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Mechanisms by which Imatinib counteracts Diabetes mellitus

putative role of PDGFR and LRP-1

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ABBREVIATIONS:

AKT	Protein Kinase B
ApoE	Apolipoprotein E
Bcr-Abl	Breakpoint Cluster - Abelson murine leukemia
c-Abl	Cellular Abelson tyrosine kinase
CD91	Cluster of differentiation 91
CML	Chronic myeloid leukemia
ERK	Extracellular signal regulated kinase
GIST	Gastrointestinal stromal tumour
Gp330	Glycoprotein 330
HFD	High fat diet
HRP	Horse radish peroxidase
JNK	c- Jun N-terminal kinases
LRP	Low density lipoprotein (LDL) receptor-related protein
P	Phospho
PAGE	Polyacrylamide gel electrophoresis
PDGFR	Platelet derived growth factor receptor
PVDF	Polyvinylidene diflouride
SDS	Sodium dodecyl sulfate

INTRODUCTION:

Diabetes mellitus:

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia and various other disorders like thirst, polyuria, blurring of vision and weight loss. Acute forms of ketoacidosis or a non-ketotic hyperosmolar state may develop which leads to coma, stupor, imbalance of carbohydrates, fat and protein metabolism. Diabetes is due to defects in insulin secretion, insulin action or both and will even lead to death in the absence of effective treatment (Report WHO consultation. 1999). Diabetes can be classified into 2 types, type 1 and type 2 diabetes. In both the cases death of the Beta (β) cells is a common feature. β cells are located in the islets of Langerhans of the pancreas. They make up ~60-65% of the islets. β cells make and release insulin, the hormone that controls blood glucose levels in the human body.

Type 1 diabetes (T1D) accounts for ~ 5 to 10% of all DM cases. The mass of β cells is reduced from 100 to 10-30% at time of diagnosis. It is thought that β-cell loss occurs gradually over years. It is a chronic autoimmune disease in which insulin deficiency and hyperglycemia occurs due to the damage or destruction of beta cells in the Islets of Langerhans.

Type 2 diabetes (T2D) accounts for more than 90% of total DM cases account for T2D. It can be found generally after 30 years old individuals with a positive diabetic family case history. Increased insulin resistance is one of the characteristic features, which leads to improper uptake of glucose into the muscle and fat cells (DeFronzo et al. 1991; DeFronzo et al. 2009) and it also involves liver, muscle and adipose tissue. This results in increased blood glucose levels. Insulin and glucagon play a crucial role in maintaining the glucose homeostasis. Symptoms could be polyphagia (increased hunger), polydispia (increased thirst), polyuria (frequent urination), weight loss and fatigue. The normal treatments are diet and exercise along with oral hypoglycemic agents.

Imatinib, Sunitinib and diabetes:

Imatinib (4-[(4-methylpiperazin-1-yl) methyl]- N- (4-methyl-3-{ [4-(pyridine-3-yl) pyrimidin-2-yl] amino } phenyl) benzamidine) or (Gleevec®, STI571) was developed to treat chronic myeloid leukemia (CML) by turning of the specific enzyme that causes the tumor cells to proliferate rather than killing the normal tissue, which was done by conservative drugs such as non-specific cell cycle inhibitors. Imatinib is a 2- phenylaminopyrimidine based ATP-competitive inhibitor of the Abl protein kinase. The ATP binding site of the kinase is the main structural component that Imatinib targets. Inactive form of Bcr-Abl is bounded and stabilized by Imatinib leading to inhibition of autophosphorylation and substrate phosphorylation.

Suntinib **(**Sutent®, SU011248**)** was designed to inhibit the members of the split-kinase domain family of receptor tyrosine kinases (RTK's), which includes the vascular endothelial growth factor receptor (VEGFR) types 1 & 2, Vascular Epidermal like Growth Factor Receptor 1 (FLT1) & Vascular Epidermal like Growth Factor Receptor 2 (FLK1/KDR); PDGFR and PDGFR-β, the stem cell factor receptor CD117 (c-Kit); the Fms like tyrosine kinase 3 (FLT3) and Rearranged during Transfection (RET) kinases. When the RTK's are inhibited, signal transduction is blocked, which affects processes like tumor growth, progression, metastasis and angiogenesis (Hanahan and Weinberg. 2000).

In T1D and T2D pathogenicity of β-cells is a characteristic feature. The possible reasons behind the cell death are unknown. It is known that patients treated with Imatinib suffering from CML were also cured from diabetes along with CML. Apart from this, lowering of blood glucose levels in patients with GIST (Little et al. 2009) and paraneoplastic syndrome (Breccia et al. 2004) was also observed in response to Imatinib. The actual reasons behind these cases are unknown, but from recent observations in NOD and streptozotocin-injected mice it is possible that Imatinib increases β-cell survival by acting against apoptosis (Bonora et al. 2005; Breccia et al. 2005). It could be due to the decrease in the JNK activation (c-Jun N-terminal kinase) in β cells. It has also been observed that JNK activation, which occurs in peripheral tissues in response to oxidative stress, increases insulin resistance and Type 2 diabetes (Billemont et al. 2008). It was shown that blood glucose levels are lowered by Imatinib- 9mg/dL and Sunitinib- 14mg/dL when treated by TKIs in diabetic and non-diabetic patients (Agostino et al. 2010).

The below picture shows the possible mechanisms how Imatinib can counteract diabetes. Imatinib inhibits c-Abl (Kharbanda et al. 1995; Sun et al. 2000), which leads to inhibition of the downstream Mitogen activated Protein Kinase, c-Jun N- terminal kinase - MAPK JNK (Hagerkvist et al. 2006) and ER stress responses (Ito et al. 2001), and increased activation of NF-*κ*B (Hagerkvist et al. 2006; 2007). PDGFR inhibition can also lead to a decrease in insulin resistance and an altered inflammatory response (Louvet et al. 2008). Inhibition of c-kit and DDR1/2- an extra cellular matrix interacting protein (Day et al. 2008) - also could lead to altered inflammatory response. This indicates that multiple mechanisms could be responsible for the Imatinib-induced β- cell survival.

