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Author: P.C. Lopes A. Fuhrmann F. Carvalho J. Sereno M.R. Santos M.J. Pereira J.W. Eriksson F. Reis E. Carvalho

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Cyclosporine A enhances gluconeogenesis while Sirolimus impairs insulin signaling in peripheral tissues after 3 weeks of treatment

PC Lopes[1], A Fuhrmann[1], F Carvalho[1], J Sereno[2,3], MR Santos[1], MJ Pereira[4], JW Eriksson[4], F Reis[2], E Carvalho[1,5]

[1]*Center for Neuroscience and Cell Biology, University of Coimbra, 3000 Coimbra, Portugal*
[2]*Laboratory of Pharmacology & Experimental Therapeutics, IBILI, Medicine Faculty, University of Coimbra, 3000-548 Coimbra, Portugal*
[3]*Institute for Nuclear Sciences Applied to Heath – ICNAS, University of Coimbra, Portugal*
[4]*Department of Medical Sciences, Uppsala University, 75185 Uppsala, Sweden*
[5]*The Portuguese Diabetes Association (APDP), 1250 203 Lisbon, Portugal*

Author's e-mails: patriciacmmlopes@gmail.com (PCL); lia2211@hotmail.it (AF); fabcarvalho27@gmail.com (FC); jose6sereno@hotmail.com (JS); martarebelosantos@gmail.com (MRS); maria.pereira@medsci.uu.se (MJP); jan.eriksson@medsci.uu.se (JE);freis@fmed.uc.pt (FR); ecarvalh@cnc.uc.pt (EC)

Corresponding Author: *Eugenia Carvalho
Center for Neurosciences and Cell Biology, University of Coimbra,
3004-517 Coimbra, Portugal.
Tel; +351 239 853 406 Fax: +351 239 853 409 E-mail address: ecarvalh@cnc.uc.pt

Abstract

Cyclosporine A (CsA) and sirolimus (SRL) are immunosuppressive agents (IA) associated with new-onset diabetes after transplantation (NODAT). This study aims to evaluate the effects of 3-weeks of treatment with either CsA (5 mg/kg BW/day) or SRL (1mg/kg BW/day) on insulin signaling and expression of markers involved in glucose metabolism in insulin-sensitive tissues, in Wistar rats.

Although no differences were observed in fasting glucose, insulin or C-peptide levels, both treated groups displayed an impaired glucose excursion during both glucose and insulin tolerance tests. These results suggest glucose intolerance and insulin resistance.

An increase in glucose-6-phosphatase protein levels (68%, $p<0.05$) and in protein-tyrosine phosphatase 1B (163%, $p< 0.05$), a negative regulator of insulin was observed in the CsA-treated group in the liver, indicating enhanced gluconeogenesis and increased insulin resistance. On the other hand, glucokinase protein levels were decreased in the SRL group (35%, $p<0.05$) compared to vehicle, suggesting a decrease in glucose disposal. SRL treatment also reduced peroxisome proliferator-activated receptor γ coactivator 1 alpha protein expression in muscle (~50%, $p<0.05$), while no further protein alterations were observed in muscle and perirenal adipose tissue nor with the CsA treatment. Moreover, the phosphorylation of key proteins of the insulin signaling cascade was suppressed in the SRL group, but was unchanged by the CsA treatment.

Taken together, these data suggest that CsA treatment enhances gluconeogenic factors in liver, while SRL treatment impairs insulin signaling in peripheral tissues, which can contribute to the development of insulin resistance and NODAT associated with immunosuppressive therapy.

Keywords: immunosuppressive agents, insulin signaling, gluconeogenesis, adipocyte, muscle, liver

List of abbreviations

Akt	Protein kinase B
AS160	Protein kinase B substrate of 160 kDa
CsA	Cyclosporine A
FKBP12	FK506-binding protein (12-kD)
FOX	Forkhead box
GK	Glucokinase
GLUT	Glucose transporter
G6P	Glucose-6 phosphate
G6Pase	Glucose-6-phosphatase
IR	Insulin receptor
IRS	Insulin receptor substrate
mTOR	Mammalian target of rapamycin
NODAT	New onset diabetes after transplantation
p70S6K	p70 ribosomal S6 kinase
PDK1	Phosphoinositide-dependent kinase 1
PEPCK	Phosphoenolpyruvate carboykinase
PI3K	Phosphatidylinositol 3-kinase
PGC1-α	Peroxisome proliferator-activated receptor- coactivator
PTP1B	Protein-tyrosine phosphatase 1B
SRL	Sirolimus

1. Introduction

Immunosuppressive therapy is used in the treatment of autoimmune diseases and after organ transplantation, to promote tolerance to allografts [1]. Two of the main immunosuppressive agents are cyclosporine A (CsA) and sirolimus (SRL). CsA is a peptide of fungal origin that forms a complex with its intracellular receptor, cyclophilin A, an important intracellular acceptor protein with peptidyl-prolyl *cis-trans* isomerases (PPIase) activity [2]. Consequently, the drug-immunophilin complex binds to and inhibits the serine-phosphatase activity of calcineurin required for T-cell activation. Prevention of the calcineurin-mediated dephosphorylation of the transcription nuclear factor of activated T-cells, blocks its translocation to the nucleus. Interleukin (IL)-2 production is inhibited and, consequently also the proliferation and differentiation of T-cells [1, 3]. On the other hands, SRL, an antifungal macrolide, binds to the 12-kD FK506-binding protein (FKBP12) and this complex inhibits the target of rapamycin (TOR) Ser/Thr kinase. As mTOR regulates mRNA translation initiation and progression from the G1 to S phase of the cell cycle, its inhibition prevents T-cell proliferation [4].

Although these immunosuppressive agents are very effective in their function, they are also responsible for the development of metabolic complications, linked to higher rates of cardiovascular disease and infections, which are the major causes of morbidity and mortality after transplantation [5-7]. One of the complications is NODAT, usually manifested in the first few months post-transplantation and varying according to the type of immunosuppressive agent, their different combinations and patient demographics [8]. NODAT is reported in 2.5 to 40% of patients that underwent renal, liver, heart or lung transplant [9]. Similar to type 2 diabetes, NODAT has been associated with impairment in glucose tolerance, insulin secretion and dysfunctional hepatic gluconeogenesis [10]. Insulin directly regulates gluconeogenesis, however in insulin resistance states it does not properly suppresses gluconeogenesis in the

liver, leading to enhanced activation of forkhead box-containing transcription factors of the FOXO subfamily, promoting increased transcription of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), rate-limiting enzymes in hepatic glycogenolysis and gluconeogenesis, respectively [11, 12]. Moreover, according to Ropelle *et al*. [13] the physical interaction of peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1α) and FOXO1 promote an important signal transduction pathway responsible for the synthesis of glucose by the liver. Furthermore, PGC-1α expression is a tissue-specific regulatory marker activated in diabetic states, as well as the fasted state. It is perhaps responsible for increased hepatic glucose production and consequently hyperglycemia [13, 14], making it a marker of interest together with its downstream targets.

On the other hand, insulin participates in many physiological processes, particularly important in maintaining glucose homeostasis. After a meal, glucose increases in circulation, stimulating the secretion of C-peptide and insulin, which inhibit glycogenolysis and gluconeogenesis, promoting at the same time glycogen synthesis and glucose uptake. Insulin binds to its cell surface receptor (IR), activating its intrinsic tyrosine kinase activity and leading to receptor auto-phosphorylation, which in turn leads to the phosphorylation of insulin receptor substrates proteins (IRS-1 – IRS-4). As a result, several downstream signaling pathways are activated, including the p85 regulatory subunit of PI3-kinase and protein kinase B (Akt/PKB). This last step activates pyruvate dehydrogenase kinase 1 (PDK1) and protein kinase C (PKC), leading to the translocation of the muscle and fat specific glucose transporter (GLUT)4 from intracellular vesicles to the plasma membrane [15]. Alterations in these signaling pathways may affect glycemia and lead to unwanted metabolic consequences like diabetes and dyslipidemia [15]. Although CsA and SRL have been linked with NODAT, the underlying mechanisms are still not completely understood. SRL has been shown to improve insulin-stimulated glucose uptake and Akt/PKB phosphorylation in L-6 muscle cells, 3T3-L1 cells

and in differentiated adipocytes [16-18], while other studies have shown reduced glucose uptake [19] and Akt/PKB phosphorylation in human mature adipocytes [20]. On the other hand, while immunosuppressive agents like CsA have been involved in the inhibition of the phosphorylation of the IR, it has not been associated with alterations in the expression or phosphorylation of proximal insulin signaling cascade proteins (Pereira *et al.*, unpublished data), [21]. Therefore, there is still a lack of consensus regarding the underlying mechanism for NODAT caused by both CsA and SRL.

Recently, we and others have reported that treatments with either CsA or/and SRL leads to metabolic alterations in liver, muscle and adipose tissue and possibly contribute to the development of dyslipidemia and insulin resistance associated with immunosuppressive therapy; however no insulin signaling studies have been performed to unravel the underlying mechanisms in these tissues [5, 19, 22-24]. Therefore, the main aim of this in vivo study is to understand how these immunosuppressive agents affect gluconeogenesis and insulin signaling in liver, muscle and adipose tissue after 3 weeks of treatment, in a rodent model.

2. Materials and methods

2.1 - Chemicals

CsA (Sandimmune Neoral®) was supplied by Novartis Pharma (Lisbon, Portugal) and SRL (Rapamune®) by Wyett Europe Ltd (Berkshire, United Kingdom) through the Pfizer Laboratories Lda (Lisbon, Portugal). Human insulin, Actrapid was kindly provided by NovoNordisk A/S (Lisbon, Portugal). Ketamine (Ketalar®, Parke-Davis) was purchase from Pfizer Labs, while chlorpromazine (Largatil®, Rhône-Poulenc Rorer) was from Vitória labs (Amadora, Portugal). The High Capacity cDNA Reverse Transcription kit was obtained from Applied Biosystems (Forest City, CA, USA) and the RNeasy® MiniKit and the QIAzol®

Lysis Reagent from QIAGEN Sciences (Germantown, MD, USA). Diethyl pyrocarbonate (DEPC) was acquired from AppliChem (Darmstadt, Germany). Methanol, isopropanol and chloroform were obtained from Merck (Darmstadt, Germany). PCR primers were designed by us, using Vector NTI Advanced 10 Software (Life technologies, Grand Island, NY, USA) and were synthesized by Integrated DNA Technologies, Inc (IDT, Coralville, IA, USA).

2.2 - Animals and treatments

Male Wistar rats, weighing ~300 g, 10 weeks old, were obtained from Charles River Lab. Inc. (Barcelona, Spain). Animal studies were conducted using protocols approved by to the National and European Community Council Directives on Animal Care. The animals were housed in a light-controlled 12 h dark/light cycles and were given standard laboratory chow (IPM-R20, Letica, Barcelona, Spain) and free access to tap water. Body weight was monitored every week [19].

