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ORIGINAL ARTICLE

Intracellular sirolimus concentration is reduced by tacrolimus in human pancreatic islets in vitro

Kristine Kloster-Jensen,[1,2] Nils Tore Vethe,[3] Sara Bremer,[4] Shadab Abadpour,[1,2] Olle Korsgren,[5] Aksel Foss,[1,2,6] Stein Bergan[3,7] and Hanne Scholz[1,2]

1 Department of Transplantation Medicine, Oslo University Hospital, Norway
2 Institute for Surgical Research, University of Oslo, Norway
3 Department of Pharmacology, Oslo University Hospital, Norway
4 Department of Medical Biochemistry, Oslo University Hospital, Norway
5 Department of Immunology Genetics and Pathology, Science for Life Laboratory, Uppsala University, Sweden
6 Faculty of Medicine, University of Oslo, Norway
7 School of Pharmacy, University of Oslo, Norway

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Correspondence:
Kristine Kloster-Jensen, Institute for Surgical Research, OUS, Rikshospitalet, Sognsvannsveien 20, 0027 Oslo, Norway.
Tel.: +47 97586887;
fax: +47 23073530;
e-mail: kkjensen@rr-research.no

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Abstract

Main problem: Islet transplantation has become a promising treatment for type 1 diabetes. However, immunosuppressive drugs used today cause islet deterioration and modification strategies are necessary. But little is known about pharmacokinetics interactions and intracellular concentrations of immunosuppressive drugs in human islets.
Methods: We determined the pharmacokinetics of tacrolimus and sirolimus in islets by measuring intracellular concentration after exposure alone or in combination at two different doses up to 48 h. A quantification technique established in our laboratory using a Micromass Quattro micro API MS/MS-instrument with electrospray ionization was used. Islets function was measured by oxygen consumption rates. Presence of drug transporters OATP1B1 and ABCB1 and metabolizing enzyme CYP3A4 in islets were quantified using real-time quantitative PCR.
Results: Islets incubated with tacrolimus and sirolimus had a significant decrease in intracellular concentration of sirolimus compared to sirolimus alone. Reduced intracellular sirolimus concentration was followed by increased p70S6k phosphorylation suggesting preservation of the mTOR-signaling pathway. Drug transporters OATP1B1 and ABCB1 and enzyme CYP3A4 were expressed in human islets, but were not involved in the reduced sirolimus concentration by tacrolimus.
Conclusion: These findings provide new knowledge of the drug interaction between tacrolimus and sirolimus, suggesting that tacrolimus has an inhibitory effect on the intracellular concentration of sirolimus in human islets.

Introduction

Pancreatic islet allotransplantation has become a successful treatment option for patients with type 1 diabetes [1]. However, the recipients require lifelong regimens of immunosuppressive therapy to prevent rejection, which negatively affect function and viability of beta-cells [2]. Calcineurin inhibitors (CNI) such as cyclosporine A (CsA) and tacrolimus prevent the translocation of the transcription factor nuclear factor of activated T cells(NFAT) into the nucleus and thereby blocks the production of cytokines essential for T-cell activation and proliferation [3]. Both CNIs are known to promote insulin resistance and have deleterious effects on human islets [4,5]. Therefore, using a regimen based on low dose of CNIs or even replacing it has been suggested for islet transplantation [6,7]. Sirolimus, an immunosuppressive drug that inhibits mammalian target of rapamycin (mTOR), is a promising drug for reducing or replacing CNIs [1]. How-

ever, studies have shown that sirolimus decreases the insulin synthesis and impairs the *in vivo* proliferation and angiogenesis of beta-cells [5,8–11].

Pharmacological interaction of immunosuppressive drugs with one another is a clinical concern due to possible synergistic toxicity. A recent *in vivo* study in rats showed interaction between CsA and sirolimus resulting in aggravation of CsA-induced nephrotoxicity and pancreas dysfunction [12]. Moreover, islets are transplanted into the hepatic vein system where the immediate concentration of orally administered drugs is higher than systemic, which potentiate increased risk of toxicity to the islets [13]. Monitoring immunosuppressive drug concentrations in peripheral whole blood or plasma is routinely performed to ensure adequate immunosuppressant effect after transplantation, but this probably correlates poorly to the tissue levels [14]. Both CNIs and mTOR inhibitors are metabolized by intestinal and hepatic cytochrome P-450(CYP)3A enzymes [15,16] and transported by the efflux pump, ABCB1 (known as P-glycoprotein [Pgp] or multidrug resistance 1 [MDR1]) [17]. Immunohistochemistry studies have demonstrated CYP3A4 and ABCB1 protein expression in human pancreatic tissue [18–20] implicating that these proteins may influence the pharmacokinetics in islets. The *SLCO1B* genes encode the organic anion-transporting polypeptides (OATP1B1 and OATP1B3), which are highly expressed in hepatocytes and mediate cellular uptake (influx) of various exogenous and endogenous substrates [21]. Interestingly, it was recently shown that OATP1B3 is highly expressed in human islets [22].

