Potential of Protein Phosphatase Inhibitor 1 as Biomarker of pancreatic beta cell injury in vitro and in vivo

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Short title: PPP1R1A as biomarker of beta cell injury

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Abbreviations: GAD65: glutamic acid decarboxylase 65kD (GAD2); IP: immunoprecipitation; PPP1R1A: protein phosphatase 1, regulatory (inhibitor) subunit 1A, inhibitor-1; STZ: streptozotocin; LC-MS/MS: liquid chromatography-tandem mass spectrometry; T1DM: type 1 diabetes mellitus; TRFIA: time-resolved fluorescence immunoassay

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Abstract

There is a need for plasma-based tests that can directly measure the extent of beta cell injury in vivo, in patients receiving islet grafts and in animal models. Here we propose protein phosphatase 1, regulatory (inhibitor) subunit 1A (PPP1R1A) as novel biomarker for acute beta cell destruction. LC-MS/MS proteome analysis of FACS-purified beta cells, tissue-comparative Western blotting and immunohistochemistry indicated relatively high molar abundance and selectivity of PPP1R1A in beta cells. PPP1R1A was discharged into the extracellular space of chemically-injured rat and human islets in vitro, proportionate to the extent of beta cell death. Streptozotocin injection in rats led to a progressive PPP1R1A depletion from the cytoplasm of disintegrating beta cells, and a marked surge in plasma levels detectable by an affinity capture method. A similar massive PPP1R1A discharge in blood was also detected in 3 patients immediately after intraportal islet transplantation. Our findings provide first proof-of-principle for PPP1R1A as real-time biomarker of beta cell destruction in animal models and patients, and warrant development of more sensitive methods for its further validation in clinical trials.
**Introduction**

Islet transplantation has the potential to improve long-term metabolic control in patients with type 1 diabetes (T1DM) and further refinement of this technique may lead towards a lasting cure (1-3). Human donor organs, however, are scarce, limiting the number of primary islet grafts that can be composed. Moreover, a substantial fraction of isolated human islets are lost in culture before transplant. In addition, 50-75% of grafted beta cells are rapidly destroyed due to hypoxia, thrombosis and inflammatory reactions (4-6). Optimizations of immune-modulatory, anti-inflammatory and surgical protocols in islet transplantation can thus lead to better therapy for more patients. These optimizations require reliable biomarkers to monitor beta cell injury. Using classical indices of glucose homeostasis (HbA1c, glycemic variability) or dynamic assays of beta cell secretory capacity, long-term outcome of islet transplantation can be reliably evaluated (7-11). What is still lacking is a direct biomarker for real-time sensitive quantification of beta cell injury in vivo. Proof-of-principle for glutamic acid decarboxylase 65kDa (GAD65) as such biomarker was provided by Waldrop et al. who reported that streptozotocin (STZ)-injured beta cells discharge GAD65 into the plasma, proportionate to the degree of beta cell loss (12). We recently found that high plasma GAD65 levels after islet transplantation predict poor long-term functional graft outcome in patients (Z.Ling, unpublished). These studies, however, also revealed shortcomings of GAD65 as biomarker: (i) GAD65 cannot be reliably used in the 30-40% of diabetic islet recipients that show high titers of circulating anti-GAD65 antibodies (13); and (ii) its relatively low molar abundance in beta cells precludes detection of minor beta cell insults, despite highly-sensitive immunoassays (14;15).
Therefore we initiated a search for alternative biomarkers to be used complementary to GAD65. In a proteomics-based screening, we previously found that rat beta cells abundantly express protein phosphatase 1, regulatory (inhibitor) subunit 1A, alias Protein Phosphatase Inhibitor-1 (PPP1R1A). (16). In combination with its reported high degree of beta cell-selectivity in rats (16;17) and mice (18;19), and its cytoplasmic localization, PPP1R1A appeared an interesting candidate for validation as biomarker of beta cell injury in vitro and in vivo.