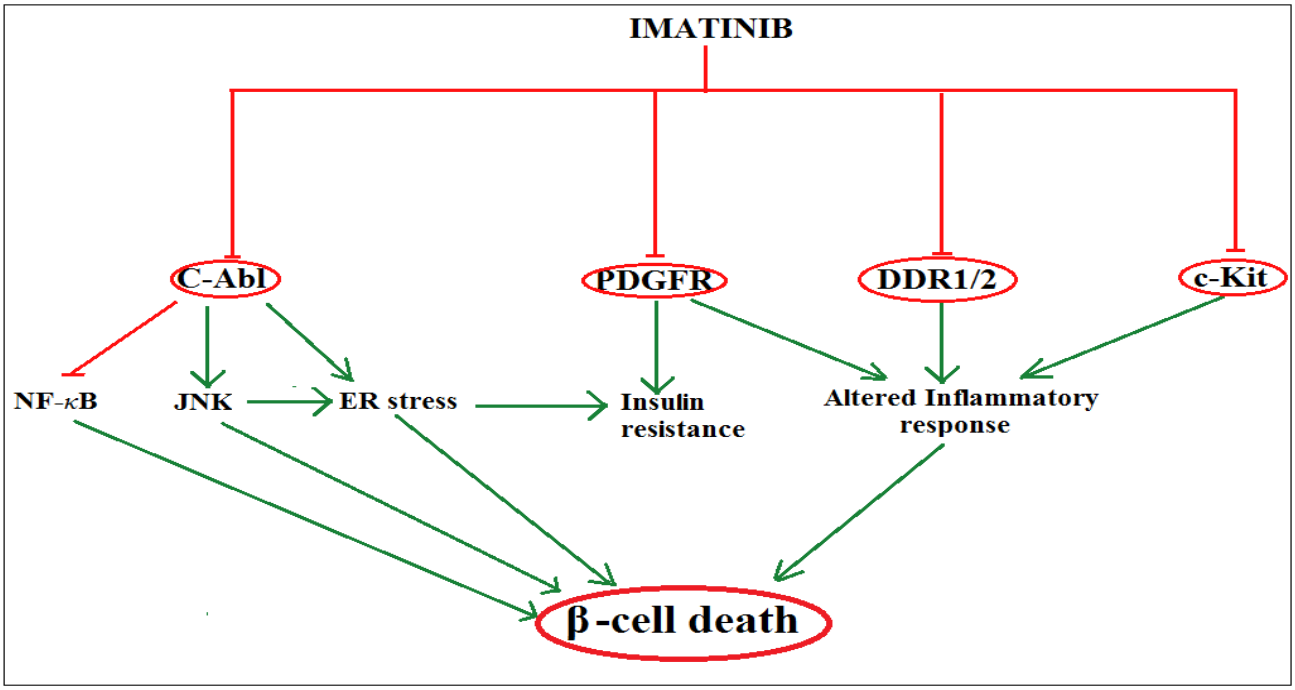

Figure 1. Possible mechanisms how Imatinib can counteract diabetes.
Based on Welsh et al. 2010.

LRP-1:

LRP-1 (Low Density Lipoprotein (LDL) Receptor-related Protein) was discovered as a large hepatic receptor, which resembled the LDL receptor (Herz et al. 1988) The receptor was called LRP-1 or CD91. LRP-1 is an endocytic and multifunctional type I cell surface membrane protein. It is a dual chain 600 kDa molecule consisting of an N- terminal 515-kDa heavy chain which is extracellular and an 85-kDa light chain, which has a transmembrane and cytoplasmic tail. The non covalent interactions in between the heavy and light chains make the heavy chain anchored to the cell surface.

LRP-1 belongs to the LDL receptor (LDLR) gene family, in which the LDLR, MEGF7, apoE receptor-2 (apoER2), gp330/megalin/LRP-2 and VLDLR are closely related; and LRP-5, LRP-6, LRP-1B and SorLa /LRP-11 are distantly related.

Ligand binding repeats or the epidermal growth factor (EGF) precursor homology domain is the characteristic common feature among all the LDLR family members. These features are important for the pH-dependent release of ligands in endosomes. The cytoplasmic tails of above mentioned receptors contains 3 NPXY motifs-recognition sites for adaptor proteins. The natural presence of these motifs indicates that these receptors could also be involved in other signal transduction events than those related to endocytosis. The reason for LRP to have diverse biological roles is that LRP can recognize multiple ligands. LRP can recognize at least 30

different ligands in a variety of protein families (Herz and Strickland et al. 2001).

Structure:

Structurally LRP-1 is arranged as (a) Cysteine rich complement type repeats (CR), (b) EGF repeats, (c) β-propeller domains, (d) transmembrane domain and a cytoplasmic domain (Figure 2).