Animals were randomly divided into three groups (n=16 per group): Vehicle (orange juice); CsA – 5 mg/kg body weight (BW)/day of Sandimmune Neoral® and SRL – 1 mg/kg BW/day of Rapamune®. The agents were diluted in orange juice as is the usual procedure in the clinic for the patients [25]. The use of a diluted form of orange juice was applied to vehicle and CsA and SRL-treated rats eliminating or highly minimizing any possible effect in glucose metabolism. Doses were chosen to have blood concentration achieved within the recommended therapeutic windows for CsA and SRL [19]. Treatments were performed daily by esophageal gavage for 3 weeks. At the end of the treatments, rats were anesthetized i.p. with 2 mg/kg/BW of a 2:1 (v:v) Ketamine solution in 2.5% Chlorpromazine. In each group, 8 animals received a bolus of insulin - Actrapid (i.p 10 U/kg) and were sacrificed 10 minutes later in order to study insulin action in vivo, in the insulin sensitive tissues. The other 8 animals received saline as a control. Blood samples were collected from the jugular vein for

biochemical analysis and liver, skeletal muscle (posterior thigh of the rat leg), perirenal and epididymal adipose tissues were rapidly harvested and frozen in liquid nitrogen for further analyses. Liver, muscle, epididymal and perirenal adipose tissues were used to perform quantitative RT-PCR and western blots. Epididymal adipose tissue was used for insulin signaling to be correlated with the insulin-stimulated glucose uptake results assessed and presented in our previous work [19].

2.3 – Glucose and insulin tolerance tests, fasting serum glucose, insulin, C-peptide and glycogen measurements

A glucose tolerance test (GTT) and an insulin tolerance test (ITT) were performed at the end of 3 weeks of treatments. A glucose solution was injected (i.p. 2 g/kg BW) after a 16 hour fasted for the GTT and for the ITT, a solution of insulin (i.p 1 U/kg BW; Actrapid) diluted in saline 0.9% (w/v) after a 6 hour period of fasting. Blood was collected from the tail vein prior to (0 min) and at the various times after injection, as indicated in figure 1 A and B. Blood glucose levels were measured using a glucometer (AccuChek Active, Roche Diagnostics®, Indiana, USA). From fasting serum samples collected on the day of sacrifice, C-peptide and insulin were determined by an ELISA kit (Mercodia, Uppsala, Sweden). Liver tissue was prepared according to the manufacturer´s instructions. Aliquots were centrifuged at 18,000 × *g* for 10 min and the supernatants were used to analyze glycogen concentration using a kit (Abnova, VWR international, Carnaxide, Portugal).

2.3.1 – Glucose clearance rate in the urine

Animals (n=6/group) were housed in metabolic cages during 24 hours and received tap water and food *ad libitum.* The 24 hour urine was collected, volume was measured and glucose

concentration was determined using Cobas Integra® 400 plus (Roche Diagnostics®, Indiana, USA), in order to calculate the glucose clearance rate.

2.6 - Liver, muscle and adipose tissue gene expression

Total RNA from liver, muscle and perirenal adipose tissue was isolated with RNeasy Mini Kit and the concentration was determined by OD260 measurement using the NanoDrop spectrophotometer (Thermo Scientific, USA). cDNA synthesis was performed using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems; Forest City, CA, USA). Relative mRNA levels were measure by RT-PCR using specific primers for each target mRNA and Sybr-green PCR mix (Quanta Biosciences, Inc., Gaithersburg, MA, USA) with a CFX Manager ^{TM} version 2.0 Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative RT-PCR results were analyzed through delta CT calculations. Relative mRNA levels for the different genes: FOXO1 (*FOXO1*); PGC1-α (*PPARGC1A*); PTP1B (*PTPN1)*; Glucose-6-phosphatase (*G6PC*); Phosphoenolpyruvate carboxykinase (*PCK*); Glucokinase (*GCK*); Insulin receptor (*INSR*); IRS-1 (*IRS-1*); GLUT1 (*SLC2A1*); GLUT2 (*SLC2A2*); GLUT4 (*GLUT4*) were determined and normalized using both glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and TATA – binding protein (*TBP*) mRNA levels. All primer sequences are available upon request.

2.5. Tissue lysates and immunoblotting

Liver, muscle, perirenal and epididymal adipose tissues were homogenized and total protein was extracted in lysis buffer (25 mM, Tris-HCl (pH 7.4), 0.5 mM EDTA, 25 mM NaCl, 1% (v/v), Nonidet P-40, 1 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$ and protease inhibitors - Sigma, St. Louis, MO, USA). Aliquots of total lysate were subjected to SDS-PAGE, transferred to a PVDF membrane and immunoblotted with the primary antibody according to

the manufacturer's instructions, and thereafter incubated with the appropriate secondary antibody. The primary antibodies ***IR Tyr1146, IRS-1, PI3Kp85, FOXO1, Akt Ser473, Akt Total, p70S6KThr 421/424, p70S6K, AS160 Ser642 and mTOR*** were purchased from Cell Signaling Technologies (Beverly, MA, USA). The primary antibodies ***Akt Thr308, G6Pase, PEPCK, GK, PTP1B*** were acquired from Santa Cruz Biotecnology, Inc. (Dallas, Texas, USA); ***GLUT1, GLUT2, GLUT4, Akt 2/β, mTOR Ser2448, AS160 total*** were purchased from Millipore (Billerica, MA, USA); and ***IRS-1 Tyr612*** was from Invitrogen (Life Technologies, Carlsbad, CA, USA). Protein expression was normalized using either α-tubulin (Cell Signaling Technologies) or the β-actin antibodies (Sigma) to avoid overlap of bands. Detection was performed by using Enhanced ChemiFluorescence **(**ECF**)** (GE Healthcare Bio-Science, Pittsburgh, PA, USA) and the generated signals were analyzed using the Image Lab^{TM} 4.1 TMsoftware (Bio-Rad Laboratories, Hercules, CA, USA).

2.6 - Statistical Analysis

Statistical analyses were performed using the GraphPad Prism software, version 5 (GraphPad Software Inc., La Jolla, CA, USA). Results are given as mean ± standard error of the mean (SEM). Differences between groups were tested by performing analysis of variance (One-Way ANOVA) and differences between basal and insulin stimulated phosphorylation were assessed through the unpaired Student-t-test. A $p<0.05$ was considered statistically significant.

3 -Results

3.1 GTT, ITT, glucose, insulin, C-peptide in serum and glycogen measurements in liver

GTTs were performed at the end of the treatments. The measurements revealed that glucose tolerance was impaired in the CsA-treated animals. The CsA-treated group displayed a peak

of glucose (18.62 ± 1.80 mmol/l) 15 minutes after the glucose bolus (2 g/kg BW, i.p.), when compared to either the vehicle group (11.16 ± 1.56 mmol/l, $p<0.001$) or the SRL-treated group (10.97 ± 1.68 mmol/l; $p<0.001$) (Fig 1A). However no significant differences were observed in the remaining time points during the GTT between the vehicle and the SRL groups. The glucose excursion curve for SRL was also impaired and the recovery kinetics of the blood glucose levels was significantly slower. Furthermore, the ITT curve revealed that insulin sensitivity was impaired in the CsA treated animals (Fig 1B). For the SRL group, a significant increase in blood glucose levels during an ITT was only observed at 60 min (3.76 ± 0.08 mmol/l) compared to the vehicle group (2.65 ± 0.14 mmol/l, $p<0.05$). Although, no significant differences were found in the AUC for the GTT (Fig. 1A), the AUC for the ITT was 67% ($p<0.001$) and 55% ($p<0.001$) increased in CsA and SRL-treated groups, compared to vehicle (Fig. 1 B). This result further confirmed reduced insulin sensitivity for both the CsA and SRL-treated animals. No significant differences were found in the glucose or insulin levels in the fasted state between groups. A non-significant decrease in fasting C-peptide levels was observed in both treated groups (Fig. 1C, D and E). The liver glycogen content was 0.61 ± 0.03 µg/µl in the vehicle group and it was not significantly different between the CsA- and the SRL-treated groups (0.55± 0.03 and 0.52 ± 0.04, respectively).

3.1.2 Glucose clearance rate in the urine

Animals in the SRL group exhibited a trend for an increased glucose clearance rate (0.14 ±0.04 ml/h/rat), compared with the CsA (0.06 ± 0.01 ml/h/rat; $p=0.06$) but no significant difference compared with the vehicle-treated group with the present study power (0.09 ± 0.01 ml/h/rat). This result suggests that an excess of glucose is present in the urine flux and is being expelled via the kidneys (Fig. 1F).

3.3 Effect of CsA and SRL on protein and gene expression in liver

3.3.1 Gluconeogenesis is modulated by either CsA or SRL

To evaluate if CsA or SRL treatment affects gluconeogenesis, we evaluated liver expression levels of some of the important key players. Although no significant changes were observed in the transcription factors PGC1-α and FOXO1 at gene level, a tendency for an increase in the protein expression was observed in the CsA group (PGC1-α $p=0.09$) (Fig. 2A). Moreover, a significant increase in protein expression for G6Pase, an enzyme that regulates hepatic gluconeogenesis, was observed in the CsA group ($p<0.05$) compared with the vehicle group (Fig.2B). No significant differences were observed for PEPCK both for gene and protein expression in the same group (Fig. 2B). The expression of GK protein, an important contributor for the formation of glycogen and responsible for the phosphorylation of glucose into glucose-6-phosphate, was not changed in the CsA group, but was significantly decreased in the SRL group (35%, $p<0.05$) (Fig. 2B).

Moreover, we evaluated PTP1B expression, an important marker that negatively regulates insulin action, and found a significant increase in protein levels in the CsA group (163%, $p< 0.05$) when compared to the vehicle group (Fig. 2A).

3.3.2 Effects of CsA and SRL on insulin signaling in the liver

To further elucidate signaling events that might promote the impaired glycemia and insulin-stimulated glucose uptake observed in our previous work [19], we evaluated the activation of important insulin signaling markers. Total IRS-1 protein level was increased in the SRL group (49%, $p<0.05$), while GLUT1 tended to decrease in the same group (Fig. 3A). No significant

changes were observed in the gene levels for IRS-1, GLUT1 and GLUT2 in both treated groups compared to vehicle (Fig. 3A). Insulin stimulation significantly increased phosphorylation of IRS-1 at Tyr612 and Akt at both Ser473 and Thr308, but no significant differences were found for IR at Tyr1146, IRS-1 at Tyr612, mTOR at Ser2448 and p70S6K at Thr421/424 (Fig 3B). SRL treatment reduced phosphorylation of all studied insulin signaling proteins, while CsA did not affect phosphorylation of any of the proteins analyzed. Total protein levels of the respective markers were not affected by either treatment.

3.4 Effects of CsA and SRL on protein and gene expression in muscle

3.4.1 SRL decreases PGC1-α in muscle.

SRL treatment reduced PGC1-*α* protein expression in muscle (~50%, $p<0.05$), compared to the vehicle group (Fig. 4A), while CsA had no effect. No changes were found for FOXO1 and PTP1B gene and protein expression with either treatment.

3.4.2 Effect of CsA and SRL on insulin signaling in muscle

We further evaluate the effects of CsA and SRL treatment on insulin signaling in muscle. No significant changes were found in gene expression for either IRS-1 or GLUT4, while GLUT1 was significantly increased in the CsA group ($p<0.03$). Furthermore, there were no significant differences for IRS-1 and GLUT1 protein levels in the SRL group, and no changes were observed in GLUT4 protein expression in either treated group (Fig. 5A). To determine whether a therapeutic dose of these IAs would affect insulin signaling in muscle, the phosphorylation of important key players were assessed. Insulin stimulation significantly

increased phosphorylation of IRS-1 at Tyr612 and Akt at both Ser473 and Thr308 (Fig 5B). SRL treatment reduced phosphorylation of IRS-1 at Tyr612, Akt at Thr308, mTOR at Ser2448 and p70S6K at Thr421/424, compared with the vehicle group. On the other hand, while no changes were observed for Akt phosphorylation on Ser473 by CsA treatment, Akt Thr308 phosphorylation was impaired. However, the total protein levels of these markers were not altered by either treatment (Fig. 5 B).