Research design and methods

**In vitro and in vivo models of beta cell injury** Freshly isolated rat islet cells (70% insulin+) and cryopreserved human (50% insulin+) islets were exposed to 5mM Streptozotocin (STZ, 30 min) and 2mM H₂O₂ (2h), respectively, and cell death monitored over the subsequent 6-24h. In vivo biomarker discharge was measured in rats injected with 60 mg/kg STZ; EDTA-plasma and pancreas were sampled at 2-24h after injection (n=3/time point). Plasma PPP1R1A was also measured in 4 T1DM patients after intraportal infusion of 1.1-4.8x10⁶ beta cells/kg bodyweight. Negative controls included patients suffering various acute organ injuries (pancreatitis, stroke and kidney transplantation, sampled at ICU < 6h from onset) and type 2 diabetics.

**Immunohistochemistry** After antigen retrieval in 10mM citric acid (pH 6.0) 5 µm sections of paraffin-embedded rat and human pancreas were stained with monoclonal rabbit anti-PPP1R1A (OriGene, USA, 1/800 for rat, 1/200 for human), monoclonal mouse anti-glucagon (Sigma, 1/500) and/or polyclonal guinea pig anti-insulin (1/1000). Secondary antibodies (1/500) were from Jackson ImmunoResearch Laboratories (USA). Pictures were taken using Zeiss Axioplan fluorescence
microscope at fixed exposure time and processed with Smartcapture software (Digital Scientific Ltd, UK).

**PPP1R1A and GAD65 measurements** PPP1R1A release was measured by immunoprecipitation: plasma and concentrated culture medium (Microcon 10kD spin columns, Millipore, USA) were incubated overnight at 4°C with Dynabeads (Invitrogen, USA) carrying anti-PPP1R1A (OriGene, USA) non-covalently coupled to Protein A Dynabeads (culture medium, 0.8µg Ab/1.5mg beads) or covalently coupled to M-270 Epoxy Dynabeads (6µg Ab/1.25mg beads per 500µL plasma). Captured PPP1R1A was eluted with 0.1M citrate (pH 3.1), detected using a polyclonal rabbit anti-PPP1R1A (F.Schuit, (18)). Intracellular PPP1R1A was quantified using recombinant human PPP1R1A as calibrator (Abcam, USA). Intensities of bands were quantified with Scion image software (Scion, Frederick, MD, USA). This assay showed intra-(inter-)assay CV% of 17% (26%), good linearity (R²=0.96, Fig.S1), acceptable recovery (80±16%) but limited sensitivity (± 2 nmol/L in plasma).

**Solid-phase detection of anti-PPP1R1A autoantibodies** was done exactly as described (20) using 50 ng PPP1R1A as bait in sera from 20 C-peptide negative T1DM patients – all showing antibodies against GAD65 and at least one other T1DM epitope - and 20 age/sex-matched healthy controls, obtained from the Belgian Diabetes Registry.

*Statistical analysis* Data are expressed as mean±SD and analyzed with One-way ANOVA or t-test. P<0.05 was considered significant.
Results:

**PPP1R1A abundance and selectivity in beta cells** In human pancreas PPP1R1A expression was restricted to the beta cells, with no protein detected in alpha cells, nor in exocrine tissue (Fig.1A). Also at whole body level in rat, PPP1R1A showed a reasonable degree of beta cell-selectivity (Fig.1B). Quantitative LC-MS/MS proteome analysis indicated that the molar abundance of PPP1R1A was similar to that of endocrine markers prohormone convertase 1 and chromogranin A, and amounted to 20% of abundant housekeeping proteins such as cyclophilin-A and glyceraldehyde-3-phosphate dehydrogenase (Fig.1C). Quantitative SDS-page using recombinant PPP1R1A standards indicated that rat and human beta cells express 24 ± 10 and 36 ± 10 attomol/cell (n=4) respectively (Fig. S1) comparing favorably to their respective GAD65 contents (0.8 ± 0.1 and 1.5± 0.1 attomol/cell), as measured by TRFIA (14).