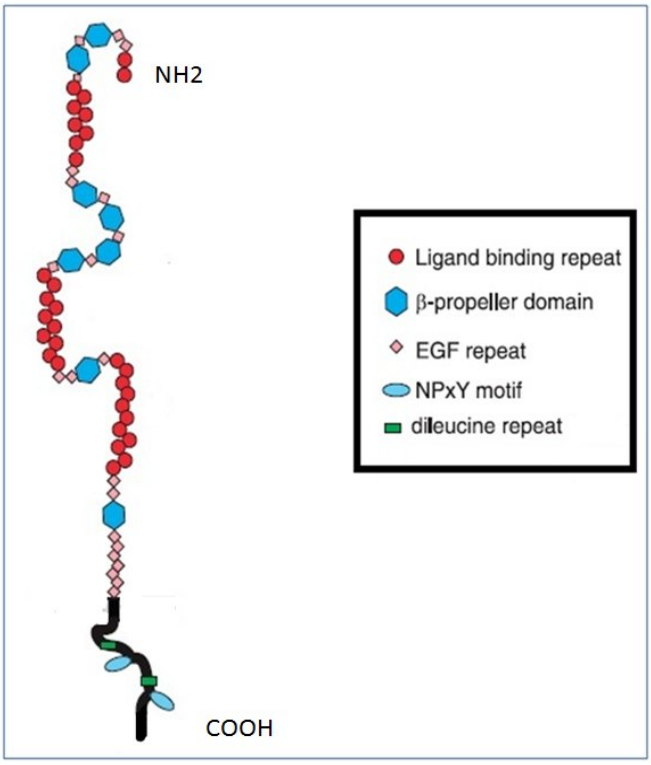

Figure 2. Structural organization of LRP1. Based on Lillis et al. 2008.

CR repeats:

CR repeats are also commonly referred to as ligand binding repeats. The folding properties of CR were first explained by NMR spectroscopic studies. (Daly et al. 1995). They described the first repeat of the LDL receptor (CR1) revealing that this module consists of a β-hairpin structure followed by a series of β-turns.

In a similar manner crystal structures of CR5 (Fass et al. 1997), CR3 (Dolmer et al. 2000), CR7 (Simonovic et al. 2001), CR5-CR6 (Jensen et al. 2006) and CR8 (Huang et al. 1999) from LRP1 and CR1-CR2 (Kurniawan et al. 2000) and CR6 (North et al. 2000) from the LDL receptor have been characterized.

LRP-1 has total of 4 clusters (I-IV), in which the CRs are localized in each cluster in variable

numbers. It has been shown in some binding experiments that the most LRP-1 ligands bind to clusters II and IV (Neels et al. 1999; Willnow et al. 1994).

EGF and β- propeller (YWTD) domains:

EGF repeats and β-propeller domains are located in LRP-1. They function as an alternate ligand for CR4 and CR5.

Transmembrane and cytoplasmic domains:

Usually LDL receptor family members possess one transmembrane and one cytoplasmic domain. The LRP-1 cytoplasmic domain consists of 100 amino acid residues and also includes 2 dileucine (LL) motifs and 2 NPXY motifs. The cytoplasmic domain interacts with numerous adaptor molecules like Protein Kinase Cα (PKCα), Postsynaptic Density Protein 95 (PSD95), M-SemF cytoplasmic domain-associated protein (SEMCAP-1), C-Jun-amino-terminal kinase-interacting protein 1 and 2 (JIP1, JIP2), engulfment adaptor protein (GULP), Talin like protein, Outer membrane Protein 25 (OMP25), Carboxyl terminal PDZ ligand of Neuronal Nitric Oxide Synthase Protein (CAPON), Phosphatidylinositol Phosphate 4,5 (PIP4,5) kinase like protein, Integrin Cytoplasmic Domain Associated Protein (ICAP1), Casitas B-Lineage (Cbl), Shc and Fe65, which all are involved in directing cellular traffic or in cell signaling events.
LRP-1 is made up of an extracellular α chain of 3924 amino acids, which is non-covalently linked to a β chain of 601 amino acids that contains an extracellular region, a single transmembrane region, a highly conserved cytoplasmic region composed of 100 amino acids including four tyrosine residues. Out of these four residues two are Tyr4473 and Tyr4507 and could possibly have Asn-Pro-X-Tyr (NPXY) motifs, which was first identified in the LDL receptor, where it is essential for clathrin-mediated internalization.

PDGF:

PDGF is very well known as a promoter/mediator of pathological cell growth, tumor development, generation of atherosclerosis, tissue fibrosis, and other conditions. It was also discovered as a growth promoter for smooth muscle cells and fibroblasts.

Structure:

It is a dimeric 30 kDa protein composed of disulphide bonded A and B polypeptide chains, which are synthesized as a precursor molecule that undergoes proteolytic processing after synthesis and dimerization. Until now 3 dimers to PDGF have been identified and purified from platelets and transformed cells. These are AA, AB and BB (Stroobant and Waterfield. 1984).

PDGFR:

The PDGFR isoforms differ in their functional properties as well as in secretary behavior. Two types of PDGFR types have been identified as α and β (Hart et al. 1988, Heldin et al. 1988). The α receptor (also called Alpha type receptor) binds to all PDGF isoforms whereas the β receptor (also called Beta receptor) binds to PDGF-BB (Beta, Beta) with higher affinity and PDGF-AB(Alpha, Beta) with lower affinity.

Relation between LRP-1 and PDGFR and role of LRP-1:

It was previously shown by Boucher et al. 2002 and Loukinova et al. 2002 that along with the PDGFR, the LRP-1 receptor works as a co-receptor in modulating signal transduction functions of the PDGFR. When PDGF binds to its receptor it induces tyrosine phosphorylation at the second NPxY motif of LRP-1 by Src and Src family kinases (Loukinova et al. 2002). This leads to the interaction of tyrosine phosphorylated LRP-1 with Shc, which promotes PDGF induced cell proliferation through Shc activated Ras signaling and mitogen activated protein kinase activation.