Extracellular concentrations of immunosuppressant will not necessarily reflect the intracellular concentrations in specific cell populations [23]. A great degree of individual variability of pharmacokinetics and pharmacodynamics (incomplete and unpredictable uptake and drug interactions) makes patient blood levels a complex and unpredictable measure unit [24]. Also, the capacity of drug accumulation within cells might depend on the intracellular expression of drug-binding proteins. The intracellular drug concentration will more accurately predict the drug effect in the cell population of interest. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) may be used to determine concentrations of tacrolimus, sirolimus, and CsA at low levels within cells with high precision and accuracy. The intracellular concentrations of sirolimus, tacrolimus, or a combination thereof in human islets have to our knowledge not been investigated previously. We used an *in vitro* approach where we examined the dose and time dependency of tacrolimus and sirolimus concentrations within human islets after exposure to each of the drugs alone or by the combination. The expression of *ABCB1*, *OATP1B1,* and *CYP3A4* in human islets was assessed in parallel.

Materials and methods

Human islet isolation and culture

Following obtained consent from the organ donor relatives, pancreata from deceased donors (mean age 49.5 ± 26.5, $n = 20$) were processed in the in the Central Laboratory of the Nordic Network for Clinical Islet Transplantation in Uppsala, Sweden. Islets were isolated according to the automated method, refined by the Nordic Network for Islet Transplantation [25]. Islet preparations used in this study exhibited 50–95% purity and a viability of 80–90%. Glucose stimulation index during dynamic perfusion was 4.1 ± 2.0. The islet preparations were qualitatively approved for human transplantation; however, the low quantity of islets made these preparations available for research. Islets were maintained in cell culture media CMRL 1066 (Mediatech Inc., Manassas, VA, USA) supplemented with 10% ABO-compatible serum, 10 mM Hepes, and 1% penicillin/streptomycin/L-glutamine (Gibco, Invitrogen, Paisley, Scotland, UK) for 1 day at 37 °C (5% CO_2), thereafter at 25 °C (5% CO_2) until the start of each experiment.

Exposure to immunosuppressive drugs

Two to five days after isolation, equal aliquots of islet preparations were placed into 90 mm Petri dishes (Sterilin, Heger AS, Norway) in cell culture media as described above, but with 2.5% ABO-compatible serum. The islets were exposed to 10 and 30 μg/l of tacrolimus (Santa Cruz Biotechnology, Dallas, TX, USA) or sirolimus (Toronto Research Chemicals, Toronto, Ontario, Canada) or the combination thereof for 24 h and 48 h at 37 °C (5% CO_2). In additional experiments, islets were exposed to sirolimus (30 μg/l) alone or combined with CsA (5 μg/ml) (Toronto Research Chemicals, Toronto, Ontario, Canada) for 24 h. All drugs were dissolved in methanol (Rathburn Chemicals Ltd, Walkerburn, UK) and diluted in cell culture medium to reach their final concentrations. Control islets were cultured and added the same volume of methanol (<0.001% of total volume) as the 30 μg/l condition but in the absence of drugs. The drug concentrations were selected based on the target trough level for each drug used in clinical practice. As portal vein peak concentrations of immunosuppressant drugs generally reach double those of systemic levels after oral administration, we used high doses of the drugs [26].

Quantification of intracellular immunosuppressant drug levels in islets

After incubation, human islets (approximately $n = 1000$) were handpicked into columns, washed twice with ice-cold phosphate-buffered saline (PBS), lysed in 150 μl water, and

homogenized by sonication. A 25 μl aliquot of the homogenate was transferred to a 1.5-ml microcentrifuge tube, and four volumes of methanol containing internal standards were added (ascomycin, D_4-everolimus reagent, and D_{12}-cyclosporine A reagent). The tube was vortexed and centrifuged, and 50 μl supernatant was mixed with 30 μl water in a LC injection vial. Drug quantification was performed with LC coupled to tandem mass spectrometry (LC-MS/MS) using an Alliance HT 2795 HPLC-coupled to a Micromass Quattro micro API MS/MS-instrument with electrospray ionization (Waters, Milford, MA, USA). A Kinetex C_{18} column (2.6 μm, 50 × 2.1 mm) was used for separation at 50 °C with a SecurityGuard ULTRA C_{18} cartridge (2.1 mm) in front (Phenomenex, Torrance, CA, USA). Mobile phase A consisted of 35% methanol in water, and mobile phase B was 100% methanol. Both mobile phases contained 2.0 mM ammonium acetate and 0.03% (v/v) formic acid. The LC gradient was as follows: 100% A until 5.5 min, then B was linearly increased to 40% between 5.5 and 9.0 min, thereafter 92% B was pumped until 12.0 min, and the column was re-equilibrated with 100% A until 16.0 min. The flow rate was 0.300 ml/min, and the injection volume was 20 μl. Precursor ammonium adducts were fragmented and monitored for tacrolimus (m/z 821 to 768), sirolimus (m/z 931 to 864), and CsA (m/z 1219.5 to 1202.5) and for their internal standards ascomycin (m/z 809 to 756), D_4-everolimus (m/z 979 to 912), and D_{12}-cyclosporine (m/z 1231.5 to 1214.5), respectively. Calibrators (tacrolimus, sirolimus and CsA) were processed in parallel with the samples, and Waters MassLynx software was used to calculate the analyte concentrations based on linear responses of peak area ratios between analyte and internal standard signals. Results were normalized to total protein content as measured by the BCA Protein Assay Kit (Thermo Scientific Pierce, IL, USA).