**In vitro validation: PPP1R1A discharge by injured rat and human beta cells** Rat islets exposed to 5mM STZ disintegrated during the subsequent 24h culture into 60-70% single dead cells (Fig.2A). This led to a depletion of the intracellular PPP1R1A (Fig.2B) and a parallel increase of extracellular PPP1R1A: 24h after injury, 20-fold more PPP1R1A was immunoprecipitated from the extracellular space than in controls. Similar results were obtained using cryopreserved human islets: exposure to H$_2$O$_2$ (2h, 2 mM) caused islet disintegration with 40-50% dead cells after 24h of culture (Fig.2A). This led to a 10-fold higher PPP1R1A discharge (Fig.2C, P<0.01) concomitant with depletion of intracellular PPP1R1A. The PPP1R1A discharge at 6h
after injury was only detected in rat cells (P<0.01), reflecting the more severe cellular injury than in human islets at this time point (Fig.2A).

**In vivo validation in rat: streptozotocin-injection causes a surge of plasma**

**PPP1R1A** Injection of a diabetogenic dose of STZ (60 mg/kg) induced hyperglycemia within 2h. Coinciding with a massive insulin discharge from the pancreas (peaking at 6h), glycemia temporarily dropped to rise again and to remain permanently elevated after 24h (Fig.3A). STZ induced progressive depletion of PPP1R1A protein from the pancreas, with little protein left after 4h (Fig.3B): in situ analysis showed that insulin-positive cells became devoid of PPP1R1A at 4h after STZ well before they disintegrated and were phagocytized (8-24h (Fig.3C)): PPP1R1A depletion from injured beta cells coincided with a sharp peak in plasma PPP1R1A between 2h and 6h after STZ with a maximal level at 4h (Fig.3D). Clearance from the circulation was verified by injection of recombinant human PPP1R1A in rats, which indicated a half-life around 15 min (Fig. 4B), less than the 2.8h reported for GAD65 (21).

**Proof-of-principle in human islet transplantation.** Engrafted islets suffer ischemic, inflammatory and mechanical stress shortly after infusion, likely causing plasma membrane disruption and inadvertent discharge of intracellular proteins. In view of its rapid clearance we investigated plasma PPP1R1A in 4 diabetic patients after intraportal islet infusion. A post-transplant PPP1R1A surge was detected in 3 patients (Fig.4A), ranging from borderline detectable – in a patient receiving only 1.1x10^6 beta cells/kg – to an unambiguous detection in two patients receiving larger grafts (2.4 and 3.9x10^6 beta cells/kg). Kinetic data in the latter 2 patients indicated a peak discharge
directly after graft infusion, followed by rapid clearance roughly compatible with PPP1R1A’s pharmacokinetic properties in rat (Fig.4B). PPP1R1A discharge appeared to be beta cell-selective, since no PPP1R1A could be detected at baseline before transplant, nor in other types of acute tissue damage such as pancreatitis, stroke or kidney transplantation (Fig.4C). Presence of anti-PPP1R1A auto-reactive antibodies in diabetic patients was ruled out using a solid-phase adsorption assay: no anti-PPP1R1A IgGs were detected in sera of 20 T1DM patients that showed B-cell reactivity to GAD65 and at least one other classical T1DM epitope, nor in age/sex-matched healthy controls (Fig. 4D).

**Discussion**

This study presents proof-of-principle that PPP1R1A can be used as real-time biomarker for pancreatic beta cell injury *in vitro* and *in vivo*, in rodents and human patients. During culture of human islets, extracellular PPP1R1A may thus be used to assess in vitro survival, while in islet graft recipients, plasma PPP1R1A may be envisaged as biomarker to evaluate the extent of beta cell graft destruction, complementary to GAD65.

PPP1R1A shows interesting properties in terms of beta cell-selectivity and molar abundance. In line with previous results (18;22), we also detected it in brain and muscle cells, albeit at a much lower level than in beta cells. This could constrain the use of PPP1R1A in patients but not invalidate it: we could confidently detect a surge of plasma PPP1R1A after STZ-induced beta cell injury in rodents but never in baseline condition. Also in humans, PPP1R1A was detected in 3 patients just after
islet transplantation, but not in control patients with acute damage to exocrine pancreas (pancreatitis), brain (stroke) or kidney (transplantation).

