmitate-Fetuin-A combination significantly increased DPP-IV expression compared to palmitate alone (Fig. 2A and D). Similar results were obtained in islets isolated from HFD mice where DPP-IV expression correlated with HFD treatment. Administration of Fetuin-A in 4 weeks' old HFD mice showed significant expression of DPP-IV, suggesting an additive effect of Fetuin-A on DPP-IV expression (Fig. 2E and F). TLR4 inhibitor CLI-095 and NF-kB inhibitor PDTC down-regulated DPP-IV expression in palmitate - Fetuin-A treated MIN6 cells (Fig. 3A and D). Inhibition of TLR4 and NFkB also restored PDX1 expression, which indicates normal physiological status of beta cells. Hence the findings suggest that palmitate-Fetuin-A mediated DPP-IV expression is regulated by TLR4-NFkB pathway.

Our previous report showed that Fetuin-A alone can stimulate NFkB phosphorylation in human adipocytes and Raw264.7 cells [14]. Other reports also confirmed that Fetuin-A alone can stimulate inflammatory cytokine expression in human THP1 monocytes [23]. Our data suggest that Fetuin-A mediated DPP-IV expression is correlated with the NFkB phosphorylation status (Fig. 4A and C). Inhibition of NFkB down-regulated the expression of DPP-IV (Fig. 4D), which suggests that Fetuin-A alone has the ability to upregulate DPP-IV expression in pancreatic beta cells through NFkB activation.

As DPP-IV is present both in the membrane bound and soluble forms [3], we checked DPP-IV levels in the media of MIN6 cells treated with palmitate and/or palmitate plus Fetuin-A. Both the treatments showed reduced shedding of membrane bound DPP-IV into the soup (Fig. 5A and B). Consistent with previous reports [24], our results showed no observable change in circulating DPP-IV levels in HFD and SD fed mice. To explain the increased release of DPP-IV into the culture media, we checked the expression status of KLK5, a protease that cleaves DPP-IV at the extracellular stalk region, releasing it into the circulation [21]. A decrease in the expression of KLK5 in palmitate and palmitate + Fetuin-A treatment in MIN6 cells (Fig. 5C and D) corroborates with the reduced shedding of DPP-IV in the MIN6 culture media, as shown above. Further studies are warranted to shed light on the beta cell secreted soluble DPP-IV in obese or type 2 diabetic subjects and their physiological relevance.

Overall, our study unravels a hitherto unknown role of FFA-Fetuin-A combination in promoting DPP-IV expression in the pancreatic beta cells through the TLR4-NFkB pathway. As both FFAs and Fetuin-A are abundant in conditions of diabesity and diabetic dyslipidemia, the regulatory influence of FFA-Fetuin-A combination

on DPP-IV expression and shedding by the pancreatic beta cells may advance our understanding about the pathophysiology of pancreatic beta cell dysfunction in people with obesity, insulin resistance and type 2 diabetes.

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Author contributions

SN designed and performed all the experiments, analyzed data and wrote the manuscript. SM analyzed the data and wrote the manuscript. Satinath Mukhopadhyay and TM conceived the idea and wrote the manuscript. RK conceived and designed the experiments, supervised the study, analyzed data and wrote the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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