Imatinib stimulates PIP₃-signaling in insulin producing cells:

Role of Src homology 2-containing inositol 5'-phosphatase interaction with c-Abl

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Abstract

It is not clear how small tyrosine kinase inhibitors, such as imatinib mesylate, protect against diabetes and beta-cell death. The aim of this study was to determine whether imatinib, as compared to the non-cAbl-inhibitor sunitinib, affects pro-survival signaling events belonging to the PI3 Kinase pathway. EndoC-bH1 cells, beta-TC-6 cells and human pancreatic islets were used for immunoblot analysis of IRS-1, Akt, ERK and beta-catenin phosphorylation. PI(3,4,5)P3 plasma membrane concentrations were assessed in endoC-bH1 and MIN6 cells using evanescent wave microscopy. SHIP2 tyrosine phosphorylation, PTEN serine phosphorylation, as well as c-Abl co-localization with SHIP2, were studied by immunoprecipitation and the proximity ligation assay. Gene expression was assessed using RT-PCR. Cell viability was measured using vital staining. Imatinib stimulated ERK(thr202/tyr204) phosphorylation in a c-Abl dependent manner. Imatinib, but not sunitinib, stimulated also IRS-1( tyr612), Akt(ser473) and Akt(thr308) phosphorylation. This effect was paralleled by oscillatory bursts in plasma membrane PI(3,4,5)P3 levels. The wortmannin-induced decrease in PI(3,4,5)P3 levels was slower in Imatinib treated cells as compared to control cells, indicating an effect on PI(3,4,5)P3 degrading enzymes. In line with this, Imatinib decreased the phosphorylation of SHIP2, but not of PTEN. C-Abl co-immunoprecipitated with SHIP2 and its binding to SHIP2 was largely reduced by imatinib, but not by sunitinib. Imatinib increased total beta-catenin levels. This was paralleled by an augmented CREM mRNA level. Imatinib increased beta-TC-6 viability in the presence of cytokines (IL-1beta+IFN-gamma) and the nitric oxide donor DETA/NO, whereas sunitinib exerted negative effects on cell viability. Our results are compatible with the view that imatinib, through inhibition of c-Abl, decreases SHIP2 activity, and that this results in enhanced PI3 kinase signaling.
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Introduction

Imatinib mesylate is a 2-phenylaminopyrimidine-based ATP-competitive inhibitor of the Abl tyrosine kinase family (1). Imatinib also inhibits the platelet-derived growth factor receptor (PDGFR), the transmembrane stem-cell factor receptor (c-Kit) and discoidin domain receptor 1 (DDR1). It is currently used to treat chronic myeloid leukemia, a disease caused by Bcr-Abl oncogene, and gastrointestinal stromal tumors that result from c-Kit mutations (2). Besides its pioneering role in oncology, imatinib has also been observed to counteract diabetes in patients (3-9) and in animal models for both Type 1 and Type 2 diabetes (10-15). Firstly, without affecting the glucose metabolism of control subjects, imatinib improves insulin sensitivity in patients and animals with insulin resistance or Type 2 diabetes (5,8,10). Secondly, imatinib enhances beta-cell survival in response to toxins and pro-inflammatory cytokines, possibly via inhibition of the pro-apoptotic tyrosine kinase c-Abl (13,14). Thirdly, imatinib probably modulates the immune system of non-obese diabetic (NOD) mice so that beta-cells are better tolerated and diabetes is both prevented and reversed (13,15). This third pathway may include imatinib-mediated inactivation of c-Kit and DDR1 (16,17).

Sunitinib is a multi-targeted receptor tyrosine kinase inhibitor, which inhibits PDGFR\(\alpha\) and PDGFR\(\beta\), c-Kit, VEGF and other tyrosine kinases (18). Similarly to imatinib, sunitinib counteracts Type 2 diabetes (7,19) and diabetes in NOD mice (15), possibly via inhibition of the PDGFR leading to lowered insulin resistance and/or modulation of the immune system (15). The effects of sunitinib on beta-cell signaling and apoptosis rates have, however, not been studied.

Insulin signaling is required for beta-cell survival, proliferation and function (20). Insulin promotes insulin receptor autophosphorylation and insulin receptor substrate (IRS) tyrosine phosphorylation. This is followed by IRS-mediated activation of phosphatidylinositol 3-kinase (PI3K) and the synthesis of phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P\(_3\)). PI(3,4,5)P\(_3\) recruits and activates the phosphoinositide-dependent kinase 1 (PDK1) and Akt/PKB, which initiates increased glucose uptake and GLUT4 translocation in fat and skeletal muscle, and proliferation, survival and improved insulin production in beta-cells (20). However, PI3K-induced signaling is antagonized by the phosphatases phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and the Src homology 2-containing inositol 5'-phosphatase 2 (SHIP2) (21,22). PTEN removes the 3'-phosphate
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from PI(3,4,5)P3 and PI(3,4)P2 thereby generating PI(4,5)P2 and PI(4)P. As a 5'-phosphatase, SHIP2 dephosphorylates PI(3,4,5)P3 into PI(3,4)P2. Enhanced activities of PTEN and SHIP2 will inhibit insulin signaling and probably accelerate the development of Type 2 diabetes. Indeed, it has been demonstrated that SHIP2+/− mice display improved insulin sensitivity and glucose tolerance, which was due to increased glucose uptake and glycogen synthesis in skeletal muscles (23). Furthermore, increased PTEN expression was found in islets of high fat diet fed and db/db mice, and deletion of PTEN in pancreatic β-cells was beneficial for maintaining β-cell mass, function and PI3K signaling (24).