3.5 Effect of CsA and SRL on protein and gene expression in adipose tissue

3.5.1 Neither CsA nor SRL affected PTP1B, PGC1-α, or FOXO1 protein levels in perirenal adipose tissue

In perirenal adipose tissue, although SRL treatment reduced the gene expression of PGC1-α (61%, $p<0.05$) compared to the vehicle group, protein levels were similar between the groups. Moreover, no changes were found for either FOXO1 or PTP1B gene or protein expression levels in this tissue (Fig. 6A).

3.5.2 Effects of CsA and SRL on insulin signaling in adipose tissue

We further evaluate the effects of CsA and SRL treatment on insulin signaling in adipose tissue. In perirenal adipose tissue, no changes were observed in IR or IRS-1 gene (Fig. 7A), or protein levels in the SRL treated group. No significant changes were either observed for GLUT1 and GLUT4 gene or protein levels in the SRL group, at the present study power. CsA treatment did not affect gene or protein expression for IR, IRS-1, GLUT1 or GLUT4 (Fig. 7A).

In epididymal adipose tissue, insulin stimulation significantly increased phosphorylation of IR at Tyr1146, IRS-1 at Tyr612 and Akt at Ser473 (Fig 7B). Treatments with both CsA and SRL significantly impaired phosphorylation of IR Tyr1146 residue compared to vehicle. On the other hand, SRL treatment reduced Akt phosphorylation at Ser473, mTOR at Ser2448 and p70S6K at Thr421/424, compared with the vehicle group (Fig. 7B). Total proteins levels were not altered by either treatment.

Discussion

The present study indicates that an in vivo 3 week-treatment of Wistar rats with either CsA or SRL impairs glucose metabolism. Treatment with CsA resulted in impaired glucose tolerance and insulin sensitivity as demonstrated during a GTT and an ITT, respectively. Moreover, treatment with CsA increased protein expression of key enzymes for hepatic gluconeogenesis, G6Pase and PEPCK, and the upstream transcription factors PGC1-α and FOXO1 were also increased in the liver, providing insight into the molecular mechanisms for the elevation of glucose in the blood. Moreover, PTP1B protein levels were also increased in the liver in the CsA-treated group, which may contribute to impaired insulin sensitivity observed during the treatment. Although SRL had no effect on the expression of genes or proteins involved in gluconeogenesis in the liver, it significantly decreased GK protein expression, an enzyme responsible for the phosphorylation of glucose to glucose-6-phosphate, possibly leading to decreased glucose disposal. In addition, the effects of these agents on activation of insulin signaling in the liver, muscle and adipose tissue were evaluated. SRL treatment reduced Akt phosphorylation in these tissues, leading to reduced AS160 phosphorylation. These effects combined might impair GLUT4 translocation, which we did not measured for lack of tissue, explaining the reduction in glucose uptake observed previously [19]. Altogether, these results

suggest that CsA and SRL modulate glucose metabolism and insulin action, although through different mechanisms, i.e. while CsA seems to enhance gluconeogenesis, SRL mainly impairs insulin signaling in peripheral tissues. These effects might contribute to the development of insulin resistance and NODAT observed during immunosuppressive therapy.

Body weight was monitored weekly as presented in our previous work [19].While, the CsA group presented a weight gain similar to vehicle, SRL gained less weight. Although food intake was not measured, other authors have observed reduced food intake and food efficiency with a higher dose of rapamycin (2mg/kg/day) [26, 27].

The CsA group presented impaired glucose tolerance during a GTT when compared to either the vehicle or the SRL-treated groups. The latter, also presented an impaired glucose excursion curve. As normal insulin action is required for clearing an oral glucose load [28], this impairment might be due to reduced insulin secretion by β-cells and/or a reduction in peripheral insulin sensitivity [5, 29] in the CsA-treated group. This was confirmed after an ITT, as even when an exogenous insulin bolus was administered, the glucose levels in the CsA group remained higher, and the rate of glucose disposal to reach basal levels was slower. This is also true for the SRL group, in particular at the 60 minutes time point where the glucose values were significantly higher than vehicle and closer to those of the CsA group. In fact, the presence of higher levels of insulin was not sufficient to decrease glucose levels similar to the ones observed in the vehicle group, suggesting marked insulin resistance in both CsA and SRL treated groups. Furthermore, to evaluate if this could be due to impaired insulin secretion from the β-cells of the islets of Langerhans, after 3 weeks of treatment with therapeutic doses, we measured insulin and C-Peptide levels. However, no differences were observed for insulin, and even thought C-peptide levels were reduced, there were not statistically significant. This condition is usually associated with induced diabetes in rats [30] and a defect in β-cells [31].

SRL is considered to be less nephrotoxic than CsA, and is presently a valid option instead of calcineurin inhibitors for the maintenance of immunosuppression [32]. Therefore, we also evaluated if the clearance of glucose rates in the urine was impaired. Surprisingly, we found a tendency for an increase in the glucose clearance rate in the SRL treated group, which might be related to an increase of glucose in the urine, in greater quantities than the renal tubule can absorb (glycosuria), and this condition has already been observed in patients under SRL therapy [33]. No difference was observed in the CsA group, but as Yale, Roy [34] have shown, it requires higher doses and duration of treatment to cause glycosuria with CsA (*e.g.*10 mg/kg BW/day for 12 weeks).

Moreover, as the development of insulin resistance has been linked to enhanced hepatic gluconeogenesis, we evaluated some of the key markers of this pathway. In our model, after 3 weeks of treatment with CsA, G6Pase protein levels were significantly increased and were accompanied by non-significant increase in protein expression for PEPCK and transcription factors PGC1-α and FOXO1, confirming an overstimulated hepatic gluconeogenesis. In the SRL group, we did not observe an increase in gluconeogenesis, as reported previously by Houde, Brûlé [26] and Lamming, Ye [35]. This apparent discrepancy might be dose-related, as the authors used a higher dose of SRL. Interestingly, although no changes were observed in GK gene expression, an enzyme responsible for producing glucose-6-phosphate, GK protein levels were decreased in the SRL-treated group. This might cause impairment in glucokinase activity, reducing the glucose disposal in the liver. However, no differences were found in glycogen content in the liver between the treated groups, in agreement with Houde, Brûlé [26] and Pfaffenbach, Nivala [36]. In addition, GK is controlled at the transcriptional level in a TORC1-dependent manner [37] and therefore assays to determine GK activity should be considered in future studies with SRL treatment. Moreover, gene and protein levels for PGC1-α and FOXO1 were also measured in muscle and perirenal adipose tissue, where their

actions are more linked to their role in mitochondrial biogenesis, myogenesis and adipocyte differentiation [38, 39]. PGC1-α expression is directly related with insulin sensitivity and is down regulated in muscle of type 2 diabetic subjects [39]. Therefore, a reduction in PGC1-α expression in the muscle of SRL-treated rats may account for the development of insulin resistance. Moreover, muscle specific mTORC1 loss is associated with a decrease in PGC1-α expression levels, and with a reduction in the expression of mitochondrial target genes including PGC-1α itself, as well as in oxidative metabolism [40-43].

Since PTP1B is a negative regulator of insulin signaling, and its deletion has been coupled with improved insulin sensitivity, we evaluated how the in vivo treatment with these agents could affect its gene and protein expression in the various tissues. Interestingly, PTP1B protein level was increased in the CsA group in the liver but not in muscle or adipose tissue. Although PTP1B gene expression in the liver was increased by SRL treatment, no changes were observed in protein expression in liver, muscle or adipose tissue. Overexpression of PTP1B may result in systemic insulin resistance in mice [44-46]. However, PTP1B activity is also regulated at the phosphorylation level, as when Akt phosphorylates PTP1B at Ser50, the enzyme shows a decrease in its ability to dephosphorylate insulin receptors [47], which was not measured in these experiments. In addition, we have recently shown that SRL treatment contributes to lipid accumulation in the liver [48], a condition known to up regulate PTP1B expression [47], and that could have contributed to the increased PTP1B overexpression. Likewise, PTP1B overexpression in the presence of excess lipids may not directly cause insulin resistance unless it is accompanied by decreased PTP1B phosphorylation [47], which might explain the similarity of the GTT results between the vehicle and the SRL group. However, to our knowledge this is the first report showing alterations on PTP1B protein expression with CsA treatment. This increase in PTP1B protein expression may be linked to an increase in insulin resistance and gluconeogenesis as liver specific PTP1B $^{-/-}$ mice have

been shown to have decreased expression of gluconeogenic genes and increased hepatic insulin signaling [46], and were able to reverse glucose intolerance [49]. Assays to determine PTP1B activity should be considered in future studies with CsA treatment, as in diabetic rats, increased PTP1B levels and activity decrease glucose uptake and insulin signaling [50].

To further elucidate the development of whole body glucose intolerance and the previously reported data showing that treatment with CsA or SRL impairs insulin-stimulated glucose uptake in epididymal adipose tissue [19, 23], we also analyzed protein expression and activation of important insulin signaling markers in muscle, liver and adipose tissue. While IRS-1 protein levels were significantly increased in the liver, but not in muscle and adipose tissue, a reduction in GLUT1 protein level was detectable in liver and muscle with the SRL treatment. No changes were observed in GLUT2 (liver) or GLUT4 (muscle and adipose tissue), the main insulin-stimulated transporter [51, 52], with either treatments. A decrease in GLUT1 protein expression with the SRL treatment might explain the reduction of the basal glucose uptake, observed by Pereira, Palming [20], Fuhrmann, Lopes [23] and Deblon, Bourgoin [27], while the increase in IRS-1 expression also observed by Takano, Usui [53] and Um, D'Alessio [54] might be a compensatory mechanism. In this study we cannot exclude the possibility that even though the GLUT4 protein expression was not different, its translocation to the membrane could be impaired, as it has been observed before in pre diabetic and diabetic states in fat [55, 56]. This experiment was not performed due to the lack of tissue, but should be addressed in future studies. Nonetheless, impaired glucose uptake in CsA-treated rats might be related with a reduced amount of GLUT4 in the plasma membrane as Pereira et al. (unpublished data) recently demonstrated that CsA treatment reduced the insulin-stimulated presence of GLUT4 in the plasma membrane of differentiated human pre-adipocytes and L6 muscle cells. On the other hand, in the SRL group, glucose uptake might be decreased due to an impairment of the insulin signaling, as already demonstrated in human

and rat insulin sensitive cells [20, 21, 57, 58]. Moreover, the decrease in PGC1-α protein expression in the muscle of the SRL-treated group might also be responsible for a decrease in insulin sensitivity, as PGC1-α increases the expression of the insulin-sensitive transporter GLUT4 in the muscle [59, 60]. Insulin initiates intracellular signaling when it binds to the insulin receptor, phosphorylating its tyrosine residues. In our work, phosphorylation of the insulin receptor at Tyr1146 residue was decreased in the SRL group both in liver and adipose tissue. Moreover SRL also impaired phosphorylation of the key protein, Akt at Ser473 and Thr308 residues in liver and adipose tissue, while no alterations were observed by the CsA treatment, previously demonstrated both in vitro *and* in vivo [61-63]. Sarbassov, Guertin [64] have also shown that mTOR kinase and rictor are essential for phosphorylation of Akt Ser473 and SRL reduces insulin phosphorylation of IRS-1 on Tyr residues [65], which is in accordance with our results at least in muscle. Moreover, Shivaswamy, Bennett [21] observed recently that SRL treatment reduces insulin-stimulated phosphorylation of Akt in liver, muscle and fat. On the other hand, insulin sensitivity may also be affected by intracellular lipid accumulation, through impairment of IRS-1-PI3K-Akt signaling pathways [63, 66], which may also be the case, as our group already demonstrated that after 3 weeks of treatment with SRL, there is an accumulation of TGs in liver and muscle [48]. Impaired Akt activation leads also to a decrease in phosphorylation of AS160, an important substrates of Akt that controls the translocation of glucose transporters to the plasma membrane. In agreement with other studies (Pereira et al., unpublished data), [20, 27] these results reveal that SRL treatment inhibits activation of Akt in response to insulin, affecting glucose metabolism in skeletal muscles and adipocytes. As expected, the mTOR pathway was blocked by SRL treatment, as evidenced by the lack of phosphorylation of its downstream target, the p70S6K.