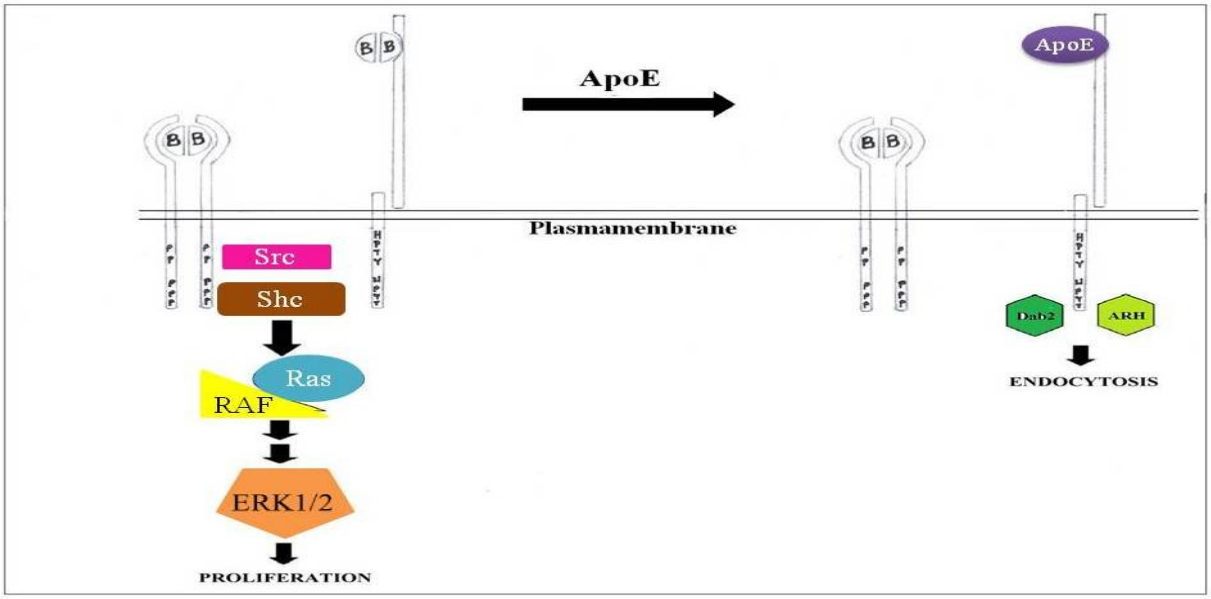

Figure 3. Picture depicting how ApoE may be involved in the dual functions of LRP-1.
Based on Herz et al. 2004.

If ApoE interacts with LRP-1, PDGF-induced tyrosine phosphorylation of LRP-1 is reduced, which could decrease signaling through Shc and promote endocytosis by interaction with other molecules like Disabled-2 (Dab2), Autosomal recessive Hypercholesterolemia (ARH) (Mishra et

al. 2002, Morris et al. 2001, He et al. 2002). Thus, we can understand that LRP-1 works like a molecular switch. The mechanism can be understood by Figure 3.

Materials and Methods:

Cell Culture: Rat INS1 832/13 cells were cultured in RPMI 1640 media (Sigma Chemicals) supplemented with 10% (v/v) fetal bovine serum (FBS)(Sigma), Penicillin, glutamine + sodium pyruvate and 50 μM ² 2-mercaptoethanol. All the cells were incubated at 5% CO_2, 37°C in a humidified air incubator.
Cells were incubated with 10 μM Imatinib and 1 μM Suntinib for 6 hours in serum free media and the cells were stimulated with 10% FBS or 100 ng/ml PDGF-BB for 5-10 minutes before immunoprecipitation.

Immunoprecipitation and Western blot: Cells were put on ice and washed with cold PBS 3 times. Cell pellets were collected and lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1/100 vol. PMSF (Sigma – Aldrich, USA) and 1/100 vol. Proteinase Inhibitor cocktail (Thermo Scientific, USA) for 30 minutes. After centrifugation the supernatant was separated into two parts, one which was used as total lysate control, and the remaining was supplemented with total LRP-1 rabbit monoclonal antibody (Abcam, UK) or a rabbit PDGFRβ antibody (kindly provided by Dr. Johan Lennartsson, LICR, Uppsala). The homogenates were kept on ice for one hour. The proteins were precipitated with Protein A Sepharose beads (G.E. Healthcare Biosciences AB, Uppsala, Sweden) and were washed with RIPA buffer 3 times and once with distilled water. SDS sample buffer (4% SDS, 20% Glycerol, 200mM Tris PH 6.8, 0.7M 2-mercaptoethanol, Bromophenol Blue) was added.

The samples were boiled for 5 to 10 minutes in a water bath and were separated by SDS-PAGE. Electrophoretically proteins were then transferred onto a polyvinyldene diflouride (PVDF) membrane (GE healthcare, UK). Blocking of membranes was done in 0.1% TBST (Tris buffered saline Tween 20) containing 2% bovine serum albumin (Sigma-Aldrich, US) and followed by incubation with primary antibodies LRP1-Rabbit monoclonal Antibody (Abcam, UK), mouse monoclonal anti-phosphotyrosine antibody 4G10 (Millipore), PDGFRβ, P-AKTSer 471 (Cell Signaling) and P-ERKTyr202Thr204 (Cell Signaling).

The membranes were mildly washed with TBST and the antigen-antibody complexes were identified by using anti-rabbit IgG, Horse radish peroxidase linked (G.E. Healthcare, UK) or anti-mouse IgG, horse radish peroxidase linked (G.E. Healthcare, UK) secondary antibodies. Immunoreactivity was detected by using Chemiluminiscent Horse radish peroxidase (Millipore Corporation, USA) substrate. The intensity of the bands was quantified using Kodak Image software. To reprobe with new antibodies, bound antibodies were removed by stripping in a

stripping solution (1M Tris pH 6.8, 0.1M 2-mercaptoethanol, 2% SDS) and subjected for shaking in a water bath for 30 min at 50°C, whereas a gentle stripping was done for only 15 minutes.

