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mRNA and Protein levels of rat pancreas specific protein disulphide isomerase are downregulated during Hyperglycemia

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Diabetes (Type I and Type II) which affects nearly every organ in the body is a multi-factorial non-communicable disorder. Hyperglycemia is the most characteristic feature of this disease. Loss of beta cells is common in both types of diabetes whose detailed cellular and molecular mechanisms are yet to be elucidated. As this disease is complex, identification of specific biomarkers for its early detection, management and devising new therapies is challenging. Based on the fact that functionally defective proteins provide the biochemical basis for many diseases, in this study, we tried to identify differentially expressed proteins during hyperglycemia. For that, hyperglycemia was induced in overnight fasted rats by intra-peritoneal injection of streptozotocin (STZ). The pancreas was isolated from control and treated rats for subsequent analyses. The 2D-gel electrophoresis followed by MALDI-TOF-MS-MS analyses revealed several up- and down- regulated proteins in hyperglycemic rat pancreas including the downregulation of a pancreas specific isoform of protein disulphide isomerase a2 (Pdia2).This observation was validated by western blot. Quantitative PCR experiments showed that the level of Pdia2 mRNA is also proportionally reduced in hyperglycemic pancreas.

Keywords: Beta cell, Diabetes, Insulin, Protein disulphide isomerase (Pdi)

Diabetes can be characterized by increase in blood glucose levels i.e., hyperglycemia, resulting from defects in insulin secretion, insulin action, or both[1]. The World Health Organization (WHO) has declared diabetes as one of the most threatening life-style disorders[2]. International Diabetes Federation, Brussels recent report states that globally 415 million adults (51.86% males) are affected by diabetes as of 2015 which it estimates to go up to 642 million by 2040. People living in urban areas account for 64.99% of total population[3]. In India, there are about 69.2 million cases presently which may rise to 115 million in next two and half decades[3]. Of the 3 types (TIDM, TIIDM and Gestational DM), type I & II DM are associated with only partially characterized destruction of pancreatic β-cells[3,4]. Among children, globally, there are more than half a million affected by TIDM. United States tops the list with 84100 children followed by India with 70200 of them[3].

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Natural flavonoids, such as rutin, apart from its anti-diarrhoeal activity, have been reported to be an effective hypoglycemic as well as hypolipidemic agent. It is also known to improve the histo-architecture of β islets and reverse hypertrophy of hepatocytes[5-7]. Another flavonoid, hyperin, commonly found in many plants, has been shown to improve the function of pancreatic islets and increasing glycolysis and decreasing gluconeogenesis[8]. In addition, oleanane-type triterpenoid saponin from the roots of *Momordica cymbalaria* Fenzl has also demonstrated to have potential antidiabetic activity by means of insulin secretion that can be attributed to modulation of calcium channel and β cell rejunavation[9]. Kalsi and Grewal who studied the TIIDM treatment with oral drugs related their poor availability to efflux transport of intestinal P-glycoprotein and suggested that natural compounds such as fumagillin and piperine have potential to control such efflux and improve their efficacy[10]. Early insulin compared to late one, is reported to significantly reduce biochemical markers like glucose, triglyceride, glycated hemoglobin, thiobarbituric acid reactive substances, AGE products and ratio of reduced and oxidized glutathione in diabetic rats. Further,

it provides benefits of early glycaemic control in preventing neuropathy and cataract development[11].

Several approaches such as genomics, transcriptomics, proteomics and metabolomics are being applied to identify more specific biomarkers of this disease for early detection, management and devising new therapies of diabetes[12-15]. Generally, genetic mutation and/or aberrant gene expression leading to defects in the function and/or expression of protein(s) may underlie a disease. Hence, to determine the profile of differentially expressed proteins may be the most important and useful approach in developing diagnostic and therapeutic techniques[16]. Despite all these attempts, many aspects of this disease being complex in nature are yet to be elucidated. Studies using animal model are important because cell culture system was not able to fully define the systemic/metabolic complexity of diabetes[15,17]. Naturally occurring broad spectrum antibiotic, streptozotocin (STZ; N-nitro derivative of glucosamine) is a chemical that is particularly toxic to the pancreatic, insulin producing beta cells in mammals[18-22]. It enters pancreatic β cells through glucose transporter 2 (GLUT2) channels in the plasma membrane and causes hyperglycemia[18,20].

