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Data Article

A dataset describing glycolytic inhibitors overcoming the underestimation of maximal mitochondrial oxygen consumption rate in oligomycin-treated cells

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ABSTRACT

Determination of oxygen consumption is one of the most valuable methodologies to evaluate mitochondrial (dys)function. Previous studies demonstrated that a widely used protocol, consisting of adding the ATP synthase inhibitor oligomycin before mitochondrial respiratory uncoupling by sequential addition of a protonophore (e.g., carbonyl cyanide 3-chlorophenyl hydrazone [CCCP]), may lead to underestimation of maximal oxygen consumption rate (OCR_{max}) and spare respiratory capacity (SRC) parameters in highly glycolytic tumor cell lines. In this dataset, we report the effects of the glycolytic inhibitors 2-deoxy-D-glucose, iodoacetic acid, and lonidamine on overcoming the underestimation of OCR_{max} and SRC in oligomycin-treated cells. We propose a protocol in which 2-deoxy-D-glucose is added after oligomycin and just before the sequential addition of CCCP to avoid underestimation of OCR_{max} and SRC parameters in A549, C2C12, and T98G cells. The oxygen consumption rates were determined in intact suspended cell lines using a high-resolution oxygraph device. The data can be used in several fields of research that require characterization of mitochondrial respiratory parameters in intact cells.

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Specifications Table

Subject	Biochemistry
Specific subject area	Cell biology, Mitochondrial bioenergetics
Type of data	Graph
How data were acquired	Data were obtained from *in vitro* experiments with cultured immortalized cell lines using a high-resolution oxygraph (OROBOROS Oxygraph-2k, Innsbruck, Austria) and a fluorescence spectrophotometer (Hitachi F-7000, Hitachi, Tokyo, Japan).
Data format	Raw data analyzed and processed.
Parameters for data collection	Oxygen consumption rate and mitochondrial membrane potential were analyzed in suspended intact cells [1,2]. Measurements were performed under basal conditions, after ATP synthase inhibition by oligomycin, and under mitochondrial respiratory uncoupling by CCCP.
Description of data collection	Cultured cells (A549, C2C12, and T98G) were suspended and treated with the glycolytic inhibitors 2-deoxy-D-glucose (40 mM), iodoacetic acid (200 µM), or lonidamine (400 µM). Maximal oxygen consumption rate (OCR_{max}) and spare respiratory capacity (SRC) were determined by sequential additions of CCCP (400 nM each addition) in the presence or absence of 1 µg/mL oligomycin. Mitochondrial membrane potential was also determined in some experiments with T98G cells treated with or without 2-deoxy-D-glucose.
Data source location	Laboratory of Bioenergetics and Cell Metabolism Faculty of Medical Sciences, University of Campinas Campinas - State of São Paulo Brazil
Data accessibility	The raw data are available in Mendeley Data repository. Data identification number: doi: 10.17632/33ybs25n7m.1 Direct URL to data: data.mendeley.com/datasets/33ybs25n7m/1
Related research articles [1,2]	J.S. Ruas, E.S. Siqueira-Santos, I. Amigo, E. Rodrigues-Silva, A.J. Kowaltowski, R.F. Castilho, Underestimation of the maximal capacity of the mitochondrial electron transport system in oligomycin-treated cells. PLoS One. 11(3):e0150967 (2016). doi: 10.1371/journal.pone.0150967 J.S. Ruas, E.S. Siqueira-Santos, E. Rodrigues-Silva, R.F. Castilho, High glycolytic activity of tumor cells leads to underestimation of electron transport system capacity when mitochondrial ATP synthase is inhibited. Sci Rep. 8(1):17,383 (2018). doi: 10.1038/s41598–018–35,679–8

Value of the Data

- These new data are related to obtaining mitochondrial respiratory parameters in a single experiment in intact cells, without underestimating any parameters.
- The data may benefit researchers who want to evaluate mitochondrial respiratory parameters in highly glycolytic cells.
- The data can be used in several research fields that require characterization of mitochondrial respiratory parameters in highly glycolytic cells.