Taken together, these data indicate that CsA affects glucose metabolism, by increasing gluconeogenic factors in liver and SRL mainly by impairing the insulin signaling cascade

pathway in peripheral tissues, which ultimately can affect glucose uptake (Figure 8). These effects might contribute to the development of insulin resistance after immunosuppressive therapy, and caution is required when choosing the therapy to apply to patients, in order to prevent the development of NODAT.

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Authors declare no conflict of interests.

520 ***References***

521 [1] Smith JM, Nemeth TL, McDonald RA. Current immunosuppressive agents: efficacy, side effects,
522 and utilization. Pediatr Clin North Am. 2003;50(6):1283-300.
523 [2] Sieber M, Baumgrass R. Novel inhibitors of the calcineurin/NFATc hub - alternatives to CsA and
524 FK506? Cell communication and signaling : CCS. 2009;7:25.
525 [3] Sarwal MM, Yorgin PD, Alexander S, Millan MT, Belson A, Belanger N, et al. Promising early
526 outcomes with a novel, complete steroid avoidance immunosuppression protocol in pediatric renal
527 transplantation. Transplantation. 2001;72(1):13-21.
528 [4] Chung J, Kuo CJ, Crabtree GR, Blenis J. Rapamycin-FKBP specifically blocks growth-dependent
529 activation of and signaling by the 70 kd S6 protein kinases. Cell. 1992;69:1227-36.
530 [5] Øzbay LA, Smidt K, Mortensen DM, Carstens J, Jorgensen KA, Rungby J. Cyclosporin and
531 tacrolimus impair insulin secretion and transcriptional regulation in INS-1E beta-cells. British journal
532 of pharmacology. 2011;162:136-46.
533 [6] Watt KD. Metabolic syndrome: is immunosuppression to blame? Liver Transpl. 2011;17 Suppl
534 3:S38-42.
535 [7] Subramanian S, Trence DL. Immunosuppressive agents: effects on glucose and lipid metabolism.
536 Endocrinol Metab Clin North Am. 2007;36(4):891-905; vii.
537 [8] Dirks NL, Huth B, Yates CR, Meibohm B. Pharmacokinetics of immunosuppressants: a
538 perspective on ethnic differences. International journal of clinical pharmacology and therapeutics.
539 2004;42:701-18.
540 [9] Pham PT, Pham PM, Pham SV, Pham PA, Pham PC. New onset diabetes after transplantation
541 (NODAT): an overview. Diabetes Metab Syndr Obes 2011;4:175-86.
542 [10] Hecking M, Werzowa J, Haidinger M, Horl WH, Pascual J, Budde K, et al. Novel views on new-
543 onset diabetes after transplantation: development, prevention and treatment. Nephrology, dialysis,
544 transplantation : official publication of the European Dialysis and Transplant Association - European
545 Renal Association. 2013;28:550-66.

[11] Pajvani UB, Shawber CJ, Samuel VT, Birkenfeld AL, Shulman GI, Kitajewski J, et al. Inhibition of Notch signaling ameliorates insulin resistance in a FoxO1-dependent manner. Nature medicine. 2011;17:961-7.

[12] Nakae J, Biggs WH, 3rd, Kitamura T, Cavenee WK, Wright CV, Arden KC, et al. Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. Nature genetics. 2002;32:245-53.

[13] Ropelle ER, Pauli J, Cintra DE, Frederico MJS, De Pinho RA, Velloso LA, et al. Acute exercise modulates the Foxo1/PGC-1α pathway in the liver of diet-induced obesity rats. J Physiol 2009:2069–76.

[14] Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, et al. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. Nature. 2001;413:179-83.

[15] Rhodes CJ, White MF. Molecular insights into insulin action and secretion. European journal of clinical investigation. 2002;32 Suppl 3:3-13.

[16] Berg CE, Lavan BE, Rondinone CM. Rapamycin partially prevents insulin resistance induced by chronic insulin treatment. Biochemical and biophysical research communications. 2002;293:1021-7.

[17] Tremblay F, Gagnon A, Veilleux A, Sorisky A, Marette A. Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology. 2005;146:1328-37.

[18] Tremblay F, Marette A. Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. J Biol Chem. 2001;276:38052-60.

[19] Lopes P, Fuhrmann A, Sereno J, Pereira MJ, Nunes P, Pedro J, et al. Effects of cyclosporine and sirolimus on insulin-stimulated glucose transport and glucose tolerance in a rat model. Transplant Proc. 2013;45:1142-8.

[20] Pereira MJ, Palming J, Rizell M, Aureliano M, Carvalho E, Svensson MK, et al. mTOR inhibition with rapamycin causes impaired insulin signalling and glucose uptake in human subcutaneous and omental adipocytes. Mol Cell Endocrinol. 2012;355:96-105.

573 [21] Shivaswamy V, Bennett RG, Clure CC, Ottemann B, Davis JS, Larsen JL, et al. Tacrolimus and
574 sirolimus have distinct effects on insulin signaling in male and female rats. Translational research : the
575 journal of laboratory and clinical medicine. 2013.
576 [22] Böhmer AE, Souza DG, Hansel G, Brum LM, Portela LV, Souza DO. Long-term cyclosporine
577 treatment in non-transplanted rats and metabolic risk factors of vascular diseases. Chem Biol Interact.
578 2010;185(1):53-8.
579 [23] Fuhrmann A, Lopes P, Sereno J, Pedro J, Espinoza DO, Pereira MJ, et al. Molecular mechanisms
580 underlying the effects of cyclosporin A and sirolimus on glucose and lipid metabolism in liver,
581 skeletal muscle and adipose tissue in an in vivo rat model. Biochemical pharmacology. 2014;88:216-
582 28.
583 [24] Shivaswamy V, McClure M, Passer J, Frahm C, Ochsner L, Erickson J, et al. Hyperglycemia
584 induced by tacrolimus and sirolimus is reversible in normal sprague-dawley rats. Endocrine
585 2010;37(3):489-96.
586 [25] Schena FP, Pascoe MD, Alberu J, Del Carmen Rial M, Oberbauer R, Brennan DC, et al.
587 Conversion from calcineurin inhibitors to sirolimus maintenance therapy in renal allograft recipients:
588 24-month efficacy and safety results from the CONVERT trial. Transplantation 2009;87(2):233-42.
589 [26] Houde V, Brûlé S, Festuccia W, Blanchard P, Bellmann K, Deshaies Y, et al. Chronic rapamycin
590 treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and
591 impairing lipid deposition in adipose tissue. Diabetes. 2010;59(6):1338-48.
592 [27] Deblon N, Bourgoin L, Veyrat-Durebex C, Peyrou M, Vinciguerra M, Caillon A, et al. Chronic
593 mTOR inhibition by rapamycin induces muscle insulin resistance despite weight loss in rats. Br J
594 Pharmacol. 2012;165(7):2325-40.
595 [28] Ferrannini E, Bjorkman O, Reichard GA, Jr., Pilo A, Olsson M, Wahren J, et al. The disposal of
596 an oral glucose load in healthy subjects. A quantitative study. Diabetes. 1985;34:580-8.
597 [29] Hjelmesaeth J, Hagen LT, Asberg A, Midtvedt K, Størset O, Halvorsen CE, et al. The impact of
598 short-term ciclosporin A treatment on insulin secretion and insulin sensitivity in man. Nephrol Dial
599 Transplant. 2007;22(6):1743-9.

600 [30] Amin KA, Awad EM, Nagy MA. Effects of panax quinquefolium on streptozotocin-induced
601 diabetic rats: role of C-peptide, nitric oxide and oxidative stress. International journal of clinical and
602 experimental medicine. 2011;4:136-47.
603 [31] Palmer J, Fleming G, Greenbaum C, Herold K, Jansa L, Kolb H, et al. C-peptide is the
604 appropriate outcome measure for type 1 diabetes clinical trials to preserve beta-cell function: report of
605 an ADA workshop, 21-22 October 2001. Diabetes. 2004;53:250-64.
606 [32] Klawitter J, Bendrick-Peart J, Rudolph B, Beckey V, Haschke M, Rivard C, et al. Urine
607 metabolites reflect time-dependent effects of cyclosporine and sirolimus on rat kidney function.
608 Chemical research in toxicology. 2009;22:118-28.
609 [33] Franz S, Regeniter A, Hopfer H, Mihatsch M, Dickenmann M. Tubular toxicity in sirolimus- and
610 cyclosporine-based transplant immunosuppression strategies: an ancillary study from a randomized
611 controlled trial. Am J Kidney Dis. 2010;55:335-43.
612 [34] Yale JF, Roy RD, Grose M, Seemayer TA, Murphy GF, Marliss EB. Effects of cyclosporine on
613 glucose tolerance in the rat. Diabetes. 1985;34:1309-13.
614 [35] Lamming D, Ye L, Katajisto P, Goncalves M, Saitoh M, Stevens D, et al. Rapamycin-induced
615 insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. Science.
616 2012;335(6076):1638-43.
617 [36] Pfaffenbach KT, Nivala AM, Reese L, Ellis F, Wang D, Wei Y, et al. Rapamycin inhibits
618 postprandial-mediated X-box-binding protein-1 splicing in rat liver. The Journal of nutrition.
619 2010;140:879-84.
620 [37] Dai W, Panserat S, Mennigen JA, Terrier F, Dias K, Seiliez I, et al. Post-prandial regulation of
621 hepatic glucokinase and lipogenesis requires the activation of TORC1 signalling in rainbow trout
622 (Oncorhynchus mykiss). The Journal of experimental biology. 2013;216:4483-92.
623 [38] Amat R, Planavila A, Chen SL, Iglesias R, Giralt M, Villarroya F. SIRT1 controls the
624 transcription of the peroxisome proliferator-activated receptor-gamma Co-activator-1alpha (PGC-
625 1alpha) gene in skeletal muscle through the PGC-1alpha autoregulatory loop and interaction with
626 MyoD. J Biol Chem. 2009;284:21872-80.