Akt controls β-catenin levels and activity by phosphorylating and inhibiting glycogen synthase kinase 3 (GSK3) (25). When non-phosphorylated by Akt, GSK3 phosphorylates β-catenin at positions ser33, ser37 and thr41, which leads to β-catenin ubiquitination and degradation. Thus, Akt activation is known to be associated with increased β-catenin stability and activity. In addition, β-catenin can be phosphorylated at other sites than ser33, ser37 and thr41, in which case nuclear translocation may occur. For example, PKA-induced phosphorylation of β-catenin at ser675 induces β-catenin accumulation in the nucleus (26). β-catenin is found both in adherens junctions, where it binds cadherins and establishes a link to the cytoskeleton, and in nucleus, where it binds T cell factor and lymphoid enhancer factor transcription factors and promotes transcription of pro-survival genes. In β-cells, it has been shown that Akt activation results in increased β-catenin activity (27), that β-catenin activity protects against cytokine- and thapsigargin-induced cell death (28) and that lack of β-catenin in early life is related to severe deregulation of glucose homeostasis (29).

In cells other than β-cells imatinib has been observed to affect insulin signaling, probably via inhibition of c-Abl (30). Thus, it is possible that imatinib promotes β-cell survival and improved function via modulation of IRS-1-, PI3K-, Akt-, ERK- and β-catenin signaling, at basal conditions, during stimulation with insulin or under stressful conditions. The aim of this study was therefore to compare the effects of the c-Abl inhibitor imatinib with those of the non-c-Abl inhibitor sunitinib on β-cell signaling events, and to correlate signaling events to β-cell survival.
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Materials and Methods

Materials

Imatinib mesylate was generously provided by Novartis. Sunitinib was from LC Laboratories. Diazoxide, insulin, and LY294002 were from Sigma. Lipofectamine 2000 was obtained from Invitrogen. Dipotassium Bisperoxo(5-hydroxypyridine-2-carboxyl)oxovanadate (V) (bpV(HOpic)) and diethylenetriamine NONOate (DETA/NO) were from Cayman Chemicals.

Cell culture

Human EndoC-bH1 cells were cultured in ECM/fibronectin-coated plates in low-glucose DMEM with supplements as previously described (ref). Murine beta-TC-6 cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% (vol/vol) fetal bovine serum (FBS, Sigma-Aldrich), 2 mM L-glutamine, benzylenicillin (100 U/ml), and streptomycin (0.1 mg/ml) (WS). HEK293T cells were grown in DMEM medium (Gibco) with 10% (vol/vol) FBS (Sigma-Aldrich), 2 mM L-glutamine, benzylenicillin (100 U/ml) and streptomycin (0.1 mg/ml). For MIN6 cell culture, 50 μM β-mercaptoethanol was added to the media. Human pancreatic islets were cultured in CMRL1066 media containing the same supplements as WS.

For knock-down of c-Abl, EndoC-bH1 cells were incubated over-night in Opti-MEM (Gibco) with Lipofectamine 2000 complexed with either control siRNA (Sigma) or c-Abl specific siRNA (30 nM) (Sigma) according to the instructions of the manufacturer. Liposome/siRNA complexes were removed the next day by a medium change. Cells were analyzed by immunoblotting two days after start of the transfection procedure.

Immunoblotting

Cells were washed in ice-cold phosphate buffer saline (PBS), lysed in SDS sample buffer, boiled for 5 min and separated by SDS-PAGE. Proteins were electrophoretically transferred onto a Hybond-P membrane (GE Healthcare). Membranes were incubated with the following primary antibodies: 4G10 anti-phosphotyrosine and IRS-1(tyr612) antibodies (Millipore), Phospho-beta-catenin(Ser675), anti-phospho-PTEN(Ser380), SHIP2, phospho-ERK(thr202/tyr204), phospho-Akt(thr308) and
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phospho-Akt(ser473) antibodies (Cell Signaling Technology), total-ERK antibody (Santa Cruz), total beta-catenin antibody (Abcam) and PTEN and c-Abl antibodies (Oncogene and Santa Cruz). Bound antibodies were removed from filters by incubating for 40 min at 55°C in 2% (wt/vol) SDS, 100 mM Tris, pH 6.8 and 0.5 M β-mercaptoethanol. The immunodetection was performed as described for the ECL immunoblotting detection system (GE healthcare) and using the Kodak Image station 4000MM. The intensities of the bands were quantified by densitometric scanning using Kodak Digital Science ID software (Eastman Kodak, Rochester, NY).