In this study, we explored the differentially expressed proteins in the pancreas obtained from rats that were made hyperglycemic upon low-moderate dosage of STZ as compared to the controls using proteomic techniques such as 2-D Gel Electrophoresis and Mass Spectroscopy.

Materials and Methods

Animal Model and Treatments

The animals taken for this study were 8-10 weeks old male Wistar rats weighing 100-150 gm body weight. The experimental protocol was approved by Animal ethics committee of the University. All animals were kept under 12 h night and day routine and fed standard laboratory chow *ad libtum*. Hyperglycemic conditions were induced in rat according to protocol mentioned before[22]. Briefly, ‘low-to-moderate’ regimen of STZ treatment which is extensively used with optimum effect i.e., effective in generating hyperglycemia but lesser cytotoxicity was applied[20,22]. Streptozotocin (Sigma Aldrich) was used at a concentration of 45 mg/kg of rat and injected intraperitoneally after 16 h of fast. Three days post injection, the blood glucose and body weight of the treated and control rats were measured and tabulated (Table 1). Hyperglycemic (≥250 mg/dl) and vehicle control rats were sacrificed and their pancreases removed for further analysis. All experimental procedures were done following guidelines of our Institutional animal ethics committee.

Table 1—Body weight and blood glucose of rat before and after Streptozotocin administration

Rat	Initial weight	Final weight	Initial Blood glucose	Final Blood glucose *
Control	116 ±10.1	152 ± 9.5	50 ± 3.6	67 ± 3.8
Treated	142 ± 5.8	118 ± 4.6	63 ± 5.2	472 ± 33.6

The data are expressed as mean ± SEM (standard error of mean) of 12 rats. *P <0.05, when compared to control

Immunohistochemistry

Processing of pancreatic tissue for immunohistochemistry (IHC) was done according to the protocol discussed in a previous article[23]. In short, pieces of pancreatic tissue were fixed in formal buffer (pH=7). Paraffin blocks of the pancreatic tissue were done. About 5 µm sections of these blocks were made and placed on slides. After de-paraffinization and rehydration, the sections were incubated in primary antibody at 1:100 dilution followed by corresponding Alexa flour tagged secondary antibody at 1:200 dilution and then counterstained with DAPI for visualizing the nuclei. Fluorescent images were captured by using Nikon Eclipse Ti microscope.

Protein extraction and 2D-PAGE analysis

All the procedures were done following manufacturer’s (GE Healthcare) protocol. The protein lysates of the tissues for 2D Gel were made by homogenizing and sonicating on ice in the lysis buffer (7M Urea, 2M Thiourea, 4% CHAPS, 50mM DTT, 2% 2D Ampholyte). Protein was estimated using Bradford method. Briefly, 100 µg of protein extract was analyzed by isoelectric focusing using 7 cm 3-10 L GE IPG strips which were rehydrated at 24°C for overnight. Next day, focusing was carried out in Ettan IPG phor 3 which was monitored by IPG phor software (GE Healthcare). For the second dimensional separation, the IPG strips were placed on the top of 10% SDS- PAGE and run at 120V. The protein spots were visualized by silver staining. The experiment was performed at least thrice using independently prepared tissue samples to confirm reproducibility. The resulting images were analyzed using Image Master 2D Platinum. The number of matched spots that showed change in percent intensity ≥ or ≤ 25 in treated as compared with the control was chosen for further study.

Mass Spectrometry

Proteins spots were picked up manually. The spots were further processed for mass spectrometry, using in-gel tryptic digestion method according to Promega's protocol and subjected to identification using ABSCIEX- 4800 MALDI TOF TOF. In short, the spots were treated by 1:130 mM potassium ferricyanide and 100 mM sodium thiosulphate. Then, the spot in gel was shrunken using 50% acetonitrile in 25 mM ammonium bicarbonate buffer. Reduction was done using 2 mg/mL DTT and alkylation was done using 10 mg/mL iodoacetamide (Sigma-Aldrich). Again, the gel was shrunken with 50% acetonitrile in 25 mM ammonium bicarbonate buffer followed by digestion with 4 ng/µL trypsin (Promega) for 16 h at 37°C. The peptides were extracted from the gel piece, pooled, lyophilized and kept at −20ºC for further use.