Oxygen consumption assay

Oxygen consumption rate (OCR) was determined using the Seahorse extracellular flux analyzer XF24 (Seahorse Bioscience, Billerica, MA, USA) [27]. Islets were treated with tacrolimus (30 μg/l), sirolimus (30 μg/l), or the combination thereof for 24 h before the experiments. Briefly, 80 islets were handpicked at the day of assay and plated into each wells of the manufactory islet plate preloaded with 400 ml unbuffered assay medium containing 3 mM glucose, 0.8 mM Mg^{2+}, 1.8 mM Ca^{2+}, 143 mM NaCl, 5.4 mM KCl, 0.91 mM NaH2PO4, phenol red 15 mg/ml (Seahorse Bioscience) for 1 h at 37 °C in air. Four wells were kept empty as controls in every experiment. Screens were carefully put on top of the depression of all wells with tweezers, and the plate was pictured to normalize for the possible different islet numbers in each well. The OCR was measured at basal glucose levels (3 mM) as well as with high glucose (20 mM), and results were expressed as percentage of baseline.

Gene expression analysis of ABCB1, CYP3A4, and OATP1B1

Following treatment with immunosuppressant drugs, 200 handpicked islets were collected by centrifugation for 1 min at 220 g and washed twice in ice-cold PBS. Reverse transcription and real-time qPCR analysis of the target genes *ABCB1*, *OATP1B1, CYP3A4* was performed as previously described [28]. Briefly, total RNA was reverse-transcribed into complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The cDNA synthesis was followed by amplification of the target genes and three reference genes, aminolevulinate delta-synthase 1 (*ALAS1*), beta-2-microglobulin (*B2M*), and ribosomal protein L13a (*RPL13A*), using the LightCycler® 480 instrument (Roche Applied Science, Penzberg, Germany). All PCR runs included analysis of cDNA from a liver biopsy as a positive control and calibration point between runs, as well as analysis of untreated islets (control).

Detection of p70S6K phosphorylation

Equal amounts of islet cell lysates (measured by total protein concentration) from the experiments described above were utilized to assess the phosphorylation of p70S6K (Thr421/Ser424) by fluorescence-based quantitative measurement on a Bio-Rad platform (Bio-Rad Laboratories, Hercules, CA, USA). Data were expressed as ratio to control islets.

Immunofluorescence

Sixty to eighty human islets were dissociated into single cells with TripLE express (Gibco, Invitrogen), washed in PBS, and spun to microscope slides (SuperFrost®Plus, Menzel, Braunschweig, Germany) by centrifugation at 800 rpm for 8 min. The cytospin slides were fixed and permeabilized by 4% paraformaldehyde and 0.3% Triton X-100 in PBS, respectively. Double immunofluorescence staining was performed for insulin and ABCB1(Pgp) or glucagon and ABCB1(Pgp). Section was incubated with polyclonal guinea pig anti-insulin 1:500, polyclonal rabbit antiglucagon 1:50 (DAKO, Glostrup, Denmark), monoclonal mouse anti-Mdr1(PgP) 1:25 (Santa Cruz Biotechnology, Inc, Heidelberg, Germany) overnight at 4 °C in humidified chamber. After washing with 1x Tris-buffered saline with tween (TBST), slides were incubated with AlexaFluor 488 Goat-Anti-Guinea pig 1:300 in combination with AlexaFluor 488 Donkey-Anti-Rabbit 1:300, or AlexaFluor

594 Donkey-Anti-Mouse 1:300 for 1 h at room temperature and thereafter mounted with Slow Fade Gold Antifade Reagent with DAPI for nuclear staining (Life Technologies AS, Oslo, Norway). Images were taken by the Axio Observer Inverted Microscope (Carl Ziess AS, Oslo, Norway) with ZEN lite software.

Statistical analysis

Data are expressed as mean ± SD. Comparison of results across groups was performed by one-way analysis of variance (ANOVA); when significant differences were found, comparisons between groups were performed with two-tailed unpaired Student's t-tests using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was considered with $P < 0.05$.

Results

Intracellular concentration of tacrolimus in human islets

To evaluate the accumulation of tacrolimus in islets, we compare 24 h exposure of low (10 μg/l) and high (30 μg/l) dose of tacrolimus to islets. We found a 1.4-fold increase of the intracellular tacrolimus concentration despite a 3.0-fold increase in drug exposure dose ($P = 0.0308$; Fig. 1). Investigating islets exposed to low (10 μg/l) dose of tacrolimus for 24 and 48 h we found that at 48 h the intracellular concentration of tacrolimus was significantly increased by 2.0-fold ($P = 0.0005$; Fig. 1). Islets treated with a combination of low-dose tacrolimus and sirolimus also increased intracellular concentration of tacrolimus ($P = 0.0063$) at 48 h compared to 24 h drug exposure. However, intracellular tacrolimus concentration in the drug combination did not change compared to tacrolimus alone, independent of dose or exposure time (Fig. 1). Intracellular tacrolimus concentration was undetectable in control islets (data now shown).