Transfection and gene silencing: Rat INS-1 832/13 cells were plated one day before transfection so that they will be 60-80% confluent at the time of transfection. On the second day transfection mixture was prepared into two groups; the control → non-specific siRNA (anti-miR negative control, Ambion, USA) and the experimental LRP-1 group → LRP-1 siRNA (Custom siRNA, Sigma-Aldrich USA). Both groups were combined with Lipofectamine reagent (Lipofectamine^{TM} 2000 Reagent, Invitrogen, USA) and incubated for 30 min at room temperature. Serum free medium was added to both the group mixtures in equal volumes. The medium from cell cultures were removed and was replaced with the freshly prepared Lipofectamine reagent mixtures. Cells were incubated for 2 hours at 5% CO_2 and 37^{o}C. Then growth medium containing 2X the normal concentration of serum was added without removing the transfection mixture and left for 2 days at culture conditions. Then serum free media was added by removing the medium from the cell cultures and again incubating the cells for 1 hour. Thereafter cells were stimulated with ApoE-Human VLDL native Protein (Genway Biotech Inc., USA) and rrPDGF-BB [recombinant rat (E.coli derived), R&D Systems, USA] for 5 minutes at 37^{0}C. The media was then immediately removed and the cells were washed with PBS. SDS sample buffer was finally added along with 1/100vol. Proteinase Inhibitor cocktail 100X (Thermoscientific USA).

The samples were collected separately into Eppendorf tubes and were boiled for 10-15 minutes in a hot water bath and were separated by SDS-PAGE. Electrophoretic transfer and blocking of membranes was done similarly as explained above.

RESULTS:

Effects of Imatinib and Sunitinib on LRP1 expression and tyrosine phosphorylation in INS-1 Cells:

To study the effects of Imatinib and Sunitinib on LRP-1 expression and tyrosine phosphorylation, INS-1 cells were treated with Imatinib and Sunitinib for 6 hours in serum free media, and then stimulated with FCS for 10 min or PDGF-BB for 5 min. LRP-1 expression was analyzed by immunoprecipitation and immunoblotting analysis. We observed strong expression of the LRP-1 protein at all conditions and LRP-1 tyrosine phosphorylation at basal conditions in INS-1 cells (Figure 4). Both serum and PDGF-BB stimulation resulted in a significant increase in LRP-1 tyrosine phosphorylation (Figure 4). This effect was completely abolished by Imatinib or Sunitinib.

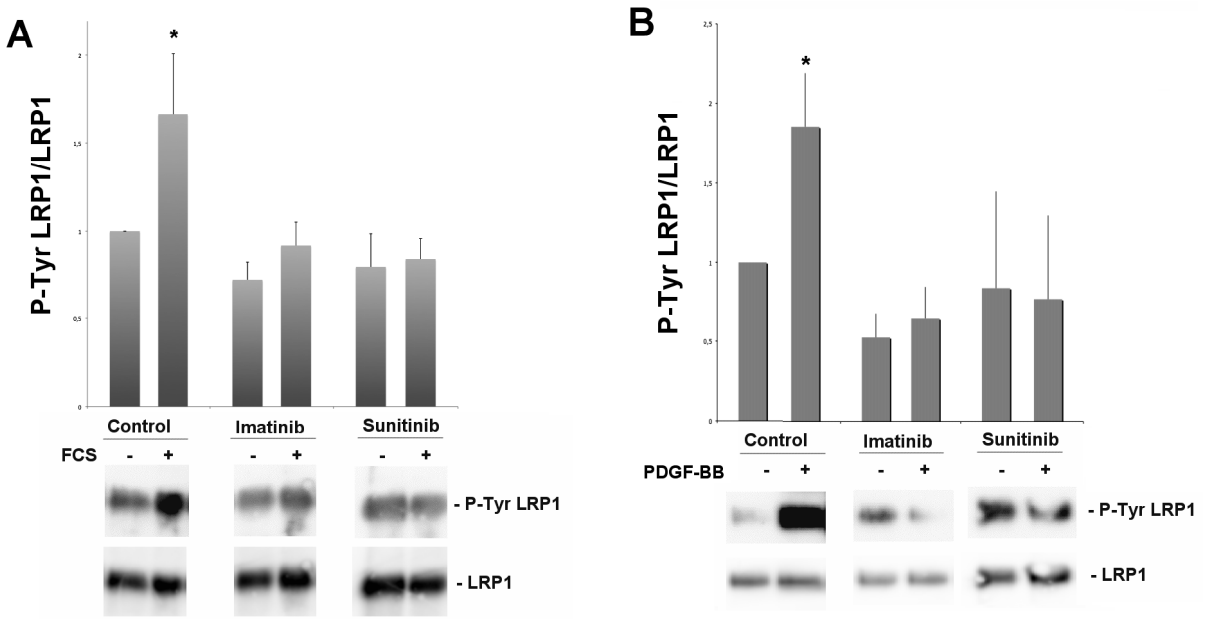

Figure 4. Effects of (FCS) (A) and PDGF-BB (B) on LRP-1 expression and tyrosine phosphorylation. INS-1 cells were treated with Imatinib (10 μM) and Sunitinib (1μM) for 6 hours, stimulated with (FCS) for 10 min in (A) and PDGF-BB for 5 min in (B). Cells were then harvested and immunoprecipitated using LRP-1 specific antibodies, later analyzed with 4G10 and total LRP-1 antibodies. Membranes were stripped in between frequent antibody incubations. Results are means ± SEM for 5 independent experiments.

Total Protein level of LRP-1:

To know whether the total LRP-1 protein was affected or not by the TKI's in every sample, I tried to calculate the average ratio of LRP-1 expressed in the experiments done. The overall level of protein was consistent with no dramatic change (Figure 5).