Immunoprecipitation

After treatment with drugs, cells were washed with cold phosphate buffered saline (PBS) three times, scraped and centrifuged. The cell pellets were collected and lysed in RIPA buffer supplemented with 1 mM PMSF and Halt™ protease and phosphatase inhibitor cocktail for half an hour. After centrifugation, supernatants were supplemented with PTEN (A2B1, Santa Cruz) or SHIP2 (C76A7, Cell Signaling Technology) antibodies and kept on ice for one hour. PTEN and SHIP2 proteins were then precipitated with Protein G- or Protein A sepharose. The samples were boiled for 5 min in SDS-sample buffer and separated by SDS-PAGE.

Plasma membrane PI(3,4,5)P$_3$ determinations

Human general receptor for phosphoinositides 1 fused to a tandem construct with 4 molecules of GFP (GFP$_4$-Grp1) was used as a translocation biosensor for the plasma membrane PI(3,4,5)P$_3$ concentration (31). MIN6 cells (passage 17–30) were seeded onto poly-L-lysine-coated 25-mm glass coverslips to achieve 50% confluency on the day of transfection. Transient transfection was performed with 2 mg of plasmid DNA and Lipofectamine 2000 in a ratio of 1:2.5 in 1 ml of DMEM during 4h. Prior to experiments, the cells were transferred to a buffer containing 125 mM NaCl, 5.9 mM KCl, 1.28 mM CaCl$_2$, 1.2 mM MgCl$_2$, and 25 mM HEPES (pH 7.40) and incubated for 45 min at 37 °C. The plasma membrane concentration of GFP$_4$-Grp1 was recorded with an evanescent wave microscopy setup built around an E600FN microscope (Nikon) with a 40X, 0.8-NA objective as previously described (32). Excitation light of 488 nm was provided by an argon laser and the GFP
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fluorescence detected at 525/25 nm (center wavelength/half-bandwidth nm) by an Orca-ER camera (Hamamatsu) under MetaFluor software control (Molecular Devices). Images were acquired by 50–100-ms exposure every 5 s.

RNA Isolation and cDNA synthesis

The total RNA was extracted using the Ultraspec™ RNA isolation system reagent (Biotecx Laboratories, US) according to the instruction of the supplier. cDNA was synthesized using SuperScript™ III Reverse Transcriptase kit (Invitrogen, US) and oligo-dT-primers according to manufacturer’s protocol.

Real-time RT-PCR

Semi quantitative real time-PCR was performed using the Lightcycler instrument (Roche) and the SYBR® Green JumpStart™ Taq Ready Mix™ (Sigma-Aldrich). The following primers (Eurofins MWG) were used: G6PDH: forward 5´-ATTGACCACCTACCTGGGCAA-3´, reverse 5´-GAGATACAATTCAACACTTGTGACCT-3´. CAMP responsive element modulator (CREM): forward 5´-ACAGTTCCCAGTCTCTCTGTCATGAAT-3´, reverse 5´-ACGTCATGTCATCAGGATACACTTCCT-3´. Thioredoxin interacting protein (TxnIP): forward 5´-CCTAGAAGAGCAGCCTACAGGTGA-3´, reverse 5´-AGTTGGCTGGCTGGGGCGAT-3´. Relative CREM and TxnIP expression was calculated using the formula: 2^(-ΔΔCt) or 2^(-crossing pointG6PDH - crossing point CREM,TxnIP).

Evaluation of cell viability

Beta-TC-6 cells were cultured in 96-well plates and were treated with imatinib (10 μM) or sunitinib (1 μM and 10 mM) for 6 hours, and then were incubated with the cell death agents IL-1β (50 U/ml), IFN-γ (1000 U/ml), DETA/NO (1 mM), hydrogen peroxide (0.1 mM) for 24 hours. Cell viability was measured by staining cells with propidium iodide (20 μg/ml) and bisbenzimide (5 μg/ml) for 10 min at 37°C. The medium was replaced with PBS and the red and blue fluorescence was detected using the Kodak 4000 MM image station. The ratio of red to blue was taken as a relative
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measure of cell death (necrosis and late apoptosis) and was quantified using Kodak Digital Science ID software (Eastman Kodak, Rochester, NY).