PPP1R1A shows several advantages over GAD65, an established marker of beta cell injury. First, human and rat beta cells abundantly express PPP1R1A, apparently more than GAD65. Use of different analytical techniques precludes a direct comparison of PPP1R1A and GAD65 molar abundances in beta cells. Yet, the fact that PPP1R1A was detectable in plasma with an IP assay operating in the low nanomolar sensitivity, while the GAD65 assay operates at the picomolar range is encouraging with regard to maximal attainable diagnostic sensitivity. Second, unlike GAD65, PPP1R1A is also expressed by mouse beta cells and can thus be applied in preclinical murine models (17;18). Finally, we showed that PPP1R1A is not a type 1 diabetic autoantigen, so can be measured without interference by auto-reactive antibodies. A possible disadvantage of PPP1R1A is its rapid clearance, likely a consequence of its limited molecular weight (20kD) below the glomerular filtration threshold.

Future validation of the PPP1R1A’s sensitivity as biomarker of beta cell injury – defined here as the number of beta cells needed to be simultaneously disrupted before the biomarker plasma level rises significantly above baseline – will require a high-throughput assay that can operate at low picomolar sensitivity. Such assay is needed to evaluate if baseline PPP1R1A levels can build up in plasma despite the relatively rapid PPP1R1A clearance, in patients with neuronal or muscle damage. We calculated that our GAD65 TRFIA can detect the simultaneous destruction of $20 \times 10^6$ and $40 \times 10^3$ beta cells in humans and rats respectively, amounting to approximately 5% of their normal endogenous beta cell number, and 15 % of a typical islet graft. Providing that an equally sensitive PPP1R1A sandwich assay can be developed, PPP1R1A’s apparent higher abundance in beta cells could significantly improve this sensitivity.
This could not only facilitate its application to monitor chronic allo-rejection of islet grafts, for which GAD65 showed borderline efficacy (5), it could also create the experimental conditions to explore the largely enigmatic dynamics of preclinical autoimmune destruction type 1 diabetes, provided that these instances of beta cell destruction involve at least some degree of biomarker discharge into the plasma, either free or enclosed in cellular blebs or apoptotic bodies. A test that could give direct proof of ongoing beta cell destruction in subjects with high risk of progression to clinical T1DM based on their autoantibody and genetic profile, would be valuable to select those subjects that could benefit from immune-reprogramming therapy with monoclonal antibodies such as anti-CD3 (10).

There is still a long way to go before this will be possible: apart from higher analytical sensitivity towards the proposed biomarkers, the ideal assay should also be run in high-throughput and in miniaturized mode, e.g. on dried blood spots obtained by capillary sampling during routine glycemic monitoring.

In conclusion, current pilot study establishes PPP1R1A as novel biomarker beta cell injury in vitro and in vivo, with favorable properties in terms of beta cell-selectivity and abundance that warrant its further validation in (pre)clinical trials.

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Author contributions: G.M. designed the study and wrote the manuscript; L.J., B.B. GK, JA and GM researched data; F.G., D.P., B.K., F.S. and Z.L. reviewed/edited the manuscript. The authors have no duality of interest to declare. G.M. is the guarantor of this work and as such, has full access to all the data in the study, and takes responsibility for the integrity of the data and the accuracy of data analysis.

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**Figure legends**

**Figure 1: selectivity and abundance of PPP1R1A in rat and human beta cells (A)**

Human pancreas stained for PPP1R1A (green) and insulin or glucagon (red). Selected images are representative for 3 different organs. **(B)** Western blotting of PPP1R1A expression in rat tissues. Bars represent mean ± SD of 3 biological replicates. **(C)** Relative molar protein abundances in rat alpha and beta cells (n=3) measured by label-free quantitative LC-MS/MS (16). Proteins are denoted by their official NCBI Gene Symbol: ACTB, beta actin; GAPHD, glyceraldehyde-3-phosphate dehydrogenase; PPIA, cyclophilin-A; CHGA, chromogranin A; PCSK1 and PCSK2, prohormone convertases 1 and 2; PPP1R1A, protein phosphatase 1, regulatory (inhibitor) subunit 1A
**Figure 2: in vitro discharge of PPP1R1A protein by injured rat and human beta cells** Panel (A) illustrates the *in vitro* cytotoxicity model: pulse exposure of rat islets to STZ and human cryopreserved islets to H₂O₂ induced a progressive disintegration of living islets (Hoechst+, blue) into single dead (propidium iodide+, red) cells. This caused increase of PPP1R1A in culture medium of both rat (B) and human (C) islets, quantified by immunoprecipitation and Western blotting. Increase of precipitated PPP1R1A discharge was accompanied by progressive depletion of intracellular PPP1R1A (right Y-axis, normalized to cyclophilin-A, PPIA). Bars indicate mean ± SD, *P<0.01.