Intracellular concentration of sirolimus in human islets

Intracellular sirolimus concentration in islets exposed to low (10 μg/l) and high (30 μg/l) dose of sirolimus for 24 h did not increase (Fig. 2). However, we found a 2.24-fold increase in intracellular concentration of sirolimus after exposure to a low (10 μg/l)-dose sirolimus for 24 h compared to 48 h ($P < 0.0001$; Fig. 2). Contradictorily, the intracellular concentration of sirolimus was significantly reduced by 0.5-fold in islets after treatment for 24 h with the combination of tacrolimus and sirolimus compared to sirolimus alone at a low (10 μg/l) dose ($P < 0.0001$; Fig. 2). A similar reduction was also found when the islets were treated with a high (30 μg/l) dose of the combination tacrolimus and sirolimus compared to sirolimus alone ($P = 0.0004$; Fig. 2). After 48 h exposure, a 0.4-fold reduction in intracellular sirolimus concentration was seen in islets treated with the combination compared to sirolimus alone at a low (10 μg/l) dose ($P = 0.0007$; Fig. 2).

When comparing the low (10 μg/l)-dose sirolimus at 24 and 48 h, we found a 2.2-fold ($P < 0.0001$) increase in intracellular concentration of sirolimus. This increase in intracellular sirolimus concentration was also observed in the combination of tacrolimus and sirolimus ($P = 0.0003$) at 48 h compared to 24 h. The intracellular concentration of sirolimus was undetectable in control islets (data not shown).

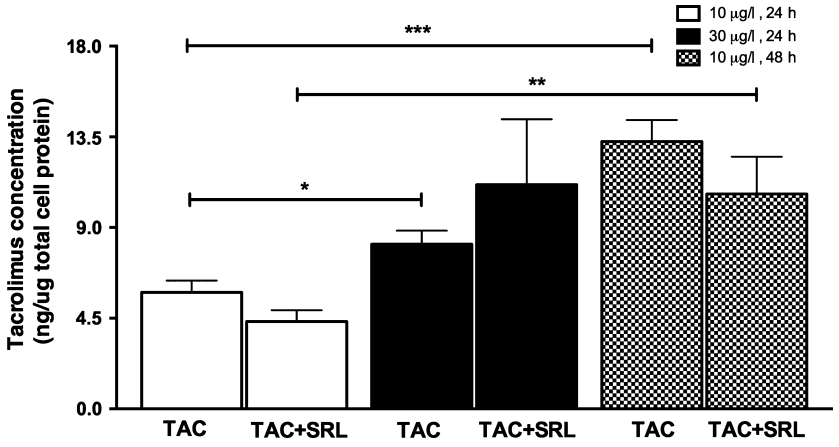

Figure 1 Intracellular concentrations of TAC in human islets. Human islets were cultured with TAC (10 or 30 μg/l), SRL (10 or 30 μg/l), or the combination thereof for 24–48 h before the intracellular concentration of TAC was measured in islet lysate and normalized to total protein as detailed in methods. Data are presented as the mean ± SD, $n = 6$ for each group. TAC, tacrolimus; SRL, sirolimus; *$P < 0.04$; ** $P < 0.007$; *** $P < 0.0006$.

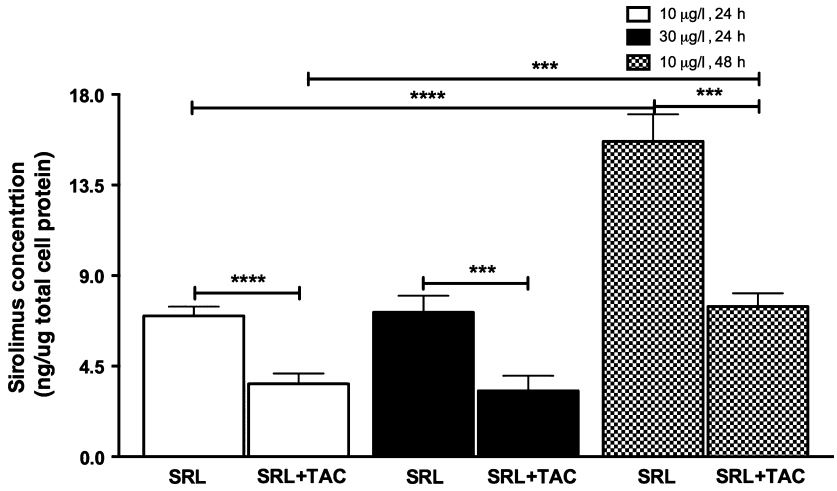

Figure 2 Intracellular concentrations of SRL in human islets. Human islets were cultured with TAC (10 or 30 µg/l), SRL (10 or 30 µg/l), or the combination thereof for 24–48 h before the intracellular concentration of SRL was measured in islet lysate and normalized to total protein as detailed in methods. Data are presented as the mean ± SD, $n = 6$ for each group. TAC: tacrolimus; SRL: sirolimus; *** $P < 0.001$; **** $P < 0.0001$.

The intracellular sirolimus concentration in human islets is not affected by CsA

To investigate whether another CNIs, CsA would demonstrate the same interaction with the intracellular sirolimus concentration in islets as tacrolimus, we performed six additional experiments where islets were exposed to CsA (5 µg/ml) or sirolimus (30 µg/l) alone, or the combination thereof for 24 h. Islets incubated with the drug combination CsA and sirolimus did not show different intracellular concentrations of sirolimus (Fig. 3a) or CsA (Fig. 3b) compared to islets incubated with the drug alone. These results suggest that the inhibitory effects on the sirolimus concentration by tacrolimus are specific and do not involved other CNIs inhibitors such as CsA.