RESULTS

Effects of imatinib and sunitinib on Akt, ERK and IRS-1 phosphorylation

Imatinib has been reported to affect insulin receptor-signaling in non-beta-cells (30). Phosphorylation of IRS-1 on tyr612 serves as a docking signal to SH2-domains and can be considered as a marker for receptor tyrosine kinase-induced IRS-1 activation (33). Following insulin or IGF-1-induced IRS-1 activation, both Akt(thr308/ser473) and ERK(thr202/tyr204) become phosphorylated as down-stream signaling events (34,35). Therefore, to establish whether Imatinib controls receptor tyrosine kinase signaling also in beta-cells, we analyzed the effects of Imatinib on ERK phosphorylation in human EndoC-bH1 cells. We observed that a 20 min exposure to 10 μM of Imatinib increased ERK phosphorylation, and that the increase was even stronger after 3 hours at both 2 and 10 μM of the drug (Figure 1). Treatment of cells with c-Abl specific siRNA resulted in a 59% decrease in c-Abl protein levels (Figure 1). In c-Abl knock down cells ERK phosphorylation was elevated at basal conditions, and no increase in response to Imatinib could be observed (Figure 1). We also probed for Akt and IRS-1 phosphorylation in EndoC-bH1 cells, but were unsuccessful in obtaining quantifiablesignals. Instead, we observed clear phospho-Akt and phosho-IRS-1 signals when using beta-TC-6 cells. We therefore analyzed the effects of imatinib, sunitinib and the non-specific PTEN inhibitor bpV(HOpic) on IRS-1(tyr612), Akt(thr308), Akt(ser473) and ERK(thr202/tyr204) phosphorylation in this murine beta-cell line. At basal conditions imatinib enhanced IRS-1(tyr612), Akt(thr308), Akt(ser473) and ERK(thr202/tyr204) phosphorylation (Fig. 2A-D). Sunitinib stimulated only the phosphorylation of ERK(thr202/tyr204) at basal conditions (Fig. 2C). The nitric oxide donor DETA/NO decreased Akt(ser473) and ERK(thr202/tyr204) phosphorylation (Fig. 2A+C). Imatinib and sunitinib attenuated the decrease of ERK(thr202/tyr204) phosphorylation during nitrosative stress (Fig. 2C). Insulin increased moderately Akt(ser473), Akt(thr308), ERK and IRS-1 phosphorylation. Imatinib and sunitinib enhanced Akt(ser473) and
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ERK(thr202/tyr204) phosphorylation in the presence of exogenous insulin (Fig. 2A+C). BpV(HOpic) increased Akt(ser473) phosphorylation in insulin-stimulated cells, but did not affect ERK or IRS-1 phosphorylation (Fig. 2A-D).

Similarly to betaTC-6 cells, human islets responded to imatinib with increased Akt(ser473), ERK(thr202/tyr204) and IRS-1( tyr612) phosphorylation (Fig. 3). When combined with insulin, imatinib did not further increase Akt, ERK or IRS-1 phosphorylation (Fig. 3).

**Effects of imatinib and sunitinib on plasma membrane PI(3,4,5)P3 levels**

Higher plasma membrane PI(3,4,5)P3 levels usually parallel increased IRS-1 and Akt phosphorylation rates. To monitor EndoC-bH1 and MIN6 plasma membrane PI(3,4,5)P3 concentration changes in real time we used evanescent wave microscopy and a green fluorescent protein-tagged PI(3,4,5)P3-binding protein domain. In unstimulated MIN6 cells exposed to 3 mM glucose the PTEN inhibitor bpV(HOpic) induced a gradually increasing level of PI(3,4,5)P3, which was rapidly reversed by the PI3K inhibitor LY294002 (Fig. 3A). Imatinib evoked oscillatory increases in the PI(3,4,5)P3 levels that appeared with an interval of 3-4 minutes (Fig. 3B). These bursts were of high amplitude and were observed in a majority of the analyzed cells (29 out of 48 individual cells in three independent experiments). The PI(3,4,5)P3 oscillations were not affected by the ATP-sensitive potassium channel opener diazoxide (Fig. 3B). Sunitinib did not affect PI(3,4,5)P3 levels, but addition of insulin promoted an intermediate increase in PI(3,4,5)P3 when added after the sunitinib exposure period (Fig. 3C).

**Effects of imatinib and sunitinib on SHIP2 tyrosine phosphorylation in human embryonic kidney 293T cells**

An increased plasma membrane concentration of PI(3,4,5)P3 may result, in part, from a lowered SHIP2 activity. Tyrosine phosphorylation has been demonstrated to increase SHIP2 enzymatic activity (36). We presently used HEK293T cells to study the effects of imatinib and sunitinib on SHIP2 tyrosine phosphorylation because a commercially available antibody that successfully precipitates SHIP2 is only available for human cells, and because no human beta-cell line has been
generated that is suitable for immunoprecipitation experiments. SHIP2 was successfully precipitated from the HEK293T cells and neither imatinib nor sunitinib affected levels of precipitated SHIP2 (Fig. 4A). However, when probing the membranes with the 4G10-antibody, a 60% decrease in SHIP2 tyrosine phosphorylation was observed in response to a 20 min imatinib exposure. In contrast, sunitinib did not affect the tyrosine phosphorylation of SHIP2 (Fig. 4A).