Western blot analysis

Western blotting was done using protocol mentioned earlier[24]. The protein lysates of tissues were made in lysis buffer (50 mM NaCl, 1 mM MgCl2, 50 mM Tris, 1 mM DTT, 1 mM PMSF, 1X Protease inhibitor). Briefly, 60 µg of protein per sample was electrophoresed in a 10% SDS-PAGE, and electroblotted onto PVDF membranes. The membranes were blocked with 5% BSA in PBS, incubated with anti- Pdia2 antibody at 1:1000 dilution and then alkaline phosphatase conjugated secondary antibody (Sigma Aldrich) at 1:10,000 dilution. Protein band detection was performed by adding 5 bromo 4 chloro 3 indolyl phosphate/nitroblue tetrazolium (BCIP/NBT; Himedia) to the membranes. β-actin was used for loading control.

RNA extraction and cDNA synthesis

Total RNA from pancreas was prepared by the Trizol reagent according to Sigma Aldrich's protocol and was used for cDNA synthesis by reverse transcription. About 1 µg of total RNA was incubated with 0.2 µg of Random hexamer (Fermentas) at 65°C for 5 min, chilled on ice, briefly centrifuged and placed on ice. This mixture was then used in the reverse transcription reaction following protocol obtained from the manufacturer (Fermentas).

Semi quantitative PCR

In a total volume of 20 µL, 1 µL of cDNA was incubated with 2 µL of Taq DNA polymerase buffer, 3 pmole of forward and reverse primers, 0.25 mM deoxynucleotide triphosphates, and 2.5 unit of Taq DNA polymerase. The primers used for Rat Pdia2 amplification were: 5′-ACTAAGAAGTATGCGCCT GTG-3′ (forward) and 5′-CGTCAAAAGCCACC TGCTCAAAA-3′ (reverse) and for Rat 18S rRNA were: 5′-GTAACCCGTTGAACCCCATT-3′ (forward) and 5′-CCATCCAATCGGTAGTAGCG-3′ (reverse). The primers were designed such that both the primers must be separated by large intronic region in the Pdia2 DNA sequence decreasing the chance of genomic DNA contamination. Each sample was amplified by PCR using 40 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) with an initial denaturation at 94°C for 5 min and the final extension was at 72°C for 5 min for Pdia2 and an endogenous control 18S rRNA. To measure PCR products semi quantitatively, 1 µL of cDNA product was amplified by using 30, 33, 35, 37, 40, 45 cycles for each target under the same conditions described above.

Real time PCR

Real Time PCR was done using the same primers which were used during Semi quantitative PCR, for both Rat Pdia2 and Rat 18S rRNA. The cDNA was made using same protocol mentioned in the RNA extraction and cDNA synthesis section before. Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas) and Real Time PCR system (Model 7500 Fast, Applied Biosystem) were used for subsequent study. R_n values were calculated from fluorescence of the reporter dye (SYBR Green) divided by the fluorescence of a passive reference dye (ROX) for each sample. C_t value for each sample was automatically computed from the respective R_n value by the software (7500 Ver. 2.0.6). ΔC_t values were calculated by subtracting C_t values of endogenous control (18S) from that of target cDNA (Pdia2). The $\Delta\Delta C_t$ value was calculated by subtracting ΔC_t value of a treated sample from that of the corresponding untreated one. Finally, the fold change of PdiA2 gene expression between treated and control pancreas was computed using following equation:

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Results and Discussion

Immunohistochemical analyses using anti-insulin antibody of the isolated pancreas, clearly shows regions of islets in the treated as well as in control samples (Fig. 1 A and B). Increased presence of activated caspase-3 in STZ-treated pancreas compared to the control samples illustrates enhanced apoptotic death of beta cells in the treated pancreas (Fig. 2 A and B). Tissue sections stained with DAPI show