Combined treatment with tacrolimus and sirolimus increased phosphorylation of p70S6K in human islets compared to sirolimus alone

To better understand the biological relevance of the observed decrease in intracellular sirolimus concentration when islets were exposed to the drug combination sirolimus and tacrolimus, the mTOR-dependent phosphorylation of p70S6K (Thr421/Ser424) was quantified. The phospho-p70S6K levels in islets were as expected significantly decreased by sirolimus (30 µg/l), but not by tacrolimus (30 µg/l) compared to control islets (Fig. 4a). Importantly, when islets were exposed to the drug combination of tacrolimus and sirolimus, the levels of phospho-p70S6K were significantly higher compared to the levels in islets exposed to sirolimus alone (Fig. 4a). In parallel experiments, we investigated whether CsA could reverse the sirolimus-mediated reduction of phospho-p70S6K levels. First, we observed no effect of CsA (5 µg/l) on the phospho-p70S6K levels in islets compared to control. Next, the combination of CsA and sirolimus did not reverse the sirolimus-mediated reduction of phospho-p70S6K levels (Fig. 4b). In line with the observations of intracellular drug concentrations, CsA did not interact with the sirolimus-mediated inhibition of p70S6K phosphorylation (Fig. 4b).

Combined treatment of sirolimus and tacrolimus does not further reduce the glucose-stimulated oxygen consumption rate in islets

Oxygen consumption rate (OCR) is considered a valid measurement of the metabolic function of islets [27,29]. Tacrolimus and sirolimus have known detrimental effects on islets insulin secretion [8,10,26]; we investigated the glucose-stimulated OCR at basal (3 mM glucose) and stimulated (20 mM glucose) levels after 24 h culturing with the drug in combination compared to the drug alone. Interesting, we found a similar decrease in glucose-stimulated OCR in islets treated with the combination of high-dose (30 µg/l) tacrolimus and sirolimus and each drug alone (Fig. 5), suggesting that the reduced intracellular sirolimus concentration by tacrolimus in islets could contribute to a threshold of toxicity due to the combination treatment.

ABCB1, OATP1B1, and CYP3A4 are expressed in human islets

The expression of drug transporters in pancreatic islets could influence the availability of immunosuppressive

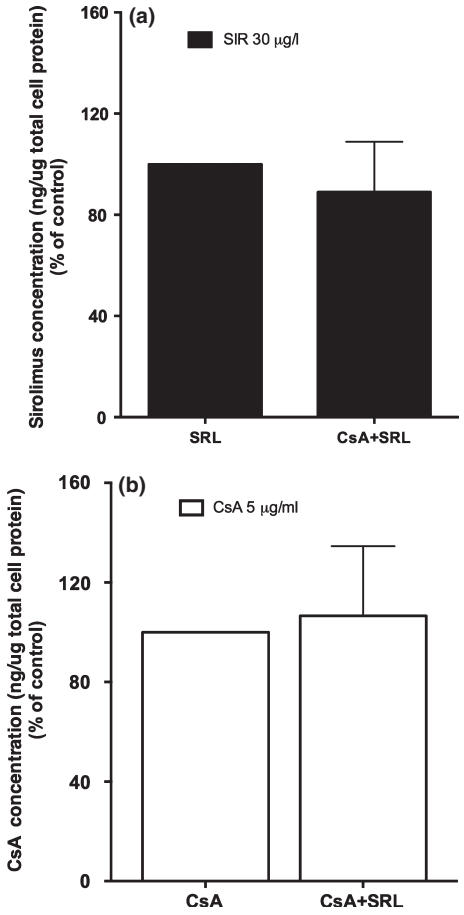

Figure 3 Effect of CsA on intracellular concentration of SRL in human islets. Human islets were cultured with the combination of SRL (30 μg/l) and CsA (5 μg/ml), or the drug alone for 24 h before the intracellular concentration of SRL (a) or CsA (b) was measured in islet lysate and normalized to total protein as detailed in methods. Data are calculated as percentages of control and are presented as mean ± SD, $n = 6$ for each group. CsA, cyclosporine A; SRL, sirolimus.