Effects of imatinib and sunitinib on c-Abl co-immunoprecipitation with SHIP2

Because the c-Abl inhibitor imatinib, but not the non-c-Abl inhibitor sunitinib, affected the tyrosine phosphorylation of SHIP2, we next analyzed whether c-Abl co-immunoprecipitates with SHIP2. For this purpose, SHIP2 was immunoprecipitated from HEK293T cells exposed to imatinib and sunitinib for different time periods. We observed that c-Abl co-immunoprecipitated with SHIP2 and its binding to SHIP2 was largely reduced by imatinib treatment for 20 min (Fig. 4B). However, no obvious effect of sunitinib on c-Abl binding to SHIP2 was found (Fig. 4B). We also attempted to study SHIP2 co-immunoprecipitation with c-Abl, but we were unsuccessful in precipitating c-Abl from both HEK293T cells and rodent beta-cell lines. Nevertheless, the imatinib-sensitive interaction between SHIP2 and c-Abl indicates that SHIP2 might be tyrosine phosphorylated directly by c-Abl and that this interaction requires c-Abl tyrosine kinase activity. This prompted us to look for putative tyrosine phosphorylation sites for c-Abl in SHIP2. Using the KinasePhos 2.0 program (37) three sites for c-Abl with high SVM scores in human SHIP2 were found: position 102 (sequence LIGLYAQPN, score 0.54361); position 610 (sequence GDLNYRLDM, score 0.608056); position 777 (sequence CLEEYKKSF, score 0.5). Among these position 102 may be particularly important because it is located in the SH2 domain of the SHIP2 protein, and it has been reported that the SH2-domain exerts an auto-inhibitory function when not phosphorylated on tyrosine residues (36). We could not observe c-Abl co-immunoprecipitation with PTEN (results not shown).

Effects of imatinib and sunitinib on ser380-phosphorylation of PTEN in beta-TC-6

PI(3,4,5)P3 levels also depend on the activity of PTEN. Phosphorylation at position ser380 is known to affect stability and activity of PTEN (38). To study the effects of imatinib and sunitinib on
serine phosphorylation of PTEN, beta-TC-6 cells were treated with imatinib or sunitinib and PTEN phosphorylation was analyzed by immunoprecipitation and immunoblotting. PTEN was successfully immunoprecipitated and the levels of the protein were not affected by imatinib or sunitinib (Fig. 4C). Ser380 phosphorylation of PTEN was not affected by imatinib (Fig. 4C). Sunitinib, however, tended to increase ser380 phosphorylation after 20 min, but the effect did not reach statistical significance.

**Imatinib increased total beta-catenin protein levels and CREM mRNA**

Having observed that imatinib, but not sunitinib, activated PI3K-signaling, we next investigated whether the Akt down-stream target beta-catenin is affected by imatinib. Imatinib treatment for 1 and 6 hours increased the levels of total beta-catenin in MIN6 cells cultured with (results not shown) or without serum (Fig. 5A). Although insulin treatment did not affect beta-catenin levels significantly, there was a trend to increased beta-catenin levels in cells stimulated with insulin for 15 min (Fig. 5A). Insulin did not further increase beta-catenin levels in cells exposed to imatinib (Fig. 5A). Imatinib or insulin treatment did not induce ser675 phosphorylation at any of the culture conditions (Fig. 5A). Instead, there was a non-significant trend to decreased P-ser675-beta-catenin levels in response to imatinib (1h and 6h) when expressed per total beta-catenin (results not shown). To corroborate the finding that imatinib augments beta-catenin in immunoblot analysis, we also stained MIN6 cells for beta-catenin immunofluorescence. Beta-catenin specific immunofluorescence was observed in the cytoplasm/plasma membrane of control cells (results not shown). Cells exposed to glucagon-like peptide-1 (GLP-1), a known activator of beta-catenin signaling, or imatinib for 6 hours displayed stronger beta-catenin immunofluorescence, and in some cells a nuclear signal could be observed (results not shown).

The finding that imatinib increased total beta-catenin levels in insulin-producing MIN6 cells prompted us to determine whether this was the case also in human islet cells. No significant effect could be observed on ser675 phosphorylation (Fig. 5B). However, islets treated with imatinib for 6 hours contained more beta-catenin (58%) than untreated control islets (Fig. 5B). A short-term exposure (1h) did not affect total beta-catenin expression (Fig. 5B). These results indicate that imatinib stabilizes beta-catenin without affecting ser675 phosphorylation.
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To further explore whether imatinib stimulates beta-catenin signaling, mRNA levels of cAMP responsive element modulator (CREM) and Thioredoxin interacting protein (TxnIP) were measured. It has been reported that GLP-1 increases CREM and decreases TxnIP mRNA levels in insulin-producing cells (39). In line with this, we presently observed that GLP-1 treatment (6 h) induced CREM mRNA expression in MIN6 cells (Fig. 6). Moreover, imatinib treatment (6 h) also induced CREM mRNA expression (Fig. 6), whereas insulin treatment (6 h) only evoked a trend to increased CREM mRNA levels. No effect of GLP-1, imatinib or insulin was observed on TxnIP mRNA levels (Fig. 6).