1. Data Description

We evaluated the effects of the glycolytic inhibitors 2-deoxy-D-glucose (2-DG), iodoacetic acid (IAA), and lonidamine (LON) on carbonyl cyanide 3-chlorophenyl hydrazone (CCCP)-induced maximal mitochondrial oxygen consumption rate (OCR_{max}) in T98G cells (Fig. 1A). Experiments were conducted in the presence and absence of the ATP synthase inhibitor oligomycin. Dimethylsulfoxide (DMSO) was the vehicle for oligomycin. In the absence of glycolytic inhibitors, OCR_{max}

was inhibited by 29.5 ± 5.4% in oligomycin-treated cells compared with control cells (i.e., with DMSO) (Fig. 1A). When T98G cells were incubated in the presence of 2-DG, there was no inhibition of OCR_{max} by oligomycin. IAA partially prevented the inhibition of OCR_{max} in oligomycin-treated cells, while LON exerted no effect (Fig. 1A).

In the absence of glycolytic inhibitors, spare respiratory capacity (SRC), i.e., the difference between the OCR_{max} and basal OCR, was underestimated by 41.1 ± 5.8% in the presence of oligomycin (Fig. 1B). When 2-DG or IAA was present, no significant inhibition of SRC was observed in oligomycin-treated cells. LON did not prevent the inhibitory effect of oligomycin on SRC (Fig. 1B).

Next, an alternative experimental protocol was employed in intact suspended cells to obtain all mitochondrial respiratory parameters in only one experimental trace, avoiding the inhibitory effect of oligomycin on OCR_{max} and SRC. In this proposed protocol (Fig. 2B, red trace), 2-DG was added after oligomycin; next, sequential additions of CCCP were performed for OCR_{max} and SRC determinations. Fig. 2A depicts traces of OCR obtained under standard conditions in the absence of 2-DG. As previously reported [1–3] and shown in Fig. 1A, OCR_{max} was underestimated in the presence of oligomycin (Fig. 2A). In the alternative experimental protocol (Fig. 2B, red trace), the basal OCR was determined after seven to eight minutes of incubation in Dulbecco's modified Eagle's medium (DMEM). Next, oligomycin was added, allowing the determination of the OCR-fraction related to the ATP synthesis. Then, 2-DG was added, and five minutes later, sequential additions of CCCP were performed to estimate OCR_{max}. In the presence of 2-DG, the OCR_{max} inhibition by CCCP-excess addition was less pronounced (Fig. 2B).

Figs. 3–5 show data when 2-DG was added to the incubation medium before estimating OCR_{max} (panels **A**) and SRC (panels **B**) in T98G, A549, and C2C12 cells, respectively. The values of OCR_{max} inhibition depicted in panels **C** were obtained when the CCCP concentrations were twice the optimal levels for reaching OCR_{max}. 2-DG prevented the underestimation of OCR_{max} and SRC (panels **A** and **B**, respectively**)** in oligomycin-treated T98G, A549, and C2C12 cells. In the presence of 2-DG, OCR_{max} inhibition by CCCP at twice the optimal concentration was less pronounced (panels **C**). The CCCP concentrations necessary for reaching OCR_{max} are shown in panels **D**.

As shown in Fig. 6, OCR and mitochondrial membrane potential ($\Delta\Psi$) were simultaneously determined in T98G cells incubated in the presence and absence of 2-DG. Progressive dissipation of $\Delta\Psi$ was induced by sequential additions of CCCP [2]. OCR_{max} was obtained at a low $\Delta\Psi$; however, complete $\Delta\Psi$ dissipation caused partial OCR inhibition (panel **A**). $\Delta\Psi$ at OCR_{max} was lower with DMSO than in the presence of oligomycin (panel **C**). In the presence of 2-DG, OCR_{max} was reached at a similar $\Delta\Psi$ in DMSO and oligomycin conditions (panels **B** and **D**). When $\Delta\Psi$ was dissipated (i.e., $\Delta\Psi$ was nearly zero), inhibition of OCR_{max} occurred with DMSO and oligomycin; however, this inhibition was almost completely abolished when experiments were conducted in the presence of 2-DG (panel **E**). The raw data (Figs. 1–6) are available in Mendeley Data [5].