627 [39] Liang H, Ward WF. PGC-1alpha: a key regulator of energy metabolism. Advances in physiology
628 education. 2006;30:145-51.
629 [40] Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell. 2012;149:274-93.
630 [41] Bentzinger CF, Romanino K, Cloetta D, Lin S, Mascarenhas JB, Oliveri F, et al. Skeletal muscle-
631 specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy.
632 Cell metabolism. 2008;8:411-24.
633 [42] Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls
634 mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature.
635 2007;450:736-40.
636 [43] Romanino K, Mazelin L, Albert V, Conjard-Duplany A, Lin S, Bentzinger CF, et al. Myopathy
637 caused by mammalian target of rapamycin complex 1 (mTORC1) inactivation is not reversed by
638 restoring mitochondrial function. Proceedings of the National Academy of Sciences of the United
639 States of America. 2011;108:20808-13.
640 [44] Vakili S, Ebrahimi SS, Sadeghi A, Gorgani-Firuzjaee S, Beigy M, Pasalar P, et al.
641 Hydrodynamic-based delivery of PTP1B shRNA reduces plasma glucose levels in diabetic mice.
642 Molecular medicine reports. 2013;7:211-6.
643 [45] Haj FG, Zabolotny JM, Kim YB, Kahn BB, Neel BG. Liver-specific protein-tyrosine phosphatase
644 1B (PTP1B) re-expression alters glucose homeostasis of PTP1B-/-mice. J Biol Chem.
645 2005;280:15038-46.
646 [46] Delibegovic M, Zimmer D, Kauffman C, Rak K, Hong EG, Cho YR, et al. Liver-specific deletion
647 of protein-tyrosine phosphatase 1B (PTP1B) improves metabolic syndrome and attenuates diet-
648 induced endoplasmic reticulum stress. Diabetes. 2009;58:590-9.
649 [47] Obanda DN, Cefalu WT. Modulation of cellular insulin signaling and PTP1B effects by lipid
650 metabolites in skeletal muscle cells. The Journal of nutritional biochemistry. 2013;24:1529-37.
651 [48] Lopes PC, Fuhrmann A, Sereno J, Espinoza DO, Pereira MJ, Eriksson JW, et al. Short and long
652 term in vivo effects of Cyclosporine A and Sirolimus on genes and proteins involved in lipid
653 metabolism in Wistar rats. Metabolism: clinical and experimental. 2014;63:702-15.

654 [49] Owen C, Lees EK, Grant L, Zimmer DJ, Mody N, Bence KK, et al. Inducible liver-specific
655 knockdown of protein tyrosine phosphatase 1B improves glucose and lipid homeostasis in adult mice.
656 Diabetologia. 2013;56:2286-96.
657 [50] Wu Y, Ouyang JP, Wu K, Wang SS, Wen CY, Xia ZY. Rosiglitazone ameliorates abnormal
658 expression and activity of protein tyrosine phosphatase 1B in the skeletal muscle of fat-fed,
659 streptozotocin-treated diabetic rats. British journal of pharmacology. 2005;146:234-43.
660 [51] Leto D, Saltiel AR. Regulation of glucose transport by insulin: traffic control of GLUT4. Nature
661 reviews Molecular cell biology. 2012;13:383-96.
662 [52] Pessin JE, Saltiel AR. Signaling pathways in insulin action: molecular targets of insulin
663 resistance. The Journal of clinical investigation. 2000;106:165-9.
664 [53] Takano A, Usui I, Haruta T, Kawahara J, Uno T, Iwata M, et al. Mammalian target of rapamycin
665 pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and
666 integrates nutritional signals and metabolic signals of insulin. Molecular and cellular biology.
667 2001;21:5050-62.
668 [54] Um SH, D'Alessio D, Thomas G. Nutrient overload, insulin resistance, and ribosomal protein S6
669 kinase 1, S6K1. Cell metabolism. 2006;3:393-402.
670 [55] Carvalho E, Eliasson B, Wesslau C, Smith U. Impaired phosphorylation and insulin-stimulated
671 translocation to the plasma membrane of protein kinase B/Akt in adipocytes from Type II diabetic
672 subjects. Diabetologia. 2000;43:1107-15.
673 [56] Carvalho E, Jansson PA, Nagaev I, Wenthzel AM, Smith U. Insulin resistance with low cellular
674 IRS-1 expression is also associated with low GLUT4 expression and impaired insulin-stimulated
675 glucose transport. FASEB journal : official publication of the Federation of American Societies for
676 Experimental Biology. 2001;15:1101-3.
677 [57] Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, et al. Prolonged rapamycin
678 treatment inhibits mTORC2 assembly and Akt/PKB. Molecular cell. 2006;22:159-68.
679 [58] Kumar A, Lawrence JC, Jr., Jung DY, Ko HJ, Keller SR, Kim JK, et al. Fat cell-specific ablation
680 of rictor in mice impairs insulin-regulated fat cell and whole-body glucose and lipid metabolism.
681 Diabetes. 2010;59:1397-406.

682 [59] Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, et al. Adaptations of skeletal
683 muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB journal : official
684 publication of the Federation of American Societies for Experimental Biology. 2002;16:1879-86.
685 [60] Michael LF, Wu Z, Cheatham RB, Puigserver P, Adelmant G, Lehman JJ, et al. Restoration of
686 insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional
687 coactivator PGC-1. Proceedings of the National Academy of Sciences of the United States of America.
688 2001;98:3820-5.
689 [61] Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, et al. Akt/mTOR pathway
690 is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nature
691 cell biology. 2001;3:1014-9.
692 [62] Lungu AO, Jin ZG, Yamawaki H, Tanimoto T, Wong C, Berk BC. Cyclosporin A inhibits flow-
693 mediated activation of endothelial nitric-oxide synthase by altering cholesterol content in caveolae. J
694 Biol Chem. 2004;279:48794-800.
695 [63] Di Paolo S, Teutonico A, Leogrande D, Capobianco C, Schena PF. Chronic inhibition of
696 mammalian target of rapamycin signaling downregulates insulin receptor substrates 1 and 2 and AKT
697 activation: A crossroad between cancer and diabetes? Journal of the American Society of Nephrology :
698 JASN. 2006;17:2236-44.
699 [64] Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB
700 by the rictor-mTOR complex. Science. 2005;307:1098-101.
701 [65] Danielsson A, Ost A, Nystrom FH, Stralfors P. Attenuation of insulin-stimulated insulin receptor
702 substrate-1 serine 307 phosphorylation in insulin resistance of type 2 diabetes. J Biol Chem.
703 2005;280:34389-92.
704 [66] Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N, et al. Reduced mitochondrial
705 density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2
706 diabetic parents. The Journal of clinical investigation. 2005;115:3587-93.

707

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Figure Legends

Figure 1. Effects of vehicle, CsA, and SRL treatment during a GTT (A), an ITT (B), as well as, fasting serum glucose (C), insulin (D) C-peptide (E), and the glucose clearance rate in the urine (F). Rats were treated with CsA and SRL for 3 weeks and fasted for 16 hours before the glucose tolerance test. Glucose levels were measured at time point 0, and after an intraperitoneal injection of glucose (2 g/kg BW) at 15, 30, 60, and 120 minutes and respective area under the curve. For the ITT, rats were fasted for 6 hours and glucose levels were measured at time point 0, and after an intraperitoneal injection of insulin (1U/kg BW) at 15, 30, 45, 60 and 90 minutes and respective area under the curve. Fasting serum glucose, insulin and C-peptide levels were measured. Urine collection was done 24 hours prior to sacrifice. Data are presented as mean ± SEM (n=6-8/group) *$p<0.05$, ** $p<0.01$***$p<0.001$ vehicle vs. CsA group; $^{\&}$ $p<0.05$ vehicle vs. SRL group; $^{\#\#\#}p<0.001$ CsA vs. SRL group. CsA – Cyclosporine A; SRL – Sirolimus;

Figure 2. Gluconeogenic gene and protein expression in liver, after a 3 week-treatment period with CsA or SRL. Relative mRNA expression levels were determined by Real-time PCR (n=8). Protein expression levels were determined by western blotting (three to five independent experiments) for PGC1-α, FOXO1 and PTP1B (A) and G6Pase, PEPCK and GK (B). Data are presented as mean ± SEM. *$p<0.05$ vehicle vs. CsA or SRL group; $^{\#}p<0.05$ CsA vs. SRL group. CsA – Cyclosporine A; SRL – Sirolimus; PGC1-α - peroxisome proliferator-activated receptor γ coactivator 1, FOXO1- forkhead box O1, PTP1B -protein tyrosine phosphatase 1B, G6Pase - Glucose-6-phosphatase, PEPCK- Phosphoenolpyruvate carboxykinase, GK - Glucokinase.

734 **Figure 3**. Expression of genes and proteins of the insulin signaling pathway in liver after a 3
735 week-treatment period, with CsA or SRL. Relative mRNA expression levels were determined
736 by Real-time PCR (n=8). Protein expression levels were determined by western blotting
737 (three to five independent experiments) for IRS-1, GLUT1 and GLUT2 (A). Phosphorylation
738 levels of IRS-1 Tyr612, protein expression levels of PI3K p85 subunit and GLUT2 and
739 phosphorylation levels of pAkt Ser473 and Thr308, p70S6K Thr412/424, mTOR Ser2448,
740 and AS160 Thr642, after stimulation with insulin (B). *$p<0.05$ vehicle vs. CsA or SRL
741 group; *$p<0.05$, **$p<0.01$ basal vs. insulin; CsA – Cyclosporine A; SRL – Sirolimus; IRS-1 –
742 Insulin Receptor substrate 1; GLUT1 – Glucose transporter 1 and GLUT2 - Glucose
743 transporter 2; PI3K - Phosphatidylinositide 3-kinase.

744

745 **Figure 4**. Gene and protein expression in muscle after a 3-week treatment period with CsA or
746 SRL. Relative mRNA expression levels were determined by Real-time PCR (n=8). Protein
747 expression levels were determined by western blotting (three to five independent
748 experiments) for PGC1-α, FOXO1 and PTPB1. Data are presented as mean ± SEM, *$p<0.05$
749 vehicle vs. CsA or SRL group; $^{\#}p<0.05$ CsA vs. SRL group. CsA – Cyclosporine A; SRL –
750 Sirolimus; PGC1-α - peroxisome proliferator-activated receptor γ coactivator 1, FOXO1-
751 forkhead box O1, PTP1B -protein tyrosine phosphatase 1B.

752

753 **Figure 5**. Expression of genes and proteins of the insulin signaling cascade in muscle after a
754 3-week treatment period, with CsA or SRL. Relative mRNA expression levels were
755 determined by Real-time PCR (n=8). Protein expression levels were determined by western
756 blotting (three to five independent experiments) for IRS-1, GLUT1 and GLUT4 (A).
757 Phosphorylation levels of IRS-1 Tyr612, protein expression levels of PI3K p85 subunit,

758 GLUT4, and phosphorylation levels of pAkt Ser473 and Thr308, p70S6K Thr421/424, mTOR
759 Ser2448, and AS160 Thr642, after stimulation with insulin (B). Data are presented as mean ±
760 SEM. *p<0.05 vehicle vs. CsA group; *p<0.05 basal vs. insulin ; CsA - Cyclosporine A;
761 SRL – Sirolimus; IRS-1 – Insulin Receptor substrate 1; GLUT1 – Glucose transporter 1 and
762 GLUT4 - Glucose transporter 4; PI3K - Phosphatidylinositide 3-kinase.