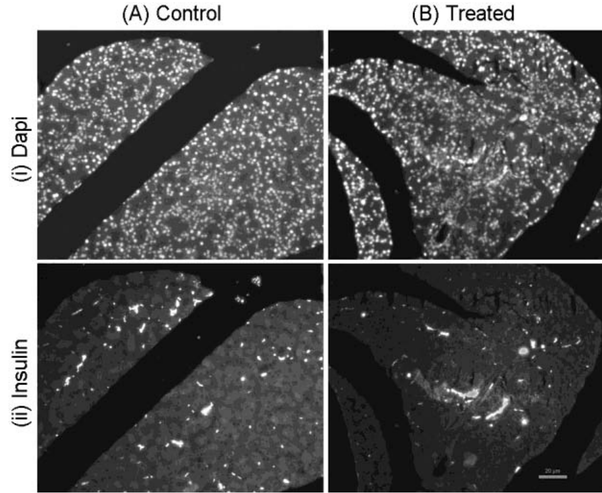

Fig. 1—Immunohistochemical analyses of (A) control and (B) STZ treated rat pancreatic samples that were stained with DAPI (i) (for nuclei) and anti-insulin antibody (ii). Magnification: 200X. White bar represents 20 µm. There was positive expression of insulin in control (A) and STZ treated hyperglycemic (B) rat pancreatic tissues.

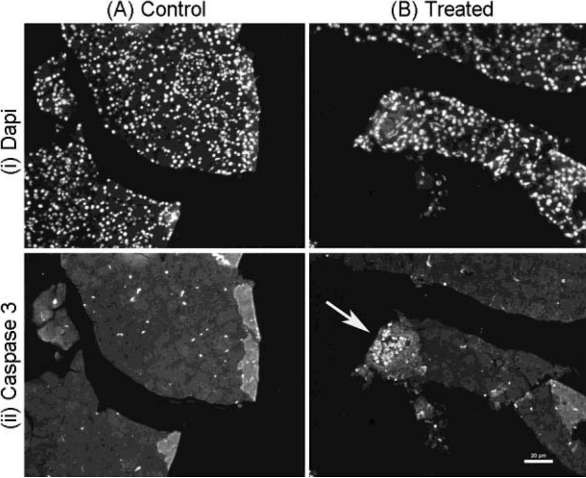

Fig. 2—Pancreatic tissue sections of (A) control and (B) STZ treated were stained with DAPI (i) (for nuclei) and anti active caspase 3 antibody (ii). Magnification: 200X. White bar represents 20 µm. There was more expression of caspase 3 in STZ treated hyperglycemic (B) rat than the control (A) rat pancreatic tissues. Arrow indicates the region enriched with active caspase 3 [B(ii)].

Table 2—Fold change in hyperglycemic rats compared to the control rats

Serial Number	Fold upregulated	Fold downregulated
1	3.6	−1.84
2	1.63	−1.84
3	1.88	−1.53
4	2.15	−1.84
5	1.51	−1.54
6	1.28	−1.31
7	1.36	−1.36
8	1.59	−1.26
9	1.6	−1.62
10	1.75	−1.46
11	1.44	−1.34
12	1.34	−1.31
13		−1.58
14		−1.2
15		−1.5
16		−1.49
17		−1.63

Table 3—Proteins identified by Mass Spectrometry

Spot	Accession number	Protein	Fold change	MW /pI
51	D3Z9K7	Pdia2	1.84	58421.92/4.77
99	P19222	Carboxypeptidase A2	1.66	47167.63/5.17
104	P19223	Carboxypeptidase B	1.47	47884.51/5.44
89	P00689	Alpha amylase	1.61	57206.7/8.19
218	P19804	Nucleoside diphosphate kinase	1.63	17385.94/6.92

substantial number of cells in the hyperglycemic comparable to the control samples proving that dosage of streptozotocin was not destructive for the cells.

To explore differential protein expression in pancreas from hyperglycemic rats, 2 D Gel Electrophoresis was carried out. Several proteins were found to be up- and down-regulated in the treated sample with respect to the control sample. Table 2 shows 12 up- and 17 down- regulated proteins in the treated samples. Some of these proteins identified in Mass Spectrometry were relevant to our study and are listed in Table 3. Figure 3 shows the protein spots as seen in the 2D gel corresponding to those mentioned in Table 3. It is shown in Table 3 that Rat Pdia2, a pancreas specific chaperone, gets 1.84 fold downregulated in the treated sample.