2. Experimental Design, Materials and Methods

2.1. Chemicals

Carbonyl cyanide 3-chlorophenyl hydrazone (CCCP; catalog #C2759), 2-DG (#D8375), DMSO (#D8418), IAA sodium salt (#I77687), LON (#L4900), oligomycin (#O4876), and sodium tetraphenylboron (TPB^-; #T4125) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetramethylrhodamine methyl ester (TMRM; #T668) was supplied by Thermo Fisher Scientific (Waltham, MA, USA). CCCP, oligomycin, LON, and TMRM stock solutions were prepared in DMSO; 2-DG, IAA, and TPB^- stock solutions were prepared in deionized water; HEPES and IAA solutions were adjusted to pH 7.4 with NaOH.

Dulbecco's modified Eagle's medium (DMEM), containing 11 mM glucose, 1.25 mM pyruvate, 4 mM glutamine, 44 mM sodium bicarbonate, and 15 mg/L phenol red, was supplied by Vitrocell (Campinas, São Paulo, Brazil). Antibiotics (1×10^4 U/mL penicillin plus 10 mg/mL streptomycin) and fetal bovine serum (FBS) were also supplied by Vitrocell. DMEM (#D5030), without glucose, pyruvate, glutamine, sodium bicarbonate, and phenol red, was purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Cell lines and culture

The human glioblastoma T98G cell line was purchased from the American Type Culture Collection (Manassas, VA, USA), the human lung adenocarcinoma A549 cell line was purchased from the "*Banco de Células do Rio de Janeiro*" (Rio de Janeiro, RJ, Brazil), and the mouse myoblast C2C12 cell line was provided by professor Leonardo Reis (UNICAMP, Campinas, Brazil). Cells were cultured as previously described [1,2] in DMEM (Vitrocell, Campinas, Brazil), containing 11 mM glucose, 1.25 mM pyruvate, 4 mM glutamine, 44 mM sodium bicarbonate, 15 mg/L phenol red, antibiotics (10^4 U/mL penicillin plus 10 mg/mL streptomycin), and 10% fetal bovine serum. On the experiment day, cells were trypsinized and resuspended ($16–32 \times 10^6$ cells/mL; >95% viability) in the standard incubation medium described below. Cell suspensions were maintained at room temperature (~23 °C) and used within 2.5 h.

2.3. Measurement of OCR in suspended cells

Cells ($2–3 \times 10^6$) were added to a standard incubation medium composed of DMEM (#D5030, Sigma-Aldrich) supplemented with 11 mM glucose, 4 mM glutamine, 1.25 mM pyruvate, 20 mM HEPES (pH 7.4), without sodium bicarbonate and phenol red. The OCR in intact suspended cells was determined at 37 °C in a 2-mL chamber of a high-resolution oxygraph (OROBOROS Oxygraph-2k, Innsbruck, Austria), as previously described [1,2,4]. The additions made to the incubation medium are described in the figure legends.

2.4. $\Delta\Psi$ measurements in suspended cells

$\Delta\Psi$ in suspended intact cells was evaluated with the fluorescent probe TMRM on a Hitachi F-7000 fluorescence spectrophotometer (Tokyo, Japan) equipped with magnetic stirring and operating with excitation and emission wavelengths of 553 and 576 nm, respectively, and a 2-second response time [2]. Slit widths were 2 nm and 5 nm for excitation and emission, respectively. T98G cells (3×10^6) were resuspended in 2 mL standard incubation medium containing 500 nM TMRM and 1 μM TPB^-. Simultaneous measurements of OCR were conducted in the chamber of the high-resolution oxygraph under identical experimental conditions.

