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Original Article

Calcineurin inhibitors suppress intestinal cellular respiration

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Abstract: The calcineurin inhibitors, tacrolimus and cyclosporine, are commonly used immunosuppressants, and transplant patients on these medications often develop gastrointestinal symptoms. This *in vitro* study investigated their effects on intestinal cellular respiration (the process of delivering nutrients and O_2 to the mitochondria, oxidation of reduced metabolic fuels, passage of electrons to O_2, and synthesis of ATP) in C57BL/6 mice. For this purpose, a phosphorescence analyzer was used to measure cellular mitochondrial O_2 consumption (k_c, μM O_2 min^{-1} mg^{-1}) in fragments from the small intestine in the presence of 10 μM drug or 1.6 μL dimethyl-sulfoxide (drug vehicle). Tacrolimus and cyclosporine significantly reduced the rate of intestinal cellular respiration by 28% and 35%, respectively. Cyclosporine had no effects on cellular respiration in renal tissue. Intestinal respiration was unaffected by the multikinase inhibitors sorafenib and regorafenib, the mTOR inhibitors sirolimus and everolimus, the PI3K/mTOR inhibitors BEZ235, GDC0980 and GSK2126458, the MEK inhibitor GSK1120212, and the P110δ inhibitor idelalisib. Thus, calcineurin inhibitors specifically impair intestinal cellular respiration. The mechanism and clinical significance of this *in vitro* event require further studies.

Keywords: Cellular bioenergetics, cellular respiration, mitochondrial function, calcineurin inhibitors, tacrolimus, cyclosporine, intestinal toxicity

Introduction

The calcineurin inhibitors, tacrolimus and cyclosporine are key immunosuppressants, which are commonly used after organ transplant [1]. Cyclosporine binds to cyclophilin and tacrolimus binds to FK binding protein 12 (FKBP12, a member of the immunophilin family that functions as protein-folding chaperones for peptides containing prolines). Both cyclosporine-cyclophilin and tacrolimus-FKBP12 directly inhibit calcineurin (a calcium/calmodulin-activated phosphatase that activates T cells). This inhibition blocks the activation of several cytokine transcription genes in T cells [2, 3]. Both drugs have frequent adverse events that include hypertension and acute nephrotoxicity due to arterial vasoconstriction [4]. Other frequent toxicities are impaired glucose metabolism and urinary tract infection, especially cytomegaloviral infection [5].

We recently showed the PI3Kd inhibitor idelalisib suppresses murine liver and lung cellular respiration [6]. In addition, the dual PI3K/mTOR inhibitors GSK2126458 and BEZ235 and the pure mTOR inhibitor sirolimus were shown to impair cellular respiration in murine kidney, liver and heart tissues [7, 8]. Thymic cellular respiration, on the other hand, was specifically inhibited by sirolimus and everolimus [unpublished data]. These results are consistent with the known contribution of mTOR to mitochondrial function and nutrient transport [10-12].

Studies on the effects of molecularly targeted agents on intestinal cellular respiration are very limited [6]. This study investigated the effects of several classes of drugs (calcineurin inhibitors, multikinase inhibitors, mTOR inhibitors, PI3K/mTOR inhibitors, MEK inhibitors, and P110δ inhibitors) on cellular respiration in the small intestine in mice. Its main purpose was to explore whether these commonly used drugs can alter cellular bioenergetics in the intestine. The other aim was to use cellular respiration as a surrogate biomarker for studying molecularly targeted drugs.