Protein disulphide isomerases being a protein thiol oxidoreductase participates in folding assembly and post-translational modification of many proteins containing disulphide bonds. As insulin and many proteins have multiple disulphide bridges, need involvement of chaperone like PDI for its maturation[25,26]. PDI have multiple isoforms among which Pdia2 being pancreas specific, has been chosen for further investigation. In this report, we have found out that a pancreas specific chaperon, protein disulphide isomerase (Pdi) denoted as Pdia2 is

downregulated in hyperglycemic condition. To verify the finding of Mass Spectrometry that the expression of Pdia2 protein is decreased in the treated pancreas, we used western blot analysis (Fig. 4 A). The quantitation of the western blot shows 1.55 fold reduction in the Pdia2 protein expression in the treated sample (Fig. 4 B).

The change in protein expression might have been caused at transcriptional or translational level. To determine the mechanism behind the downregulation of Pdia2 protein expression, we did semi-quantitative PCR. Results of semi-quantitative -PCR showed clear diminution of Pdia2 mRNA expression in the hyperglycemic rat pancreas (Fig. 5 A; compare lanes 1, 3, 5, 7, 9, 11 between control and treated). Using

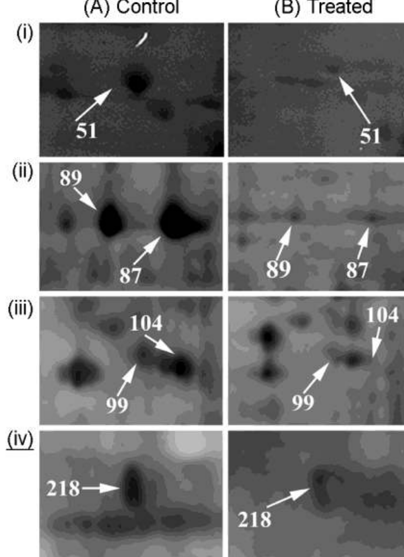

Fig. 3—Images of the protein spots in a 2D gel electrophoresis. About 100 µg of pancreatic protein lysate was run between pH 3 to 10. Panel (A) represents the control and Panel (B) represents the treated samples. Annotated protein spots were analyzed and identified by mass spectrometry are shown here; where (i) Spot 51(Pdia2); (ii) Spot 87 and 89 (Alpha amylase); (iii) Spot 99 (Carboxypeptidase A2), Spot 104 (Carboxypeptidase B); and (iv) Spot 218 (Nucleoside diphosphate kinase). The indicated spot numbers are shown in Table3.

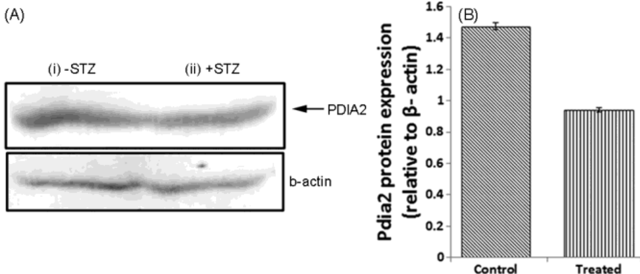

Fig. 4—(A) Immunoblot of pancreatic protein lysates from control [−STZ, panel (i)] and STZ treated hyperglycemic rats [+STZ, panel (ii)], respectively. [About 60 µg of pancreatic protein lysates was resolved by SDS-PAGE using a 10% gel, transferred to polyvinylidene difluoride membranes, and immunoblotted for Pdia2 protein using anti Pdia2 antibody. β-actin was used as the protein loading control]; (B) The band intensities from the immunoblot (Fig. 4A) of the ▨ control and the ▥ treated samples were determined by densitometric scanning and were quantified by Image J software. [The band intensities were normalized by those of β- actin. The experiment was done in triplicate. SD and SE values were used to calculate the error bars]