2.5. Statistical analysis

The data are displayed as representative traces or means + standard deviation (SD). Experiments were performed with cells from at least four passages. The unpaired Student's *t*-test was applied to analyze differences between two groups. Multiple comparisons were performed using a two-way ANOVA/Bonferroni post-hoc test for parametric data. CCCP concentrations data were considered as ranked, because they were obtained after only a few additions of predetermined CCCP concentrations, therefore these data were analyzed as non-parametric data by Kruskal-Wallis test/Dunn's post-hoc test. When a group is not denoted by * and/or #, it is understood "not statistically significant" in the respective comparison.

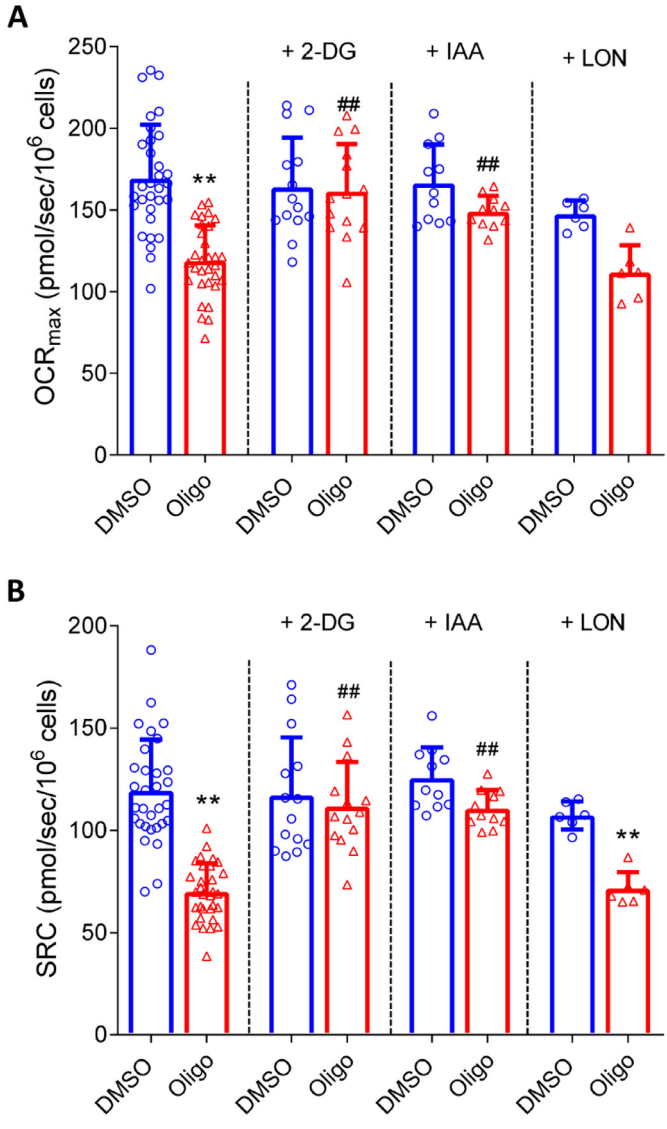

Fig. 1. Effects of glycolytic inhibitors on the underestimation of maximal mitochondrial oxygen consumption rate (OCR_{max}) and spare respiratory capacity (SRC) in oligomycin-treated cells. T98G cells (1×10^6 /mL) were resuspended in a standard incubation medium in the presence or absence of glycolytic inhibitors. **A** and **B**: Effects of the glycolytic inhibitors 40 mM 2-deoxy-D-glucose (2-DG), 200 μM iodoacetic acid (IAA), and 400 μM lonidamine (LON) on OCR_{max} (**A**) and SRC (**B**), determined in the presence and absence of 1 μg/mL oligomycin (Oligo). DMSO (the oligomycin solvent) was present at a final concentration of 0.025% (v/v). $^{**}P < 0.01$, statistically significant difference versus respective DMSO group; $^{\#\#}P < 0.01$, statistically significant difference versus respective group without glycolytic inhibitors; two-way ANOVA/Bonferroni post-hoc test.