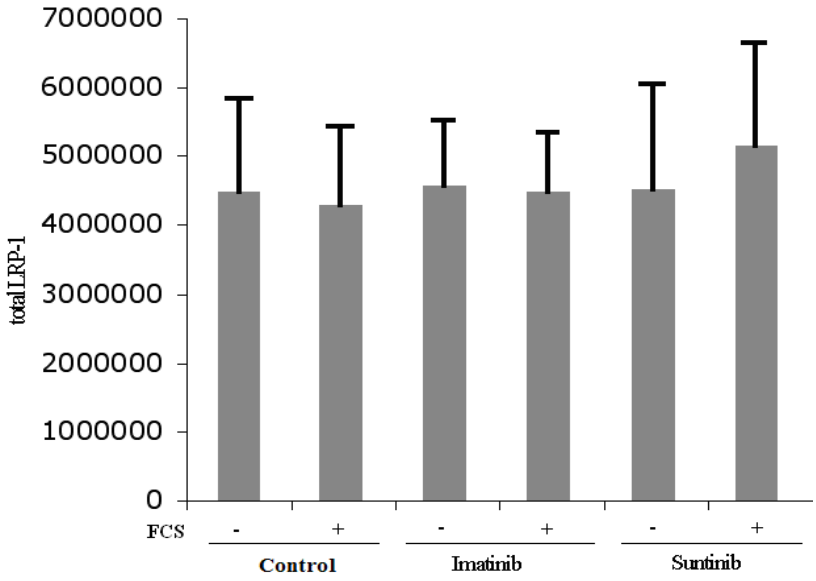

Figure 5. Effect of TKI's on total LRP-1 protein levels. INS-1 cells were treated with Imatinib (10 μM) and Sunitinib (1μM) for 6 hours, stimulated with (FCS) for 10 min. Cells were then harvested and immunoprecipitated using LRP-1 specific antibodies, later analyzed with 4G10 and total LRP-1 antibodies. Membranes were stripped in between frequent antibody incubations. Results are means ± SEM for 6 independent experiments.

Effects of Imatinib and Sunitinib on LRP-1 and PDGFR co immunoprecipitation:

Having observed that LRP-1 is expressed in insulin producing cells and that FCS and PDGF-BB-induced phosphorylation of LRP-1 is blocked by Imatinib and Sunitinib, we next studied whether LRP-1 and PDGFR co-immunoprecipitate and whether this event is affected by PDGF-BB stimulation or Imatinib/Sunitinib supplementation, INS-1 cells were treated with Imatinib and Sunitinib for 6 hours. After a 5 minute PDGF-BB stimulation LRP-1 and PDGFR were immunoprecipitated separately and analyzed by immunoblotting. The results showed that LRP-1 immunoprecipitation resulted in PDGFR co-immunoprecipitation (Figure 6A), and vice versa (Figure 6B). PDGF-BB, Imatinib or Sunitinib supplementation did not, however, consistently affect the extent of co-immunoprecipitation.

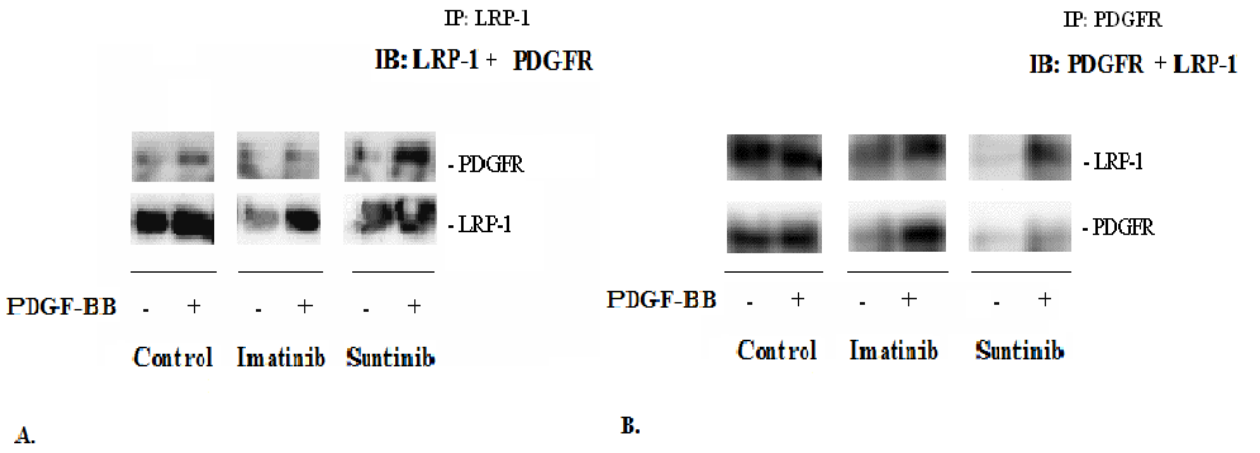

Figure 6. Effects of Imatinib and Sunitinb on co-immunoprecipitation of LRP-1 and PDGFR. INS-1 cells were either treated or untreated with Imatinib (10 μM) and Sunitinib (1 μM) for 6 hours, stimulated with PDGF-BB for 5 minutes, harvested and immunoprecipitated either with total LRP-1 antibody (A) and PDGFR antibody (B). The precipitates were then separated by SDS-PAGE, transferred onto PVDF membranes and analyzed for total LRP-1 and PDGFR.

Effects of LRP-1 down regulation on PDGF-BB and ApoE-induced ERK and Akt phosphorylation.

We next studied down-stream signaling events to the PDGFR and LRP-1 by downregulating LRP-1. To study the effects of LRP-1 downregulation on the phosphorylation of ERK and Akt, LRP-1 gene was silenced by siRNA and ERK and Akt phosphorylation in response to PDGF-BB and ApoE was studied by immunoblot analysis. INS-1 cells were stimulated with PDGF-BB or ApoE for 5 minutes two days after the siRNA treatment. At this time point LRP-1 protein levels were decreased by 35% in cells treated with LRP-1 siRNA (Figure 7).