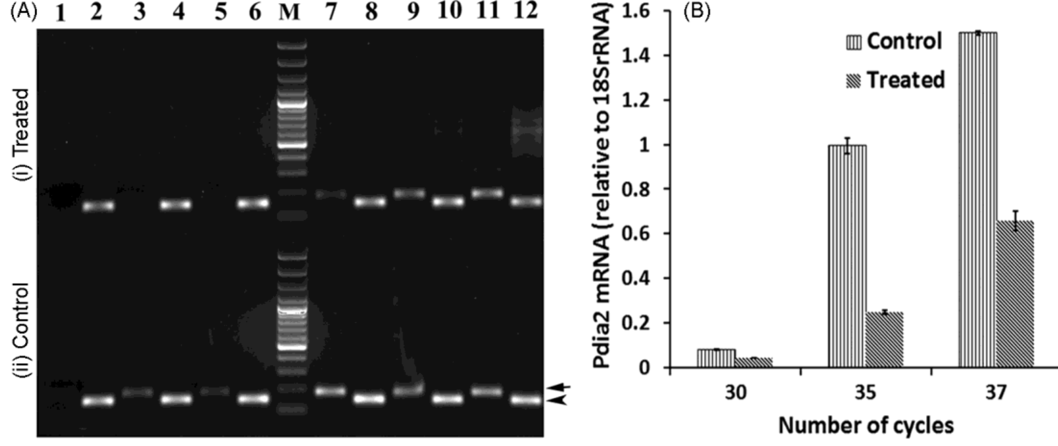

Fig. 5—(A) Semi quantitative PCR of cDNA corresponding to 18S rRNA (lanes 2, 4, 6, 8, 10 and 12) and Pdia2 (lanes 1, 3, 5, 7, 9 and 11) are shown in both the (i) treated and (ii) control samples. [Amplified products: 30 cycles (lanes 1-2), 33 cycles (lanes 3-4), 35 cycles (lanes 5-6), 37 cycles (lanes 7-8), 40 cycles (lanes 9-10) and 45 cycles (lanes 11-12) are shown in this figure. Lane M represents the marker lane. The Pdia2 amplicon is of 186bp (arrow) and 18S rRNA amplicon (arrowhead) is 151bp in size]; (B) Quantitation of amplified Pdia2 cDNA as shown in (Fig 5A) using Image J software was done where ▨ represents the control and ▥ represents the STZ treated samples. [Amplified values taken from the linear range of increase in Pdia2 cDNA were plotted against corresponding number of cycles. The band intensities were normalized by those of cDNA. SD and SE values were used to calculate the error bars]

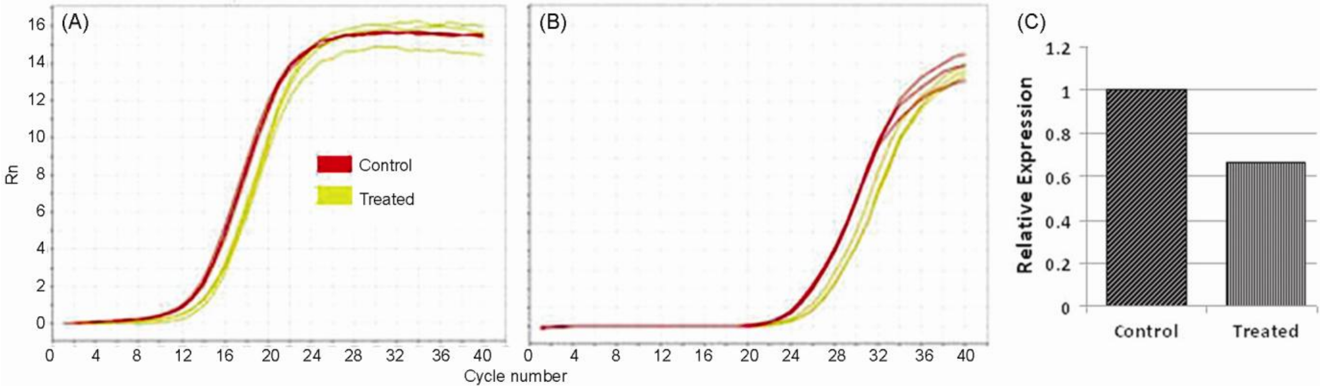

Fig. 6—Amplification plot of RT PCR of (A) 18S cDNA; (B) Pdia2 cDNA of both treated and control pancreas. In the graph red and yellow curve represents the treated 18S cDNA/Pdia2 cDNA of control and treated pancreas, respectively. X axis denoted the cycle number and Y axis represent Rn value; (C) Quantitation of amplified Pdia2 cDNA as shown in (Fig. 5A) using RT PCR. Here ▨ represents the control and ▥ represents the STZ treated samples. [The ΔC_t value of Pdia2 was normalized to that of 18S cDNA. The fold change in Pdia2 expression over control was calculated as described in the Materials and Methods section. Finally, a bar diagram of their relative expression was plotted assuming the value of control as 1]

quantitative PCR, we have demonstrated that Pdia2 mRNA is also decreased in the pancreas isolated from STZ treated rat. Densitometric scanning of the amplified band showed 1.48 fold reduction of the cDNA sample from the treated pancreas compared to the untreated control (Fig. 5B).