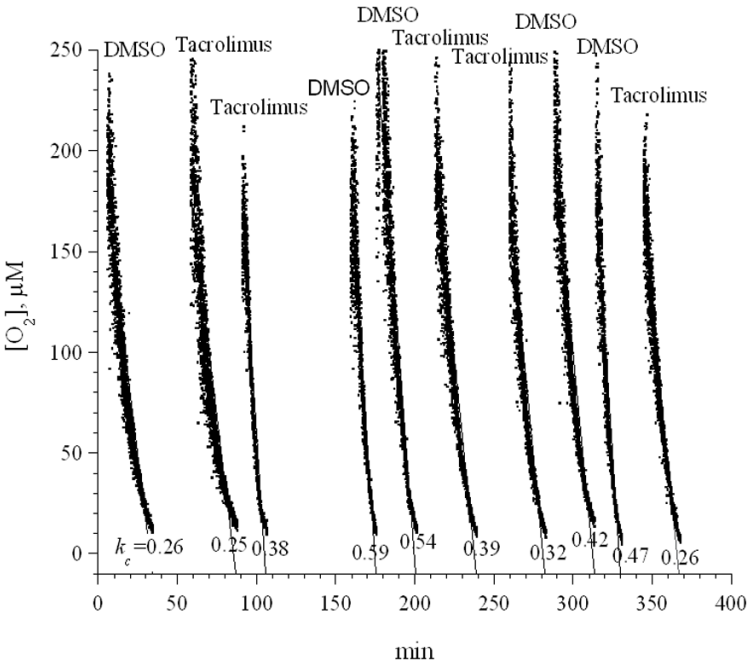

Figure 1. Effects of tacrolimus on O_2 consumption in small intestine. Representative experiments are shown. Each run represented a small intestinal fragment that was excised from the mouse and processed *immediately* for measuring cellular respiration in the presence of 10 μM tacrolimus or 1.6 μL DMSO. Rate of respiration (k_c (μM O_2 min^{-1})) was the negative of the slope of [O_2] *vs.* *t*. The values of k_c (μM O_2 min^{-1} mg^{-1}) are shown at the bottom of each run. The lines are linear fits.

Materials and methods

Reagents and solutions

Tacrolimus (FK-506, fujimycin), cyclosporine, sirolimus (rapamycin), everolimus, idelalisib (CAL-101), BEZ235, GSK2126458, GDC0980 (PI3K/mTOR inhibitors), GSK1120212 (MEK inhibitor), sorafenib and regorafenib were purchased from MedChem Express, LLC (Princeton, NJ). The drugs were dissolved in dimethyl sulfoxide (DMSO) at 5 mg/mL and stored at -20°C. Pd(II) complex of *meso*-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin (Pd phosphor) was purchased from Porphyrin Products (Logan, UT). Pd phosphor (2.5 mg/mL = 2 mM, made in dH_2O) was stored at -20°C in small aliquots. RPMI (Roswell Park Memorial Institute) 1640 medium and remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Mice

C57BL/6 (10 weeks old) mice were housed at 22°C, 60% humidity, and 12-h light-dark cycles. They had ad libitum access to standard rodent chow and filtered water. The study was approved from the Animal Ethics Committee-College of Medicine and Health Sciences (A29-13; *In vitro* assessment of the effects of nephrotoxic drugs and toxins on renal cellular respiration in mice). The methods described here were carried out in "accordance" with the approved guidelines.

Tissue collection and processing

Urethane (25% w/v, 100 μL per 10 g) was administered intraperitoneally for anesthesia. A fragment (20 to 40 mg) of the small intestine was then quickly excised (while the organ was well perfused) with a sterile scalpel (Swann-Morton, Sheffield, England) and immediately immersed in ice-cold RPMI saturated with 95% O_2: 5% CO_2. The tissue was rinsed thoroughly, weighted in the same solution, and immediately placed in the oxygen vial for measuring cellular respiration at 37°C [6-8]. The vial contained 1.0 mL RPMI, 3 μM Pd phosphor, 0.5% fat-free albumin, and 10 μM drug (treated condition) or 1.6 μL DMSO (control condition).