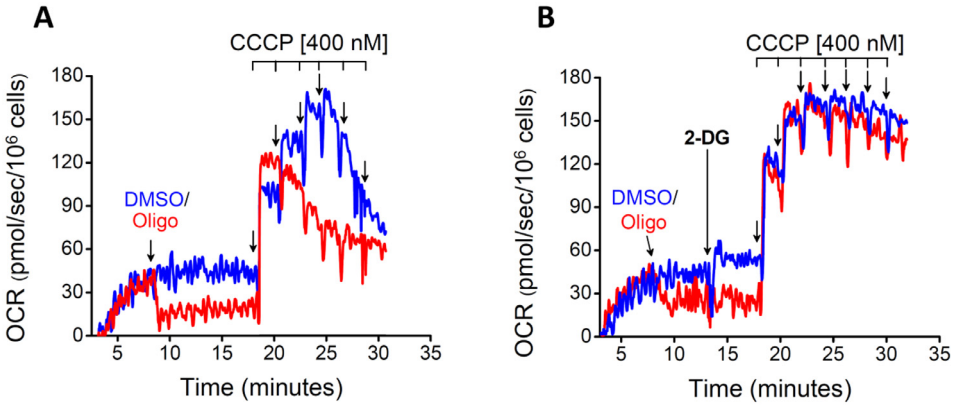

Fig. 2. Representative traces of OCR determinations in T98G cells in the presence or absence of 2-DG. T98G cells (1×10^6 /mL) were resuspended in standard incubation medium. Oligomycin (1 µg/mL; Oligo; red traces), 0.025% (v/v) DMSO (blue traces), 40 mM 2-DG, and CCCP (400 nM each addition) were added where indicated by the arrows.