763

764 **Figure 6**. Gene and protein expression in perirenal adipose tissue after a 3-week treatment
765 period, with CsA or SRL. Relative mRNA expression levels were determined by Real-time
766 PCR (n=8). Protein expression levels were determined by western blotting (three to five
767 independent experiments) for PGC1-α, FOXO1 and PTP1B. Data are presented as mean ±
768 SEM , *p<0.05 vehicle vs. SRL group; CsA – Cyclosporine A; SRL – Sirolimus; PGC1-α -
769 peroxisome proliferator-activated receptor γ coactivator 1, FOXO1- forkhead box O1, PTP1B
770 -protein tyrosine phosphatase 1B.

771

772 **Figure 7**. Expression of genes and proteins of the insulin signaling cascade in epididymal
773 adipose tissue after a 3-week treatment period, with CsA or SRL. Relative mRNA expression
774 levels were determined by Real-time PCR (n=8). Protein expression levels were determined
775 by western blotting (three to five independent experiments) for IRS-1, GLUT1 and GLUT4.
776 Phosphorylation levels of IRS-1 Tyr612, protein expression levels of PI3K p85 subunit,
777 GLUT4 and phosphorylation levels of pAkt Ser473 and Thr308, p70S6K Thr421/424, mTOR
778 Ser2448, and AS160 Thr642, after stimulation with insulin. Data are presented as mean ±
779 SEM, *p<0.05 basal vs. insulin. CsA – Cyclosporine A; SRL – Sirolimus; IR – Insulin
780 receptor; IRS-1 – Insulin Receptor substrate 1; PI3K - Phosphatidylinositide 3-kinase;
781 GLUT1 – Glucose transporter 1 and GLUT4 - Glucose transporter 4.

Figure 8. Scheme summarizing the effects of CsA and SRL on the gluconeogenesis and insulin signaling in muscle and adipose tissue. Red arrows correspond to CsA; Blue arrows correspond to SRL. ↑, increase; ↓, decrease.

Figure 1.

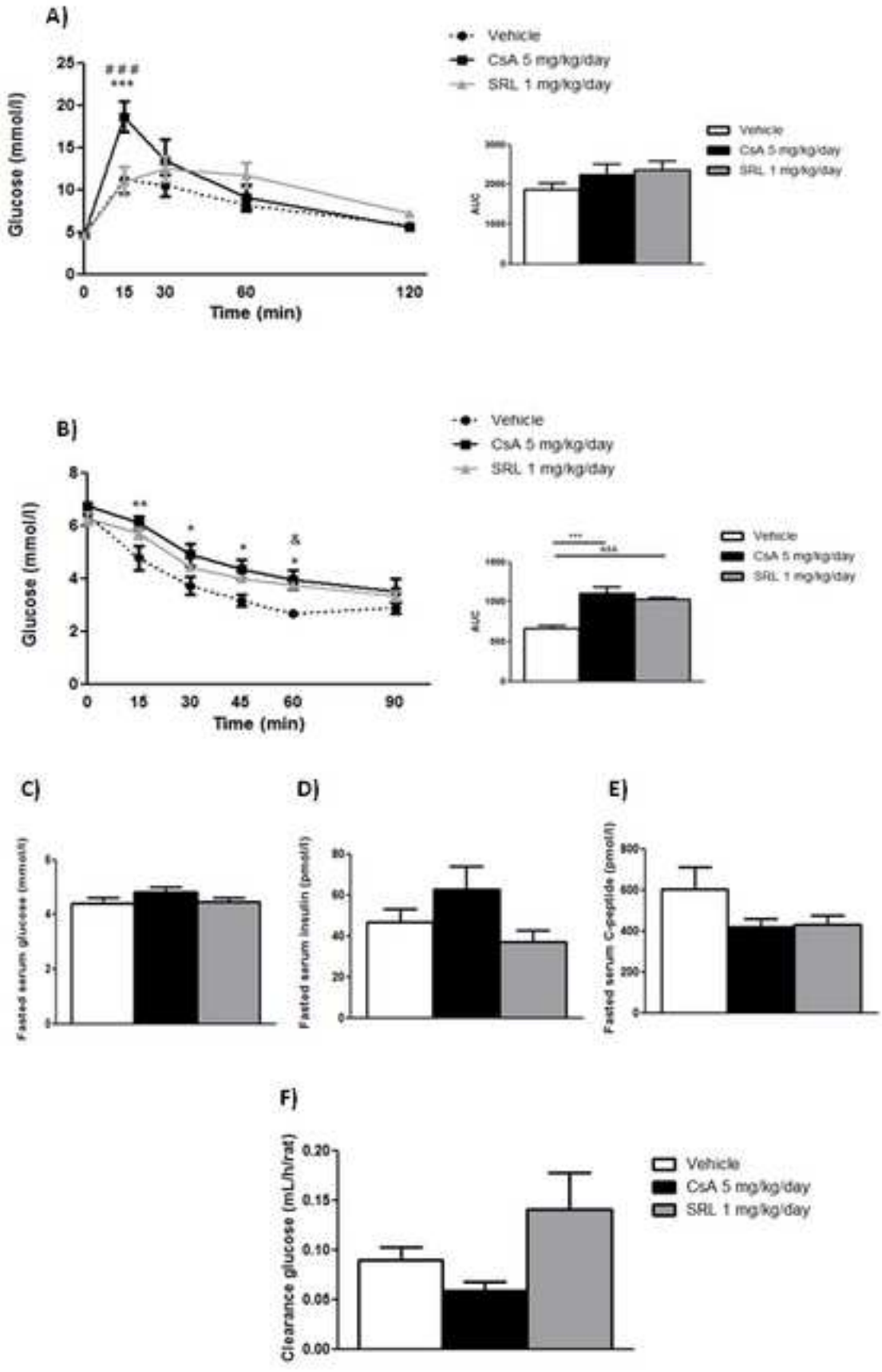


Figure 2.

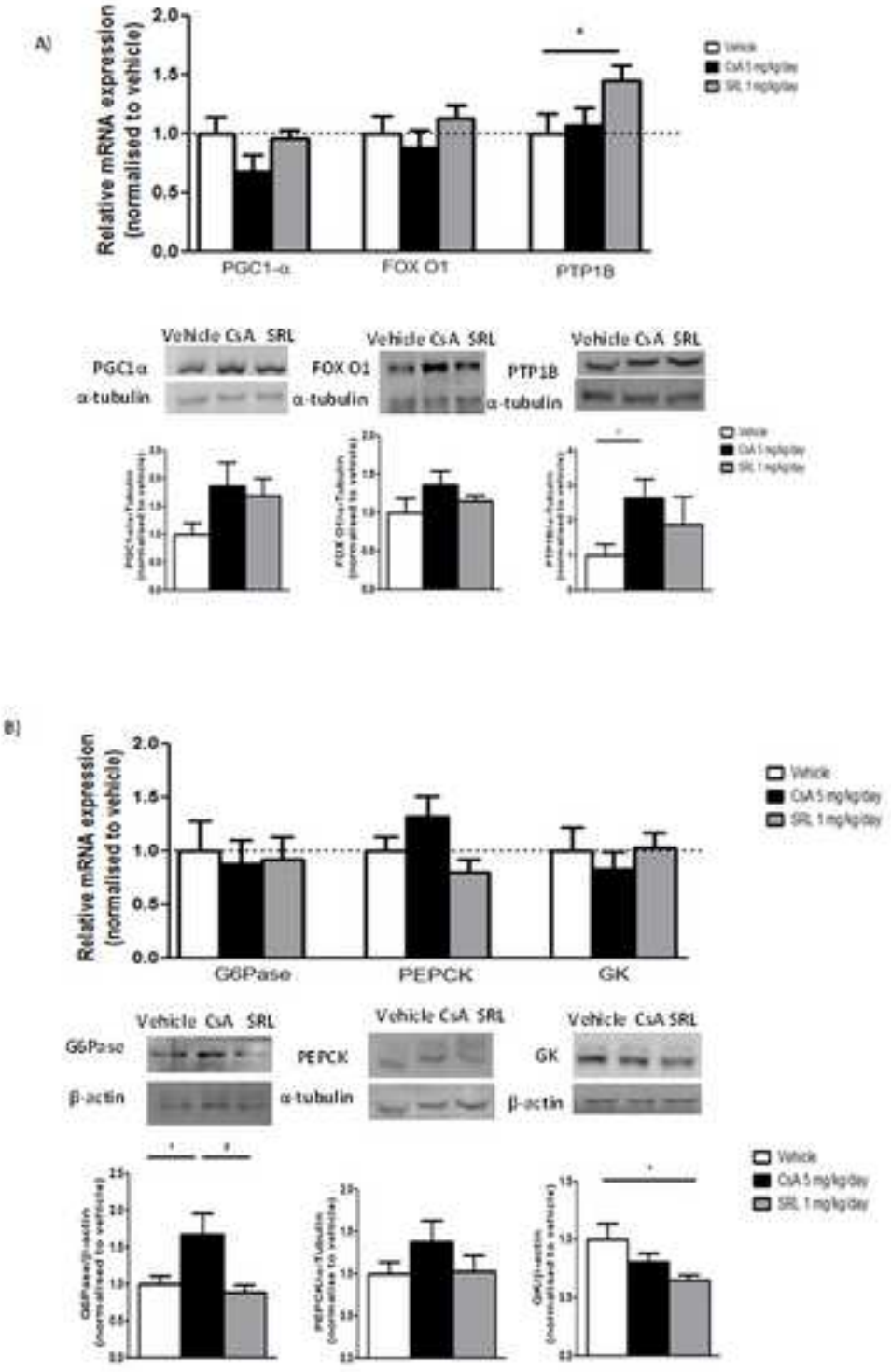


Figure 3.