Effects of imatinib and sunitinib on beta-TC-6 cell viability in response to hydrogen peroxide, cytokines and DETA/NO

Lastly, we wanted to correlate the effects of imatinib and sunitinib on signaling events with cell viability. For this purpose, beta-TC-6 cells were pretreated with imatinib or sunitinib for 6 hours and then incubated with hydrogen peroxide, cytokines (IL-1beta and IFN-gamma) and the nitric oxide donor DETA/NO for an additional 24 hours. Cytokines, DETA/NO and hydrogen peroxide enhanced cell death rates by 30%, 60% and 80%, respectively (Fig. 7). Imatinib increased survival at basal conditions by 26% and protected beta-TC-6 cells against cytokines and DETA/NO to a similar degree (Fig. 7). Sunitinib did not protect against cell death. Instead, it tended to increase cell death at a concentration of 1 mM (Fig. 7). At 10 mM, sunitinib dramatically increased beta-TC-6 cell death by itself (Fig. 7).

Discussion

We have presently observed that imatinib increases the activity of the IRS-1-, PI3K-, Akt- and beta-catenin-signaling pathway, and that this was paralleled with an improved survival at basal conditions and in response to cytokines and the nitric oxide donor DETA/NO. In other cell types than insulin producing cells c-Abl has been demonstrated to negatively regulate receptor tyrosine kinase signaling. Both trk A, the receptor for nerve growth factor, and Met, the receptor for hepatocyte growth factor,
associate with c-Abl, and this event leads to attenuated receptor signaling (40-43). Since imatinib, but not sunitinib, enhanced IRS-1 phosphorylation in insulin producing cells, it is likely that also in beta-cells, c-Abl dampens receptor tyrosine kinase signaling at basal conditions. The putative receptor tyrosine kinases that are negatively affected by c-Abl in beta-cells remain unidentified, but we speculate that the insulin-like growth factor-1 receptor and/or the insulin receptor are stimulated in response to imatinib-induced c-Abl inhibition.

The fluctuations in plasma membrane PI(3,4,5)P₃ concentrations observed in imatinib-treated cells resembled those induced by stimulatory glucose concentrations (44). The glucose-induced PI(3,4,5)P₃ elevations are secondary to insulin secretion and autocrine activation of insulin receptors and PI3K. However, we consider it unlikely that the effects of imatinib were derived from a direct action on the insulin secretion process. In a previous study imatinib did not influence insulin secretion from human and rat islets during short term (1 h) or long-term (24 h) imatinib exposure periods (13). Moreover, diazoxide, which hyperpolarizes the plasma membrane and suppresses insulin secretion by opening ATP-sensitive K⁺ channels, did not affect imatinib-induced PI(3,4,5)P₃ levels. This observation may indicate that imatinib raises PI(3,4,5)P₃ by different mechanisms than glucose and insulin. Besides by PI3K, PI(3,4,5)P₃ levels are also controlled by the PI(3,4,5)P₃ phosphatases PTEN and SHIP2. It has been reported that the c-Abl protein interacts directly with SHIP2 via its SH3 domain (45). Thus, imatinib could modulate PI(3,4,5)P₃ levels differently than glucose and insulin by not only activating PI3K, but also by controlling SHIP2 activity. Indeed, we presently observed c-Abl co-immunoprecipitation with SHIP2, and imatinib counteracted both SHIP2 tyrosine phosphorylation and SHIP2 binding to c-Abl. Although predicted c-Abl target sites are present in the SHIP2 protein, indicating that c-Abl phosphorylates SHIP2 directly, we cannot exclude the possibility that c-Abl activates some other SHIP2 phosphorylating tyrosine kinase. Because tyrosine phosphorylation augments the phosphatase activity of SHIP2 (36), these finding indicate that c-Abl binds to and phosphorylates SHIP2 leading to increased SHIP2 activity. It is conceivable that an altered SHIP2/PTEN balance, induced by imatinib, could promote the dramatic PI(3,4,5)P₃ oscillations presently observed. In this context it is noteworthy that SHIP2 inhibition in INS1E cells has been observed to stimulate Akt-, GSK3- and ERK phosphorylation, and to promote increased cell
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proliferation (46). A suppressive effect of imatinib on SHIP2 activity may not only be pertinent to the function of the insulin producing cell, but also to insulin-sensitive peripheral cells, as inhibition of SHIP2 in adipocytes has been observed to ameliorate insulin resistance (47). Thus, imatinib-induced insulin sensitivity may arise not only from inhibition of excessive PDGFR activity (5,8,10), but also from inhibition of c-Abl-induced SHIP2 activation.

PTEN phosphorylation at position ser380 was not affected by imatinib, but was possibly increased by sunitinib. Ser380 phosphorylation is considered a marker for PTEN relocalization from the plasma membrane to some other internal site with less enzymatic activity (48). However, since sunitinib did not affect PI(3,4,5)P$_3$ plasma membrane concentration, the importance the sunitinib-induced trend to increased ser380 phosphorylation remains unclear.