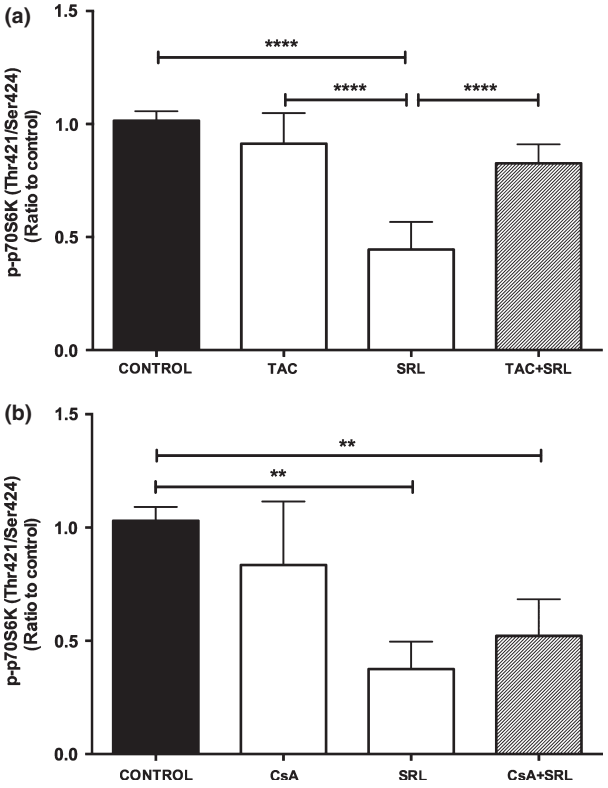

Figure 4 The effect of SRL, TAC, or CsA on phosphorylation of p70S6k in islets. Human islets were cultured with TAC (30 μg/l), SRL (30 μg/l), or the combination thereof for 24 h before the presence of p-p70s6k was assessed by the cell-signaling Bio-Plex assay in human islet cell lysate and normalized to total protein (a). In a parallel experiment, human islets were cultured with SRL (30 μg/l), CsA (5 μg/ml), or the combination thereof for 24 h before p-p70S6k was detected in the lysate and normalized to total protein. Data are calculated as ratio to control and are presented as mean ± SD, $n = 3$–6 for each group. TAC, tacrolimus; SRL, sirolimus; CsA, cyclosporine A, ** $P < 0.01$, **** $P < 0.0001$.

drugs in islets and thereby the toxicity. We investigated the drug transporters OATP1B1 and ABCB1 and the metabolizing enzyme CYP3A4 expression in islets. Real-time qPCR analysis showed that the ABCB1 gene was expressed, in human islets at the level of 52% relative to the liver control sample (Fig. 6a). The CYP3A4 and OATP1B1 were also expressed in the islets; however, the expression levels were very low (<1% relative to the liver control sample) (Fig. 6a). OATP1B1 mRNA expression was reduced by 56% after exposure to sirolimus compared to control islets or 46% compared to tacrolimus treated islets, whereas the exposure of the combination of tacrolimus and sirolimus tended to increase the expression compared to sirolimus alone (Fig. 6b).

Having shown that drug transporters and metabolizing enzyme are expressed in islets, we wanted to determine whether the drug interaction between tacrolimus and sirolimus could be related to altered expression of OATP1B1, ABCB1, or CYP3A4. However, there were no significant changes in ABCB1 nor CYP3A4 gene expression in islets after exposure to tacrolimus, sirolimus, or the combination compared to control islets (data not shown), implicating that the reduced intracellular sirolimus concentration caused by tacrolimus was not due to induction of ABCB1 or CYP3A4. Detection of protein expression of ABCB1 in human islets was also determined by immunofluorescent staining of dispersed islets. ABCB1 encodes P-glycoprotein (Pgp). Only few cells stained positive for ABCB1(Pgp) in relation to insulin or glucagon (Fig. 6c,d), suggesting this protein may not be involved in the secretory mechanisms in beta- nor alpha-cells.

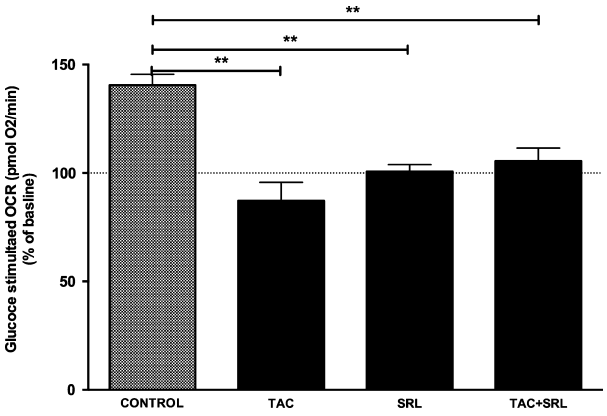

Figure 5 Oxygen consumption rates (OCR) in human islets after treatment of TAC, SIR, or SIR+TAC. Human islets were cultured with TAC (30 μg/l), SIR (30 μg/l), or a combination thereof for 24 h before the glucose-stimulated OCR was measured as indicated in methods. OCR is expressed as percentage of baseline and is presented as the mean ± SD, $n = 6$ for each group. TAC, tacrolimus; SRL, sirolimus, ** $P < 0.01$.

Discussion

Optimization of the balance between efficacy and drug-related toxicity is a main goal in clinical practice. The direct toxicity on islets by tacrolimus and sirolimus has been extensively reported [30]. However, the accumulation of the immunosuppressive drugs added to human islets alone or in combination has to our knowledge not been studied. To better understand the drug–drug interactions of the CNIs and mTOR inhibitors, we investigated the effect of a combination treatment of tacrolimus and sirolimus on the intracellular drug concentration in human islets using an *in vitro* approach. Our main findings were that intracellular islets sirolimus concentration was significantly reduced when islets were exposed to a combination of tacrolimus and sirolimus, also with impact on the p70S6K-signaling pathway. Islets function was decreased after treatment of high doses of tacrolimus and sirolimus, but the combination of the two drugs did not have additive negative effect on the islet function compared to the treatment of each drug alone. We can therefore not exclude interplay between the observed reduced sirolimus concentration by tacrolimus and islets function. The role of toxicity synergisms of the two drugs should be investigated in future mechanistic studies. We showed that human islets express the membrane transporters of ABCB1, OATP1B1, and metabolic enzyme CYP3A4, but in our study, we did not find these to be involved in the reduced sirolimus concentration by tacrolimus.