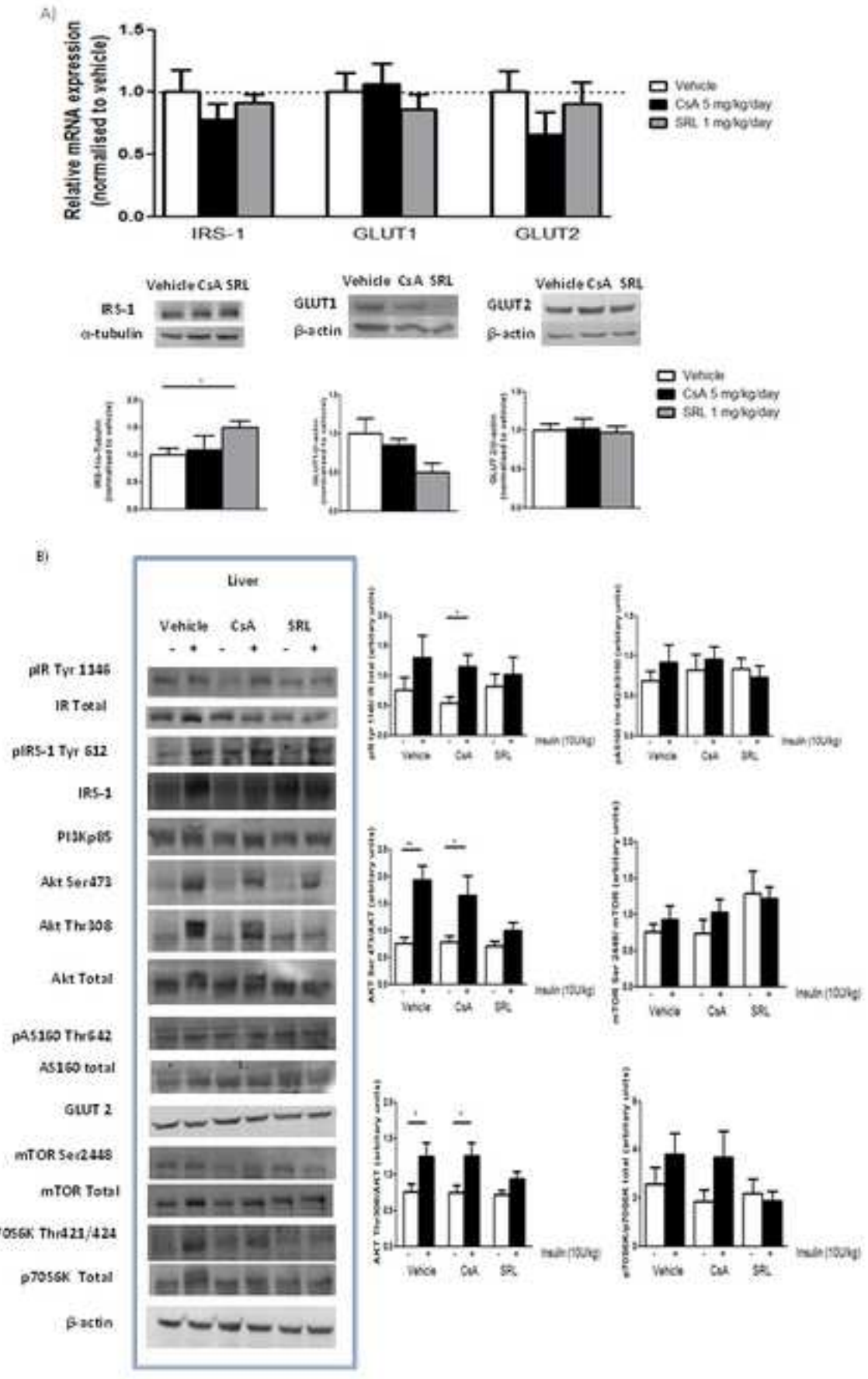


Figure 4.

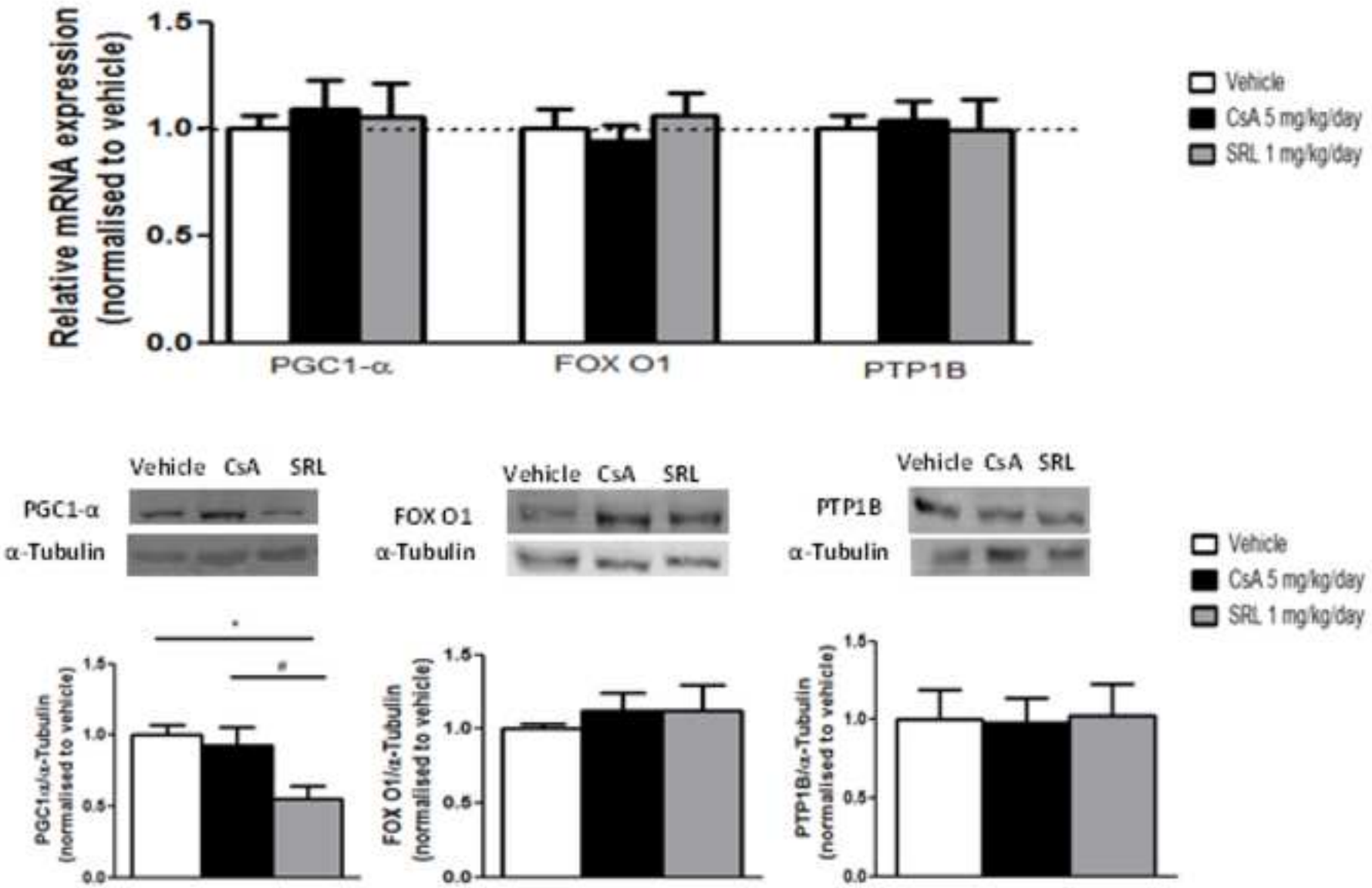

Relative mRNA expression (normalised to vehicle)
1.5
1.0
0.5
0.0
PGC1-α
FOX O1
PTP1B
Vehicle
CsA 5 mg/kg/day
SRL 1 mg/kg/day
Vehicle CsA SRL
PGC1-α
α-Tubulin
FOX O1
PTP1B
PGC1-α/α-Tubulin (normalised to vehicle)
FOX O1/α-Tubulin (normalised to vehicle)
PTP1B/α-Tubulin (normalised to vehicle)
*
#

Figure 5.

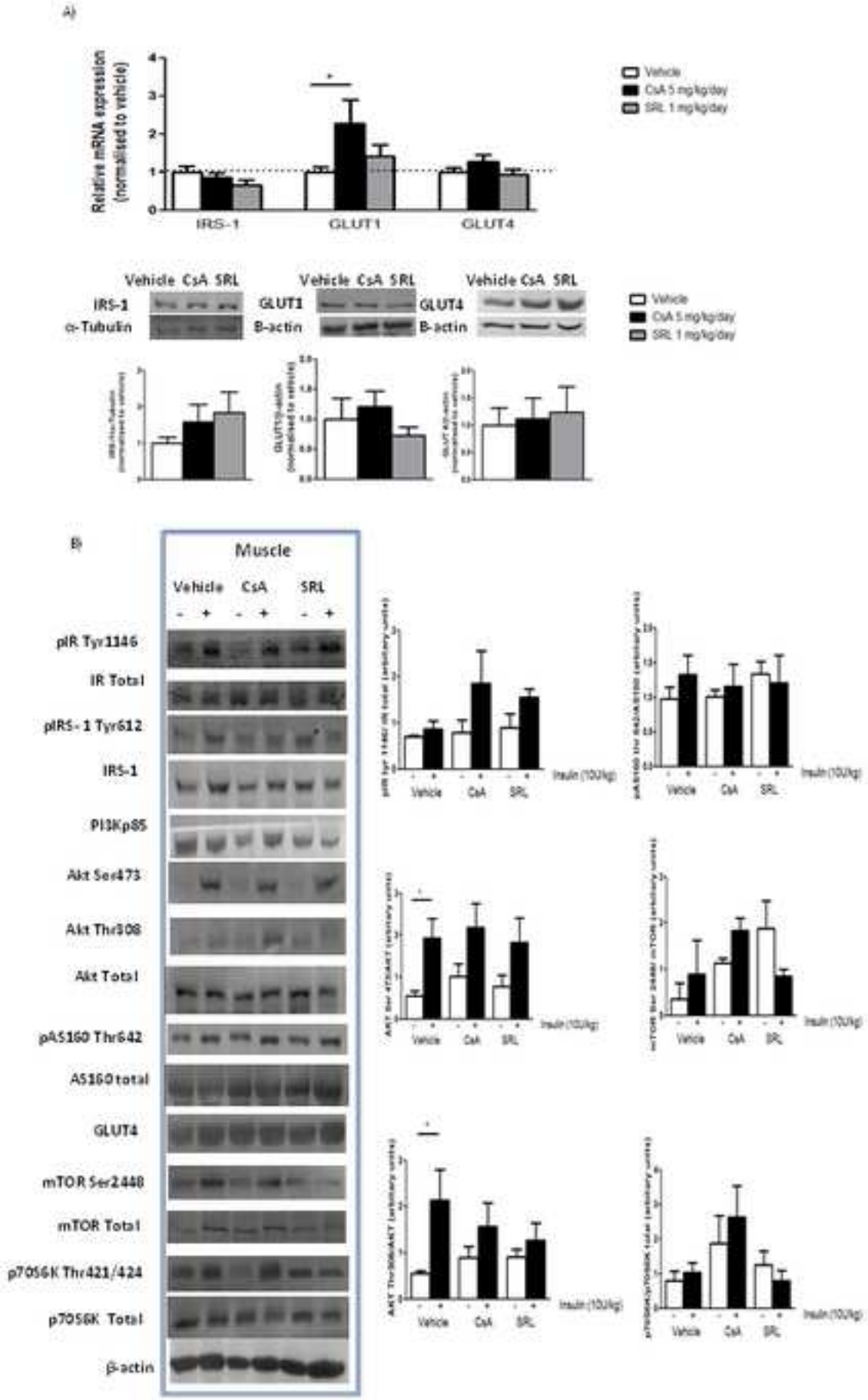


Figure 6.