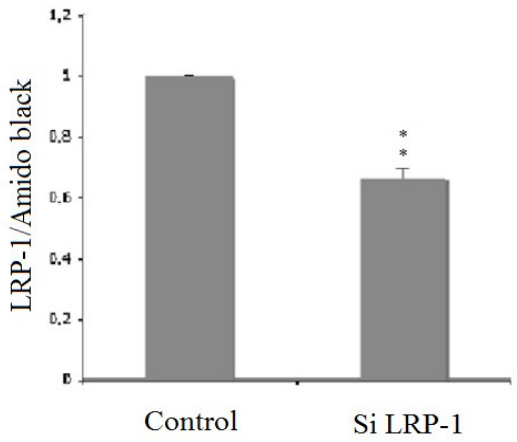

Figure 7. Effect of LRP-1 siRNA on LRP-1 expression. INS-1 cells were treated with Lipofectamine 2000 in serum free and antibiotic free medium for 2 hours at 5% CO_2, 37^o C, combined either with LRP-1 siRNA (10 ng/ml) or non-target siRNA (10 ng/ml). Samples were then harvested and separated by SDS-PAGE and then transferred onto a PVDF membrane for analysis with total LRP-1 antibodies.

Furthermore, in control cells we observed increased ERK phosphorylation in response to a combination of PDGF-BB and ApoE (Figure 8). PDGF-BB and ApoE by themselves tended to increased P-ERK in control cells, but this did not reach statistical significance. When analyzing LRP-1 siRNA treated cells, we observed a lowered ERK phosphorylation at basal conditions, after treatment with PDGF-BB or with ApoE (Figure 8). In LRP-1 siRNA treated cells the combination of PDGF-BB and ApoE did not stimulate ERK phosphorylation. Thus, LRP-1 is required for both basal and PDGF-BB + ApoE-induced ERK phosphorylation.

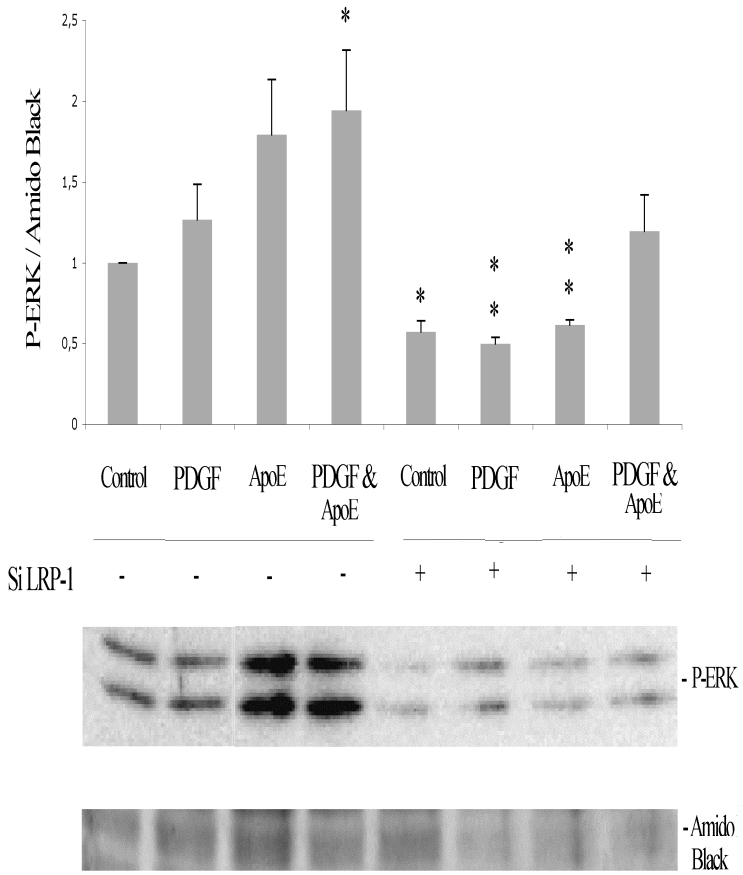

Figure 8. Effects of LRP-1 siRNA on the phosphorylation of ERK: INS-1 cells were treated with siRNA specific for LRP-1 for 2 hours. 2 x Serum media was added and cells were incubated for 48 hours, stimulated with PDGF-BB, ApoE, PDGF-BB + ApoE for 5 minutes. Cells were harvested and separated by SDS-PAGE and transferred onto a PVDF membrane for immunoblot analysis.

PDGFR/LRP-1 signaling is known to affect not only ERK, but also Akt. Using the same setup as with the ERK-experiments above, we observed that the phosphorylation of Akt was increased by ApoE and the combination of ApoE + PDGF-BB (Figure 9). PDGF-BB alone tended to increased Akt phosphorylation, but the effect was not statistically significant. In LRP-1 deficient cells Akt phosphorylation was decreased at basal conditions (Figure 9). Also the response to PDGF-BB and ApoE was attenuated in cells treated with LRP-1 siRNA. Somewhat surprisingly, Akt phosphorylation was very variable when LRP-1 deficient cells were treated with both PDGF-BB and ApoE (Figure 9).