**Figure 3: In vivo discharge of PPP1R1A protein by streptozotocin-injured rat beta cells** Panel (A) shows plasma glucose (black bars, left Y-axis) and insulin (white bars, right Y-axis) at the indicated time after STZ (60 mg/kg) injection (mean±SD, n=3 per time point). (B) STZ induced a progressive depletion of total pancreatic PPP1R1A content, attributed to (C) a disappearance of PPP1R1A protein (green) from insulin-positive (red) beta cells before the beta cell core of the islets disintegrates and can only be recognized by the residual alpha cell mantle (magenta). Images representative for n=3 animals/time point. This causes PPP1R1A to become detectable by IP (D) in plasma, with a peak discharge at 4h after STZ injection. Bars indicate mean±SD, n=3, *P<0.05 versus baseline

**Figure 4: Proof-of-principle in human islet graft recipients.** PPP1R1A discharge in plasma was investigated by IP in C-peptide negative T1DM patients after intraportal infusion of the indicated number of beta cells per kg bodyweight. Bar graphs represent the quantification of the captured PPP1R1A visualized on Western
blot. Panel (B) shows the pharmacokinetic profile in rat circulation after injection of recombinant human PPP1R1A in tail vein (average ± SD for n=3, representative blot). Panel (C) illustrates that no detectable PPP1R1A amounts could be affinity-captured from sera of patients with the indicated pathologies; serum spiked with INS1 cell lysate served as positive control (representative for n=3 patients/condition). Panel (D) shows a solid-phase adsorption test for anti-PPP1R1A reactive immunoglobulins in sera of T1DM patients (left) and age/sex matched healthy controls (right). 20 subjects were examined, 10 shown here. T1DM all showed anti-GAD65 antibodies (GADA), the titers of which (determined by % radiobinding in certified assays) correlated well with solid-phase HRP-signal against spotted rhGAD65. None of the samples showed any IgG reactivity to spotted rhPPP1R1A. Technical controls (middle) include 1:1000 dilutions of mouse monoclonal anti-PPP1R1A and anti-GAD65, or no primary antibody.

**Supplementary Figure 1 (Fig.S1): the PPP1R1A affinity capture-SDS page assay**

Panel (A) illustrates quantification of PPP1R1A cellular content in human and rat beta cells using a standard curve of recombinant human His-tagged PPP1R1A. Bar graph below indicates average ± SD of n=4 such PPP1R1A measurements, compared to parallel measurement of beta cellular GAD65 content by TRFIA. Panel (B) shows linearity in a recovery experiment with INS1 cell lysate spiked in matrix (representative for 3 such experiments); intra-and inter-assay coefficients of variation were determined by a simplified Newgard protocol for imprecision testing using 3 consecutive runs of triplicates.
selectivity and abundance of PPP1R1A in rat and human beta cells
254x190mm (300 x 300 DPI)
in vitro discharge of PPP1R1A protein by injured rat and human beta cells
In vivo discharge of PPP1R1A protein by streptozotocin-injured rat beta cells
Proof-of-principle in human islet graft recipients

254x118mm (300 x 300 DPI)
Proof-of-principle in human islet graft recipients

254x154mm (300 x 300 DPI)
A

His-rhPPP1R1A (pmol)

|   | Rat beta cells | Human islets |
|---|---------------|--------------|
| 0 |               |              |
| 0.3 |             |              |
| 0.5 |             |              |
| 1.0 |             |              |
| 2.0 |             |              |

B

Intensity

y = 0.40x + 1.69
R² = 0.96

the PPP1R1A affinity capture-SDS page assay