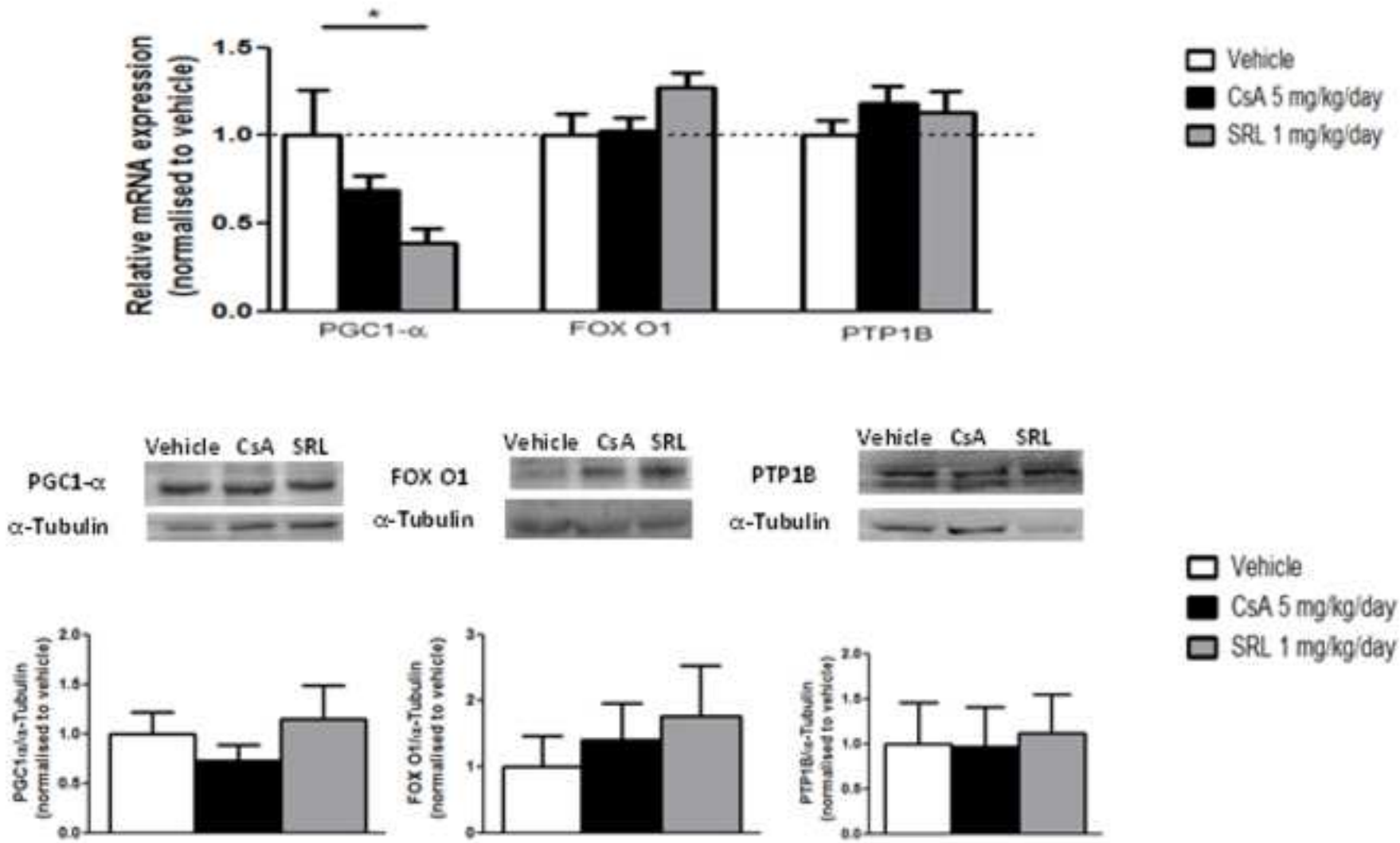

*
Relative mRNA expression (normalised to vehicle)
1.5
1.0
0.5
0.0
PGC1-α
FOX O1
PTP1B
Vehicle
CsA 5 mg/kg/day
SRL 1 mg/kg/day
Vehicle CsA SRL
PGC1-α
α-Tubulin
Vehicle CsA SRL
FOX O1
α-Tubulin
Vehicle CsA SRL
PTP1B
α-Tubulin
PGC1α/α-Tubulin (normalised to vehicle)
FOX O1/α-Tubulin (normalised to vehicle)
PTP1B/α-Tubulin (normalised to vehicle)
Vehicle
CsA 5 mg/kg/day
SRL 1 mg/kg/day

Figure 7.

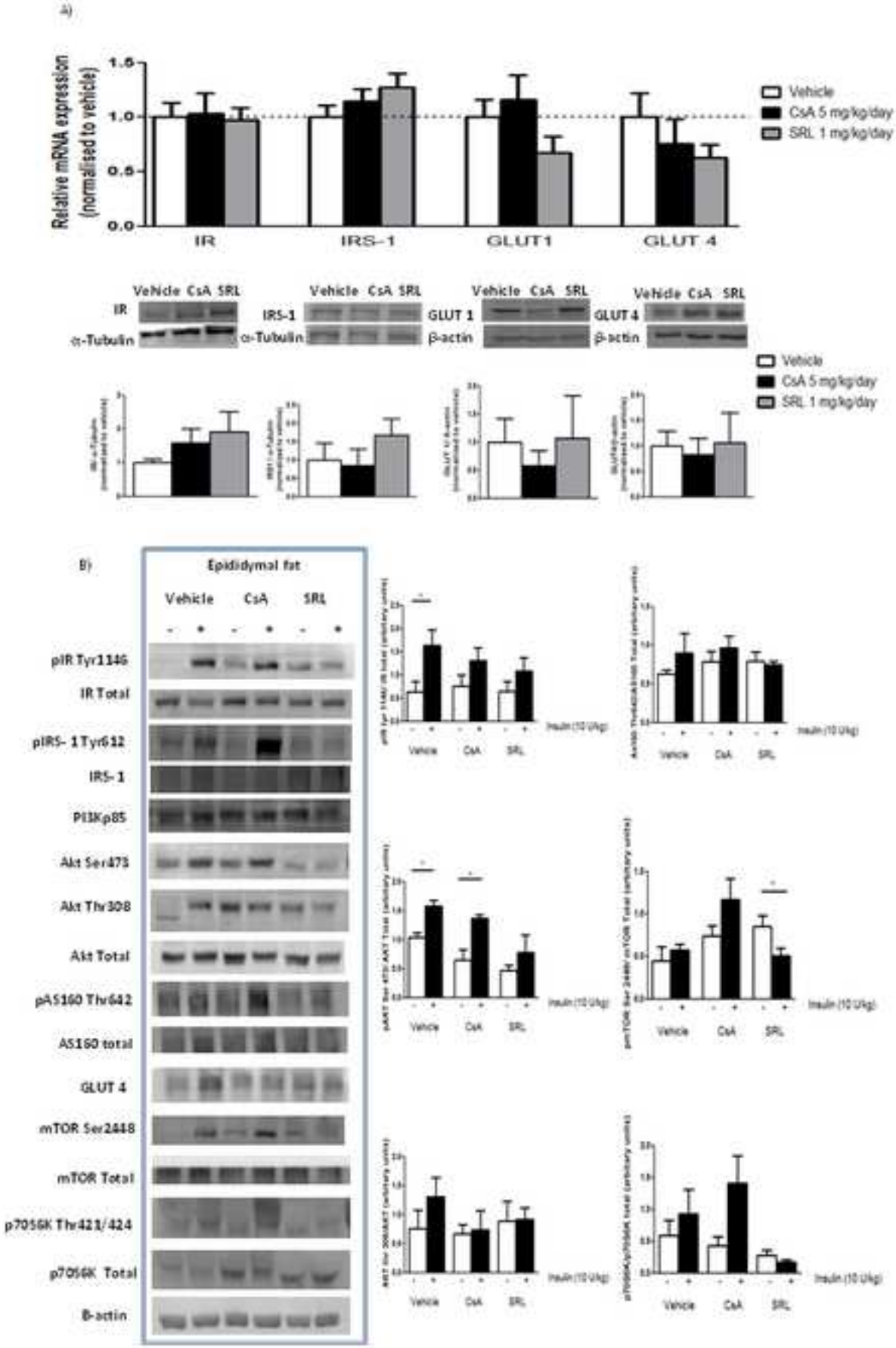
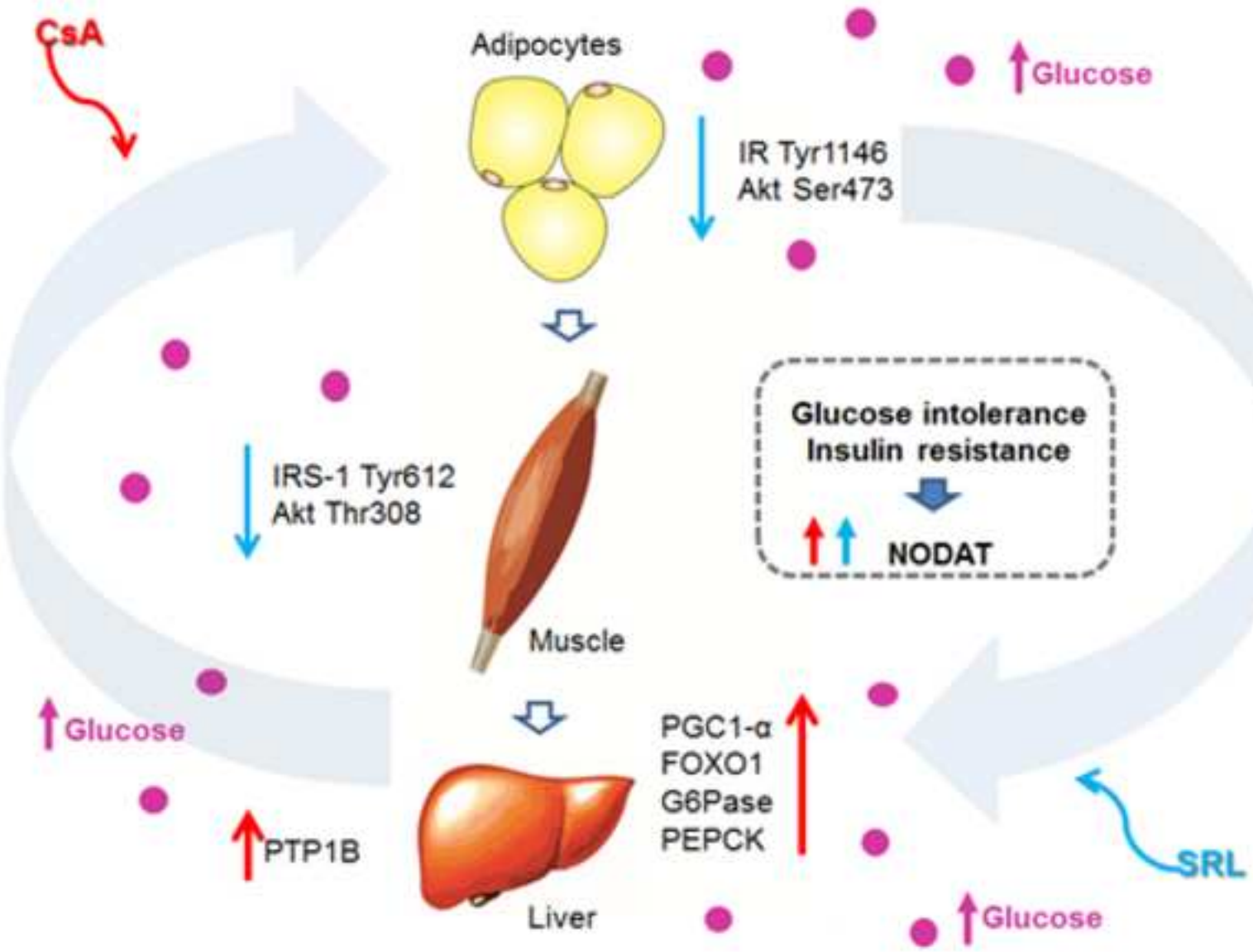


Figure 8.


CsA
Adipocytes
Glucose
IR Tyr1146
Akt Ser473
IRS-1 Tyr612
Akt Thr308
Glucose intolerance
Insulin resistance
NODAT
Muscle
Glucose
PTP1B
PGC1-α
FOXO1
G6Pase
PEPCK
SRL
Liver
Glucose

Graphical Abstract (for review)