Having observed the result of the semi-quantitative PCR, we wanted to verify that by Real Time PCR (RT-PCR) experiment using cDNA from the treated and control rat pancreas. The result showed the $\Delta\Delta$ct value to be about −0.57 (Fig. 6 A and B). That means the mRNA of Pdia2 in the treated sample was 1.48 fold lower than that in the control (Fig. 6C).

Proteomics is a powerful tool which is adopted to unravel differential protein expression in various pathophysiological conditions[27,28]. Studies using this approach have been utilized to explore different proteins that are present in whole pancreas or isolated islet from pancreas[27,28]. In this study, using similar proteomic approach several proteins were identified from pancreas of hyperglycemic rats that were up- and down- regulated with respect to the control (Table 2 & 3). Among the downregulated proteins, a pancreas specific chaperone protein disulphide isomerase, Pdia2 was chosen for further study because this enzyme was implicated previously to be involved in insulin biosynthesis[29]. Not much is reported about the downregulation of Pdia2 protein in diabetic rat pancreas. However, results from several laboratories have shown the isoforms of this enzyme to get upregulated in diabetic liver and other tissues of mammalian origin[30-33]. Among these studies only a few utilized proteomic tools to demonstrate differential expression of proteins in diabetic tissues. Earlier reports showed that similar to our finding the levels of different isoforms of HSP 90 and ER chaperone GRP78 were also reduced in diabetic rat and mini pig pancreas [27,34]. Moreover, PDI expression in liver and other organs is partially regulated by insulin[30]. But how the regulation of pancreatic isoform of PDI (which we studied) takes place is not fully elucidated. It is likely that the specific isoforms of PDI expression in pancreas and liver are distinctly regulated.

It was evident using proteomics studies[35] that downregulation of alpha amylase (AMY 2) occurred in diabetic human saliva which is similar to our result (Table 3). Our data also showed that nucleoside diphosphate kinase (NDPK-β) is downregulated in the treated pancreas; although earlier report showed its presence in β-cells, but nothing was mentioned about the level of its expression[36]. We have seen that carboxypeptidase A2 is downregulated which is also not reported. The fact that the carboxypeptidase B protein which is present in islet and involved in the processing of proinsulin, decreased during hyperglycemic condition[37], is also verified by our study (Table 3).

It can be argued that the downregulation of Pdia2 protein found and verified by proteomic study and western blot analysis could be due to loss of pancreatic β cells treated with streptozotocin. Our result rules out that possibility because the moderate dosage of streptozotocin (45 mg/kg) at which the loss

of cells in the treated pancreas was not appreciable (Fig. 1Bi and 2Bi). The reduction in protein amount could be due to regulation at either at the level of translation or transcription. The reduction in the steady state mRNA level of Pdia2 is indicative of a regulation either at the transcriptional or at the posttranscriptional level or both. The diminution of Pdia2 protein and mRNA levels in hyperglycemic rat pancreas is apparently opposite to what was shown earlier in liver of diabetic rat and human may possibly have some role in the pancreas during hyperglycemia[30-33]. Indeed, downregulation of some ER chaperons was reported by other workers[38]. Moreover, Zhang *et al*.[39] have demonstrated that overexpression of PDI in β cells led to reduction in insulin secretion and accumulation of proinsulin in the ER resulting in ER stress. Further, the hyperglycemic condition was shown to exert effect on some chaperon proteins including PDI. Additionally, expression levels of these proteins in turn regulate insulin biosynthesis and secretion[39,40]. It is also to be noted that different observations have been obtained from experiments using cultured cells and intact animal models. Taken together, our study in conjunction with earlier findings suggest that pancreas specific isoform of PDI is downregulated possibly to modulate insulin biosynthesis and secretion to cope with hyperglycemic condition. Further studies are in progress to unveil the mechanism behind this downregulation of Pdia2.

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