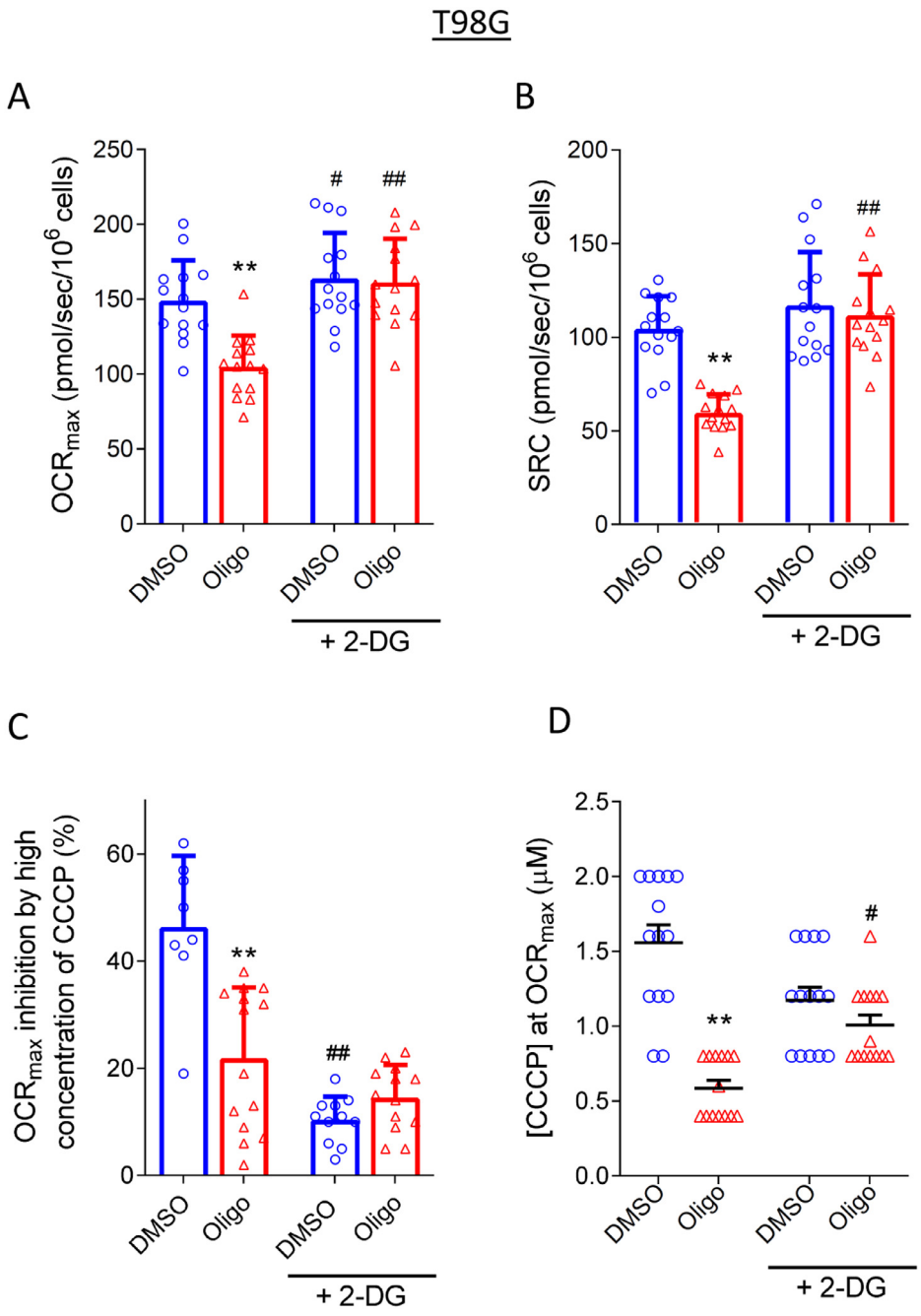

Fig. 3. 2-DG effects on the determination of OCR_{max} and SRC in T98G cells. T98G cells (1×10^6 /mL) were resuspended in standard incubation medium, and 0.025% (v/v) DMSO, 1 μg/mL oligomycin (Oligo), or 40 mM 2-DG were added to the experiments as indicated. **A** and **B**: 2-DG effects on OCR_{max} and SRC, respectively. **C**: OCR_{max} inhibition when cells were incubated in the presence of double-optimal CCCP concentrations. **D**: CCCP concentrations to reach OCR_{max}. $^{**}P < 0.01$, statistically significant difference versus respective DMSO group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, statistically significant difference versus respective group without 2-DG; two-way ANOVA/Bonferroni post-hoc test (**A-C**) or Kruskal-Wallis test/Dunn's post-hoc test (**D**).