Cellular respiration

A phosphorescence analyzer was used to monitor cellular mitochondrial O_2 consumption in fragments from the small intestine as previously described [6-9]. The Pd phosphor Pd (II) complex of *meso*-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin was used. Samples were flashed from pulsed (10/s) light-emitting diode array with peak output at 625 nm (OTL630A-5-10-66-E, Opto Technology, Inc., Wheeling, IL). Emitted phosphoresce was passed through an interference filter centered at 800 nm and detected by a Hamamatsu photomultiplier tube #928. Amplified phosphorescence decay was digitized at 1.0 MHz by a 20-MHz A/D converter using an analog/digital converter PCI-DAS

Table 1. *In vitro* effects of studied drugs on the small intestinal cellular respiration

	k_c (μM O_2 min^{-1} mg^{-1})	Inhibition (%)	*P*
DMSO	0.43 ± 0.13 (47)	-	-
Tacrolimus (Calcineurin inhibitor)	0.31 ± 0.10 (11)	28	0.003
Cyclosporine (Calcineurin inhibitor)	0.28 ± 0.04 (3)	35	0.021
Sorafenib/Regorafenib (Multikinase inhibitors)	0.34 ± 0.07 (6)	23	0.065
Sirolimus/Everolimus (mTOR inhibitors)	0.43 ± 0.19 (9)	0	1.0
BEZ235/GDC0980/GSK2126458 (PI3K/mTOR inhibitors)	0.43 ± 0.17 (9)	0	1.0
GSK1120212 (MEK inhibitor)	0.47 ± 0.02 (3)	0	0.507
Idelalisib (P110δ inhibitor)	0.51 ± 0.19 (9)	0	0.507

For each run, a fragment of the small intestine was excised from a mouse and immediately placed in the oxygen-measuring vial for determining the rate of cellular respiration in the presence of 1.6 μL DMSO or 10 μM designated drug. The values of k_c are mean ± SD. The values in parentheses are number of mice (= number of runs).

4020/12 I/O Board (PCI-DAS 4020/12 I/O Board; Computer Boards, Inc., Mansfield, MA) [6-9]. O_2 concentration, [O_2], was determined as function of time from the phosphorescence decay rate (1/τ) of Pd phosphor. The values of 1/τ were linear with dissolved O_2 ($1/\tau = 1/\tau_o + k_q[O_2]$); where $1/\tau_o$ = phosphorescence decay rate in the absence of O_2; k_q = the second-order O_2 quenching rate constant in s^{-1} μM^{-1} [13].

Cellular respiration was measured at 37°C in 1-mL sealed glass vials. The rate of respiration (*k*, in μM O_2 min^{-1}) was the negative of the slope d[O_2]/dt; its value was expressed in (k_c) μM O_2 min^{-1} mg^{-1}. Cyanide inhibited respiration, confirming O_2 was consumed in the respiratory chain [6].

Statistical analysis

Data were analyzed on SPSS statistical package (version 19), using the nonparametric (2 independent samples) Mann-Whitney test. *P*-values <0.05 were considered significant.

Results

Figure 1 shows representative runs of cellular mitochondrial O_2 consumption in small intestine fragments in the presence of tacrolimus or DMSO. Each run represented a tissue specimen that was collected from the small intestine, immersed in RPMI, and then *immediately* placed in the oxygen vial for measuring cellular respiration at 37°C in RPMI supplemented with 10 μM tacrolimus or 1.6 μL DMSO. The rate of respiration (k_c) was the negative of the slope of [O_2] *vs.* *t* plots and expressed in μM O_2 min^{-1} mg^{-1}.

A summary of all results is shown in **Table 1**. The rate of intestinal cellular respiration (k_c, mean ± SD) in the presence of DMSO was 0.43 ± 0.13 (n = 47 mice), 10 μM tacrolimus 0.31 ± 0.10 (n = 11 mice, 28% inhibition, $p = 0.003$), and 10 μM cyclosporine 0.28 ± 0.04 (n = 3 mice, 35% inhibition, $p = 0.021$), **Table 1**. Small intestine cellular respiration was unaffected by the multikinase inhibitors sorafenib and regorafenib, the mTOR inhibitors sirolimus and everolimus, the PI3K/mTOR inhibitors BEZ235, GDC0980 and GSK2126458, the MEK inhibitor GSK1120212, and the P110δ inhibitor idelalisib ($p \geq 0.065$), **Table 1**. Thus, small intestine cellular respiration was specifically suppressed by the calcineurin inhibitors.