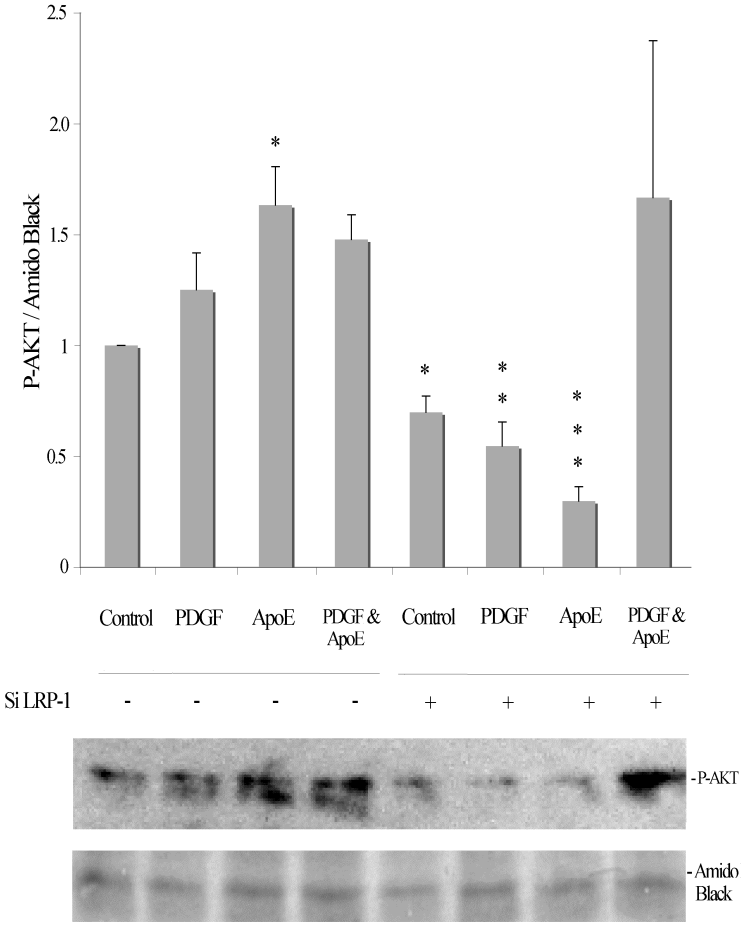

Figure 9. Effects of LRP-1 siRNA on the phosphorylation of Akt: INS-1 cells were treated with siRNA specific for LRP-1 for 2 hours. 2 x serum media was added and cells were incubated for 48 hours, stimulated with PDGF-BB, ApoE, PDGF-BB + ApoE for 5 minutes. Cells were harvested and separated by SDS-PAGE and transferred onto a PVDF membrane for immunoblot analysis.

DISCUSSION:

PDGFR and its co receptor LRP-1 were considered as the main factors for Imatinib and Sunitinib-induced protection against β-cell death.

It has been shown that LRP-1 is involved in signaling pathways. LRP-1 is known to regulate the PDGFR pathway (Herz et al. 2005). The disruption of LRP-1 expression in vascular smooth muscle cells in the mouse resulted in the elevation of PDGFRβ expression, leading to the

development of atherosclerotic lesions and destruction of elastic layer in large arteries (Boucher et al. 2003). So, it could be that LRP-1 is involved in the signaling events and LRP-1 and PDGFRβ together control the β-cells. But the mechanism of regulation is not yet completely understood. In order to study this, INS-1 cells were first studied to ensure that the LRP-1 receptor is expressed and tyrosine phosphorylated. LRP-1 tyrosine phosphorylation may indicate interaction with the PDGFR. Treatments with tyrosine kinase inhibitors Imatinib and Sunitinib were done in order to check the impact on LRP-1 phosphorylation in INS-1 cells when stimulated with PDGF-BB, and I observed a decrease by 50% with Imatinib and 45% with Sunitinb (Figure 4). By this we show that the PDGF-BB/LRP-1 interaction is involved in β-cell signaling pathways.

Immunoprecipitation is the technique which is widely used for the purification of a specific protein of interest with specific antibody. Coimmunoprecipitation experiments helped us to understand that the PDGFR and LRP-1 are part of the same protein complex, which further supports an interaction between these proteins in insulin producing cells.

PDGFR is a unique growth factor receptor, which has the capacity to promote tyrosine phosphorylation of LRP-1 (Boucher et al. 2002; Loukiniva et al. 2002). When the INS-1 cells were treated with the growth factor PDGF-BB, it induces dimerization of either PDGF α or β receptors, which subsequently activates their tyrosine kinase activity (Claesson-Welsh et al. 1994). PDGF-β receptor down-regulation was clearly observed in LRP-1 deficient fibroblasts when stimulated with PDGF-BB (10ng/ml) (Herz et al. 2005). In the same manner reduced activity of PDGFR was observed when LRP-1 gene was knocked down even when stimulated with PDGF-BB in INS-1 cells.

ApoE is a ligand for LRP-1. ApoE is an autocrine/paracrine factor regulating neural stem cell (NSCs) survival via the MAPK/ERK signaling pathway (Ahmed et al. 2011), it protects neurons from apoptosis (Vance et al. 2009) and a it is key player in preventing atherosclerosis. In my results I could observe that P-ERK and P-AKT levels in ApoE and PDGF/ApoE stimulated samples were down- regulated significantly when compared to the control (Figure 8, 9). ApoE could stimulate the AKT phosphorylation in a calcium and cAMP- dependent manner as in neuronal cells (Taniguchi et al. 2002). In a similar manner we could also observe increase in the levels of P-ERK in ApoE samples (Figure 8). The LRP-1 deletion leads to impairment of the AKT survival pathway in macrophages resulting in apoptosis (Yancey et al. 2010). The same could be observed for P-AKT in the LRP-1 gene silenced samples.

By using these interesting results, the effects of LRP-1 knockdown in human islet cells could be studied and compared to Imatinib on Akt and ERK phosphorylation, insulin production and viability. Signaling levels of Insulin can also be studied by inducing Insulin resistance simultaneously by LRP-1 knockdown or activation or by treating with Imatinib.

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