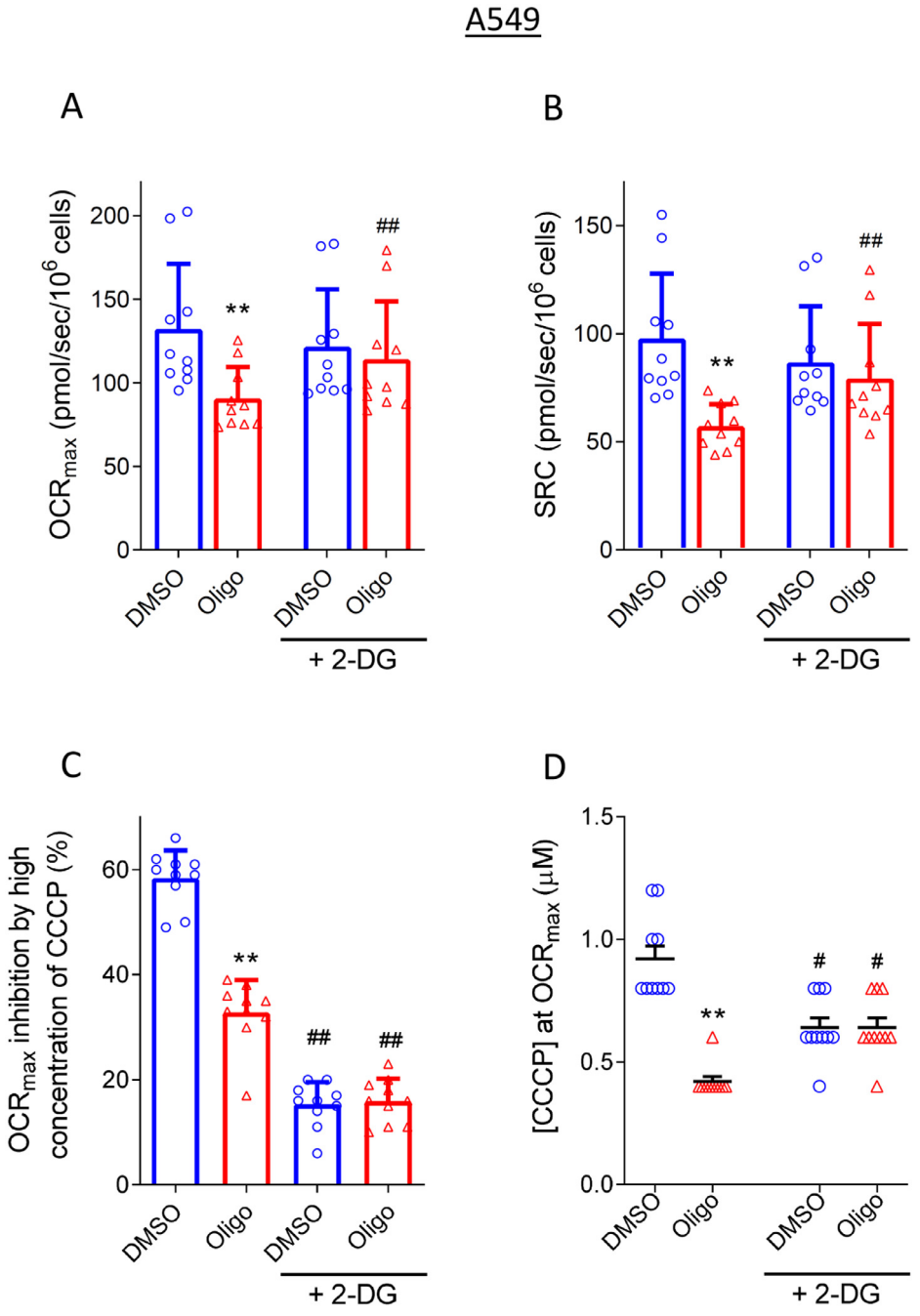

Fig. 4. 2-DG effects on the determination of OCR_{max} and SRC in A549 cells. A549 cells (1.25×10^6 /mL) were resuspended in standard incubation medium, and 0.025% DMSO (v/v), 1 µg/mL oligomycin or 40 mM 2-DG were added to the experiments as indicated. **A** and **B**: 2-DG effects on OCR_{max} and SRC. **C**: OCR_{max} inhibition when cells were incubated in the presence of double-optimal CCCP concentrations. **D**: CCCP concentrations to reach OCR_{max}. $^{**}P < 0.01$, statistically significant difference versus respective DMSO group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, statistically significant difference versus respective group without 2-DG; two-way ANOVA/Bonferroni post-hoc test (**A-C**) or Kruskal-Wallis test/Dunn's post-hoc test (**D**).