Immunosuppressive drug treatment is routinely adjusted according to serum drug concentrations, to achieve efficient therapy, and avoid adverse drug effects [31,32]. Drug entry into cells is assumed limited by permeability and diffusion along a concentration gradient where the main obstacle is the lipoprotein containing bilayer of the cell membrane. We found a poor correlation between the extracellular and intracellular drug levels of tacrolimus and sirolimus in human islets after *in vitro* exposure. One reason for this could be slow kinetics uptake of tacrolimus and sirolimus in human islets. In our study, we investigated up to 48-h exposure time and found that the intracellular volume increased, but the discrepancy between sirolimus alone and in combination with tacrolimus remains the same. We cannot rule out that the saturating threshold for sirolimus in human islets might be at a concentration below 10 μg/l. However, a previous study has shown that variations in drug clearance and absorption rates result in a wide range of sirolimus C_{min} values among patients receiving the same dose [33].

One could also argue that the constant high drug concentration seen when islets are exposed to drugs *in vitro* compared to *in vivo* cause a more toxic environment. However, *in vivo* peak portal drug concentration after islet transplantation is generally double that of systemic level after oral administration measured by peripheral blood concentration. In this study, our *in vitro* doses fare from exceeds the doses islets are exposed to *in vivo* [13].

Despite previous findings that tacrolimus has no significant effect on the systemic pharmacokinetics of sirolimus [34], we found that tacrolimus reduces the intracellular concentration of sirolimus in human islets exposed to the combination of tacrolimus and sirolimus by approximately 50%. Both tacrolimus and sirolimus are known ligands of FK506-binding protein 12 (FKBP12). A competitive binding to FKBP12 would explain the tacrolimus-mediated reduction in intracellular sirolimus in our study. Also, tacrolimus and sirolimus antagonize one another and have an affinity for the same binding site [35–37]. Measuring the intracellular drug concentration rather than blood concentrations to establish the best therapeutic dose is impossible; therefore, using *in vitro* models to verify the intracellular interactions might be the first step in the direction to study new immunosuppressive drug combinations that can decrease toxicity and prolong graft survival in islet transplantation.

The activation of p70S6K via mTOR complex 1 has been implicated in the control of islet proliferation [38] with glucose as one of the activators completely inhibited by sirolimus [24]. We used the phospho-p70S6K as a verification of the mTOR involvement and thereby the pharmacodynamics of intracellular sirolimus. Demonstrating that when tacrolimus and sirolimus are combined, tacrolimus antagonizes the inhibitory effects on phosphorylation of p70S6K caused by sirolimus [39]. Islets exposed to the combination sirolimus and CsA showed no