ERK phosphorylation was potently augmented by both imatinib and sunitinib. This indicates that it is not the inhibition of c-Abl, but possibly that of PDGFR, that promotes this effect. Indeed, both imatinib and sunitinib have been reported to stimulate ERK phosphorylation in glioma cells (49). The mechanisms by which ERK phosphorylation occurs are, however, not clarified. It is also not clear how stimulation of ERK in the present experimental setting affects beta-cell function. ERK activation is usually considered as a proliferation and survival-promoting signal, but sunitinib-induced ERK activation was presently associated with increased cell death. Thus, it is possible that ERK phosphorylation needs to be combined with increased Akt signaling in order to promote beta-cell survival, as in the case with imatinib.

Beta-catenin is an important mediator of Wnt and Akt signaling, and increased beta-catenin levels have been shown to confer anti-apoptotic and proliferative effects in various cell types. We presently observed that a 6-hour incubation with imatinib increased beta-catenin levels in both MIN6 cells and in primary human islets. In addition, the stabilization was not due to phosphorylation of beta-catenin at position ser675. This particular phosphorylation event is executed by PKA and mediates beta-catenin nuclear translocation and activation (26). Instead, it is likely that beta-catenin levels rise in response to Akt-induced GSK3 phosphorylation and inactivation, as observed in previous studies (27,46). The augmented beta-catenin levels were likely to promote significant alterations in gene expression in view of the observed increase in CREM mRNA, a beta-
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catenin/TCF7L2 target gene (39).

We have previously reported that the anti-apoptotic and inflammatory transcription factor NF-kappa B is modestly activated in insulin producing cells when challenged with imatinib (13). In addition, it is known from other cell types that the anti-apoptotic Akt pathway promotes NF-kappa B activation by inhibitor of kappa-B kinase (IKK) phosphorylation (50). Thus, there may exist a link between NF-kappa B and Akt also in insulin producing cells thereby explaining our previous and present findings that imatinib promotes activation of both pathways.

In summary, our results suggest that imatinib, by relieving beta-cells from c-Abl-mediated suppression of tyrosine kinase receptor signaling and activation of SHIP2, stimulates events that promote beta-cell survival. This is in contrast to sunitinib, which exerted negative effects on beta-cell survival. This might indicate that although sunitinib protects against diabetes in NOD mice (15), it might be a less suitable candidate for Type 1 diabetes prevention trials, which have recently been proposed for imatinib (51-53). The use of imatinib in Type 2 diabetes trials is not a realistic option considering the side-effect profile and high cost of this drug, but an improved knowledge of its mechanisms of action will hopefully help us understand how Type 2 diabetes develops and how to better treat the disease.

Acknowledgements

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Imatinib stimulates PIP3-signaling in insulin producing cells

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Legends to the Figures

FIG. 1. Effects of imatinib and sunitinib on beta-TC-6 cell Akt, ERK and IRS-1 phosphorylation. Beta-TC-6 cells were pre-incubated with imatinib (10 mM) or sunitinib (1 mM) for 6 hours or with bpV(HOpic) (PTEN inh; 100 nM) for 60 min at serum free conditions. To some of the groups DETA/NO (1 mM) or insulin (100 ng/ml) was added after the 6 hours and the incubation was prolonged with 60 min. The cells were then used for immunoblot analysis using antibodies specific to (A) Akt(thr308), (B) Akt(ser473), (C) ERK(thr202/tyr204) or (D) IRS-1(tyr612). Band intensities were quantified and expressed per total protein loading (amidoblack staining). Results are means ± SEM for 3-6 observations. * and ** denote p<0.05 and p<0.01, respectively, using Student’s paired t-test vs. corresponding control.

FIG. 2. Effects of imatinib on human islet Akt, ERK and IRS-1 phosphorylation. Human islets were pre-incubated with imatinib (10 mM) for 6 hours at serum free conditions. To some of the groups insulin (100 ng/ml) was added after the 6 hours and the incubation was prolonged with 60 min. The islets were then used for immunoblot analysis using Akt(ser473), (ERK(thr202/tyr204) or IRS-1(tyr612) specific antibodies. Results are means ± SEM for 5 observations. * denotes p<0.05 using Student’s paired t-test vs. corresponding control.

FIG. 3. PI(3,4,5)P3 plasma membrane levels in response to imatinib and sunitinib in MIN6 cells. Evanescent wave microscopy recording of GFP4-Grp1 translocation in response to (A) the PTEN inhibitor bpV(HOpic) (100 nM), (B) imatinib (10 mM) and (C) sunitinib (1 mM). After termination of bpV(HOpic), imatinib and sunitinib exposures, LY294002 (100 mM), diazoxide (250 mM) and insulin (100 nM) were added. Traces (thin and bold) are from individual cells and are from three independent experiments in which at least 10 cells per experiment were analyzed.