Discussion

This study investigated the effects of several molecularly targeted drugs on small intestine cellular respiration. The calcineurin inhibitors cyclosporine and tacrolimus both significantly suppressed cellular respiration (**Table 1**). As shown previously, cyclosporine had no effects on cellular respiration in murine renal tissue [7]. Therefore, the effects of calcineurin inhibitors on respiration appears to be tissue-specific. In addition, the other studied agents had no effects on cellular respiration in the gut (**Table 1**). Studies, however, are needed to elucidate mechanisms responsible for the observed effects of calcineurin inhibitors on small intestine cellular respiration. A lower rate of cellular respiration usually implies defects in any of the following processes: delivering nutrients and O_2 to the mitochondria, oxidation of reduced metabolic fuels, passage of electrons to O_2, and synthesis of ATP.

Reports addressing the effects of drugs on small intestine cellular respiration are very rare [6]. In this study, a typical experiment (e.g., the six-hour runs shown in **Figure 1**) represents multiple oxygen measurements. Each measurement (run) lasts about 30 min and represents a small intestine fragment that is rapidly excised from the mouse and immediately placed in the oxygen-measuring vial for determining the rate of cellular respiration in the presence of DMSO or designated drug. Marked histologic changes are expected in this *in vitro* system; thus, the assessment of treatment-induced morphologic derangements requires *in vivo* studies.

Furthermore, the experiments shown here utilized full thickness of the small intestine, which includes the mucosa, submucosa, muscularis propria and serosa. It is worth noting that our recent study on stomach mucosal biopsies from patients show normal mucosal cells consume oxygen at a rate of 0.17 ± 0.02 μM O_2 min^{-1} mg^{-1} [9]. These determinations confirm mucosal cells contribute to the overall rate of intestinal oxygen consumption. The relative rate of each intestinal layer, however, remains to be illustrated.

For each experiment (e.g., **Figure 1**), at least three mice were used for the studied drug and three for the drug vehicle DMSO. Therefore, the number of mice used for 'DMSO control' was relatively high (**Table 1**). Control runs were necessary for validating the results of the studied drugs. Fourteen mice were used to study the calcineurin inhibitors (11 tacrolimus and three cyclosporine). The coefficient of variation for cyclosporine was low sufficiently low (14%); therefore, three mice were thought to be sufficient (**Table 1**).

Therapeutic serum levels of the studied drugs differ markedly. For example, target serum levels for tacrolimus are 6.2 to 18.7 nM (5 to 15 μg/L) [14], for cyclosporine 0.17 to 0.33 μM (200 to 400 μg/L) [15], and for sirolimus 5 to 16 nM (5-15 ng/mL) [16]. In this *in vitro* study, drug effects were investigated at 10 μM concentration. However, the exposure time was relatively short (about 30 min) and the distribution of drugs in *ex vivo* tissue fragments was expected to be slow. It is worth noting, however, that much lower concentrations can be used in cell culture [10], especially if the exposure time is several hours or days.

mTOR is required for the mitochondrial oxidative function [10-12]. In one study, the mTOR inhibitor sirolimus decreased mitochondrial gene expression and oxygen consumption [12]. The study here shows sirolimus has no such effect on intestinal cellular respiration (**Table 1**). A previous study in Jurkat cells showed exposure of the cells to sirolimus for 30 min resulted in about 20% decrease in cellular respiration [10]. This effect was accompanied by accumulation of intracellular lactate and other biomarkers of anaerobic metabolism [10]. Consistently, both sirolimus and everolimus inhibited cellular respiration in the thymus (unpublished observation). In addition, sirolimus inhibited cellular respiration in murine heart, liver and kidney tissues; while tacrolimus and cyclosporine had minimum or no effects on in these organs [8]. Thus, the effects of mTOR inhibition on cellular respiration appear to be tissue-specific. Whether impaired cellular bioenergetics contributes to the immunosuppressive activity of mTOR inhibitors remains to be illustrated.

In conclusion, the results show calcineurin inhibitors lower intestinal cellular respiration in mice. The drugs, however, have no effects on cellular respiration in other organs [7]. The drugs were added directly to the tissue rather than dosing the mice. Therefore, more clinically relevant, *in vivo* studies are needed to overcome this limitation.

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Disclosure of conflict of interest

None.

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