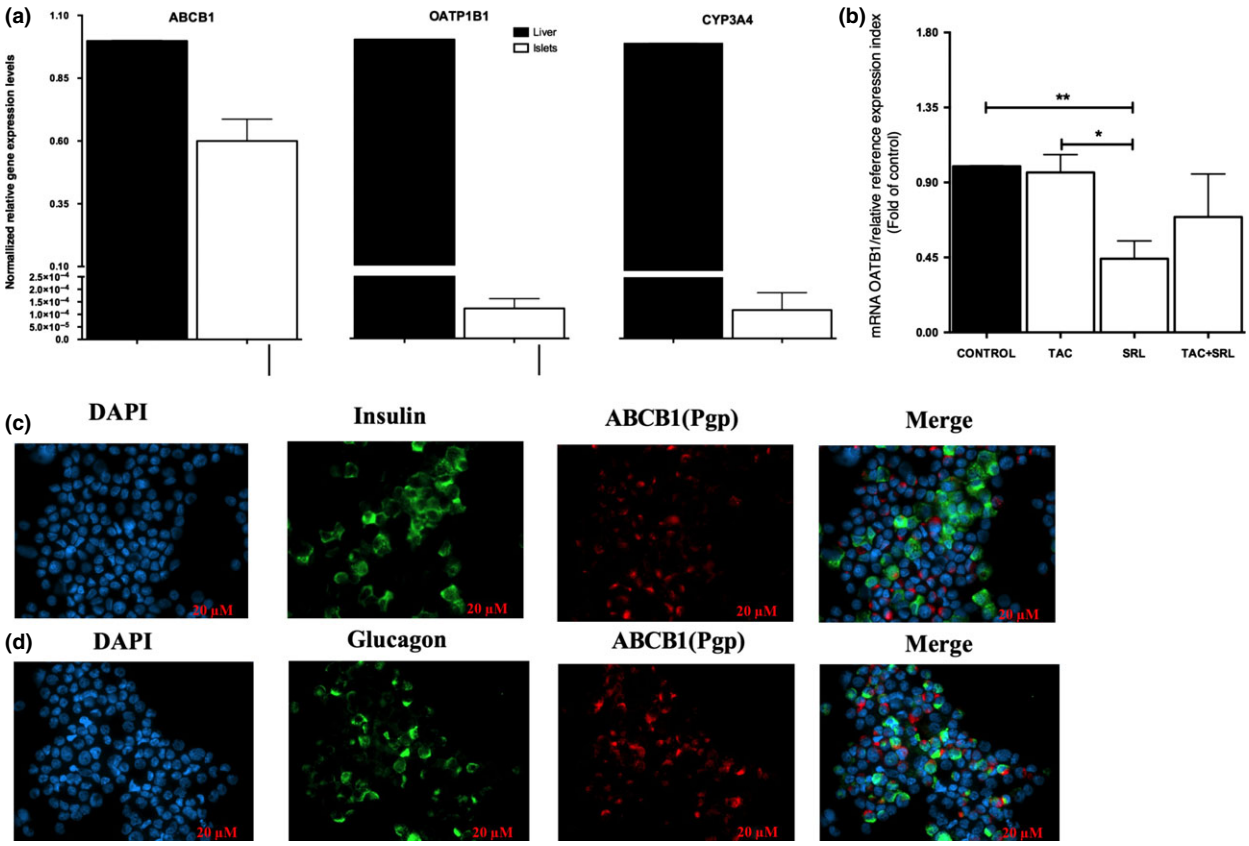

Figure 6 Expression of ABCB1 (Pgp), OATP1B1, and CYP3A4 in human islets. Human islets were cultured for 24 h before the expression of the drug transporter (ABCB1(Pgp) and OATP1B1), and the metabolic enzyme CYP3A4 was evaluated. RNA was prepared and subjected to qPCR as detailed in methods. The reference gene index is calculated by the mean of ALAS1, B2M, and *RPL13A* expression and used to normalize the expression of target genes in isolated hepatocytes relative to the mRNA level of ABCB1(Pgp), OATP1B1, and CYP3A4 in human islets (a). OATB1 mRNA expression in human islets was normalized to reference gene index after exposure to TAC (30 µg/l), SRL (30 µg/l), or a combination thereof for 24 h (b). Representative immunofluorescence image of dispersed human islets stained for insulin (green), ABCB1(Pgp) (red) and nuclear staining with DAPI (blue) (c), or glucagon (green), ABCB1(Pgp) (red) and nuclear staining wit DAPI (blue) (d). Data are presented as mean ± SD, $n = 4$–5 for each group. TAC, tacrolimus; SRL, sirolimus; *$P < 0.05$; ** $P < 0.01$.

similar effect, which indicates that tacrolimus limit the inhibitory effect of sirolimus on p70S6K activation [40].

Drug transporters and drug-metabolizing enzymes play important roles in the absorption, distribution, and elimination of many drugs [41]. The transport membrane protein ABCB1 encoding P-glycoprotein (Pgp) has been detected at the surface of the endocrine islet cells [18], and CYP3A4 has been found in both islets and acinar cells of rodents [42]. We hereby demonstrate for the first time expression of ABCB1, OATP1B1, and CYP3A4 in isolated human islets. The inadequacy in bioavailability of tacrolimus and sirolimus is due to processes involving ABCB1 in the gut [43]. As tacrolimus and sirolimus are substrates of ABCB1, the presence in islets could also involve the pharmacokinetic interaction between tacrolimus and sirolimus. A recent study did not detect OATP1B1 in pancreas tissue but that might be due to differences in research methods [22]. The quantity of OATP1B1 was significantly reduced by the presence of sirolimus, whereas the same did not occur when islets were exposed to tacrolimus. Interestingly, the combination of tacrolimus and sirolimus tended to increase OATP1B1 expression which may indicating that tacrolimus antagonizes the sirolimus inhibition of OATP1B1 expression. The clinical importance of this is not known and will be an aim for further studies. Investigate the function of these transporters in islets may be important determinants for long-term islet survival after transplantation as well as the pharmacokinetic interactions between tacrolimus and sirolimus [44].

Published data on the pharmacokinetic interaction between sirolimus and tacrolimus are limited and inconsistent. Both drugs have a narrow therapeutic window with a need for drug monitoring. The effect of tacrolimus on long-term sirolimus exposure in renal transplantation has

been observed [45] as well as a need for higher dose of sirolimus when combined with tacrolimus than with CsA [46]. Sirolimus monotherapy is not sufficient to suppress rejection in an islet graft [47]. Our findings could offer an explanation to why the islets toxicity of the drug combination does not exceed that of each drug alone and why higher dose of sirolimus is needed when administered with tacrolimus than with CsA [46].

In conclusion, this study shows that the intracellular concentration of sirolimus in human islets is decrease when given in combination with tacrolimus, and this drug interaction influences the p70S6K-signaling pathway. Although we confirm that the drug transporter ABCB1 is expressed in human islets both at mRNA and protein levels, no involvement in the drug–drug interaction of tacrolimus and sirolimus was found. OATP1B1 and CYP3A4 are also expressed in islets, however, at low levels. Their roles in the pharmacokinetic processes of human islets are unknown.

Authorship

K.K-J, H.S.: designed and conducted the study, researched data, and wrote the manuscript. N.T.V., S.B.: participated in the conduct of the study, researched data, and reviewed/edited the manuscript. S.A.: designed and conducted the immunohistochemistry analysis. S.B, O.K, A.F: contributed to the design of the study, the discussion, and reviewed/edited the manuscript.

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