FIG. 4. Effects of imatinib and sunitinib on tyrosine phosphorylation of SHIP2 in human embryonic kidney 293T cells (A), c-Abl co-immunoprecipitation with SHIP2 (B) and (Ser380)-phosphorylation
Imatinib stimulates PIP₃-signaling in insulin producing cells

of PTEN (C). (A) HEK 293T cells were either left untreated or treated with imatinib (10 µM) or sunitinib (10 µM) for 20 min, 60 min and 180 min. Cells were solubilized and proteins were immunoprecipitated with SHIP2 antibody. SHIP2 tyrosine phosphorylation was analyzed by immunoblotting with anti-phosphotyrosine and SHIP2 antibodies. Values of phospho-protein bands were related to those of SHIP2 bands. Data are presented as means ±SEM for 3-4 experiments. *denotes p < 0.05 using Student’s paired t-test. (B) Human embryonic kidney 293T cells were either left untreated or treated with imatinib (10 µM) or sunitinib (10 µM) for 20 min, 60 min and 180 min. Cells were solubilized and proteins were immunoprecipitated with a SHIP2 antibody. c-Abl co-immunoprecipitation was detected with a c-Abl antibody. One representative blot out of three experiments is shown. (C) Beta-TC-6 cells were either left untreated or treated with imatinib (10 µM) or sunitinib (10 µM) for 20 min, 60 min and 180 min. Cells were solubilized and proteins were immunoprecipitated with a PTEN antibody. PTEN phosphorylation was analyzed by immunoblotting with phospho(Ser380)-PTEN and PTEN antibodies. Values of phospho-protein bands were related to those of PTEN bands. Data are presented as means ±SEM for 3-4 experiments. *denotes p < 0.05 using Student’s paired t-test vs. corresponding control.

FIG. 5. Imatinib increases beta-catenin protein levels in MIN6 cells and human islets. (A) MIN6-cells cultured without serum were either left untreated or treated with imatinib (10 µM) for 1 or 6 hours. In indicated groups the cells were stimulated with insulin (100 nM) during the last 15 min. The cells were then analyzed by immunoblotting using by total beta-catenin and phospho-beta-catenin(Ser675) antibody. Results from immunoblots were quantified using densitometry. Total beta-catenin and phospho-beta-catenin bands were related to total protein loading (amidoblack staining). Data are presented as means ±SEM for 3 individual observations and * denotes p < 0.05 vs. untreated group using Student’s t-test. (B) Human pancreatic islets cells were left untreated (control) or treated with imatinib (10 µM, 1 or 6 h) in serum free media. Data are presented as means ±SEM for 4 individual observations and *, denotes p < 0.05 vs. untreated group using Student’s t-test vs. corresponding control.
**FIG. 6.** Effects of GLP1, insulin and imatinib on relative CREM and TxnIP mRNA levels in MIN6 cells. MIN6 cells were treated with GLP-1 (100 nM), insulin (100 nM) and imatinib (10 µM) for 6h at serum free conditions. CREM and TxnIP mRNA levels were semi-quantified by real-time RT-PCR. Results are presented as means ±SEM for 3-5 individual observations and *, denotes p<0.05 statistical significant differences vs. control using Student’s t-test vs. corresponding control.

**FIG. 7.** Effects of imatinib and sunitinib on beta-TC-6 cell viability. Beta-TC-6 cells were either left untreated or treated with imatinib (10 µM) or sunitinib (1 µM or 10 mM) for 6 hours, and then incubated with cytokines (IL-1beta, 50 U/ml; IFN-gamma, 1000 U/ml), DETA/NO (1 mM) or hydrogen peroxide (0.1 mM) for 24 hours. Cells were then stained with propidium iodide (20 µg/ml) and bisbenzimide (5 µg/ml) for 10 min at 37°C. Then the medium was replaced with PBS and the red and blue fluorescence was detected with the Kodak 4000MM image station. Results were quantified using densitometry and the ratio of red to blue was taken as a relative measure of cell death. Graphs are shown as means ±SEM from 3-5 experiments. *denotes p < 0.05 using Student’s paired t-test vs. corresponding control.
Imatinib stimulates PIP$_3$-signaling in insulin producing cells

Figure 1
Imatinib stimulates PIP$_3$-signaling in insulin producing cells
Imatinib stimulates PIP₃-signaling in insulin producing cells

Figure 2
Imatinib stimulates PIP\(_3\)-signaling in insulin producing cells

Figure 3
Imatinib stimulates PIP$_3$-signaling in insulin producing cells

Figure 4

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Imatinib stimulates PIP_3-signaling in insulin producing cells
Imatinib stimulates PIP_3-signaling in insulin producing cells

**Figure 6**

![Bar graph showing relative expression of CREM/G6PDH and TxnIP/G6PDH in different conditions: Control, GLP-1 6h, Imatinib 6h, Insulin 6h. Notable increase in CREM/G6PDH expression in GLP-1 6h and Imatinib 6h conditions.](image-url)
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