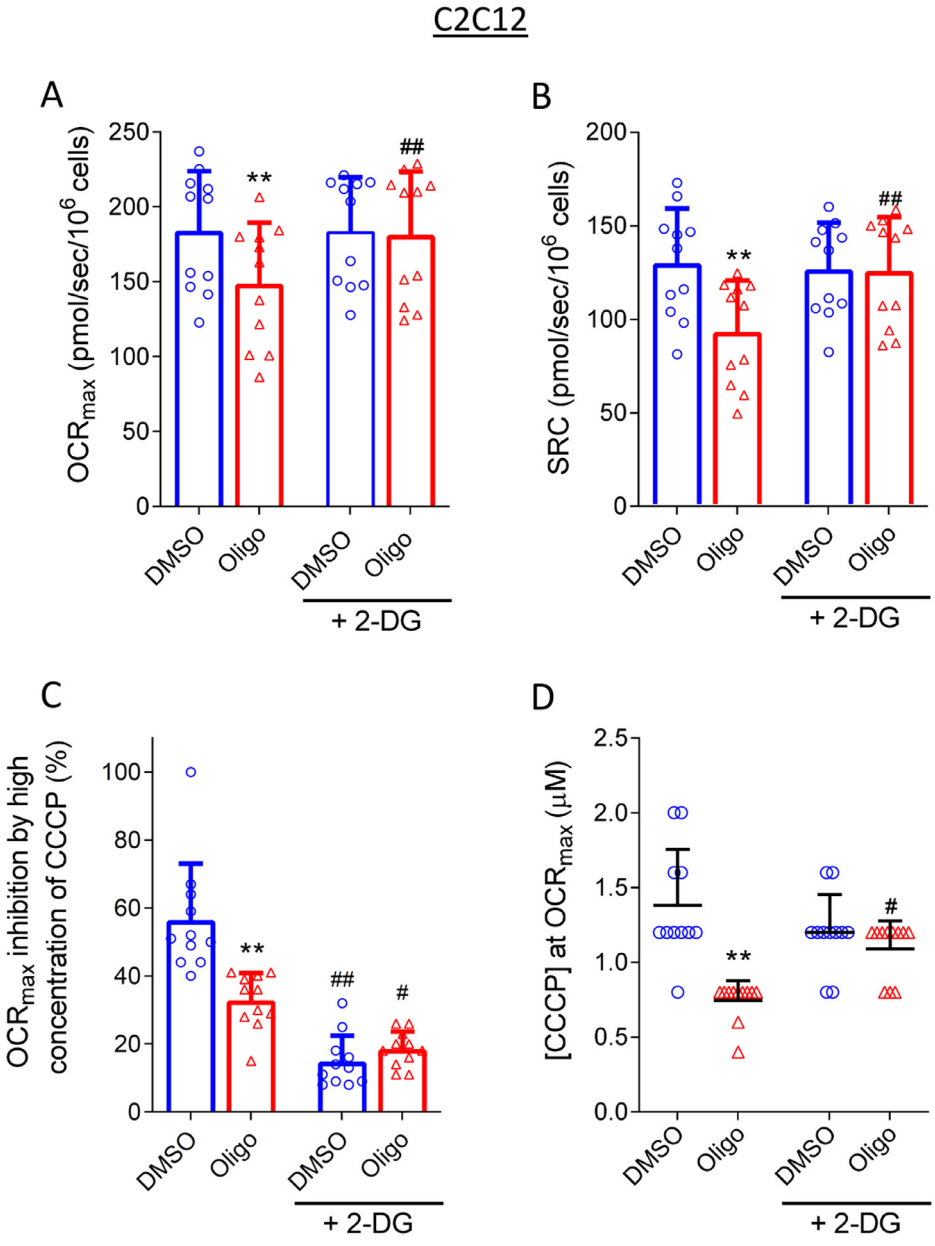

Fig. 5. 2-DG effects on the determination of OCR_{max} and SRC in C2C12 cells. C2C12 cells (1×10^6 /mL) were resuspended in standard incubation medium, and 0.025% (v/v) DMSO, 1 μg/mL oligomycin or 40 mM 2-DG were added to the experiments as indicated. **A** and **B**: 2-DG effects on OCR_{max} and SRC. **C**: OCR_{max} inhibition when cells were incubated in the presence of double-optimal CCCP concentrations. **D**: CCCP concentrations to reach OCR_{max}. $^{**}P < 0.01$, statistically significant difference versus respective DMSO group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, statistically significant difference versus respective group without 2-DG; two-way ANOVA/Bonferroni post-hoc test (**A-C**) or Kruskal-Wallis test/Dunn's post-hoc test (**D**).

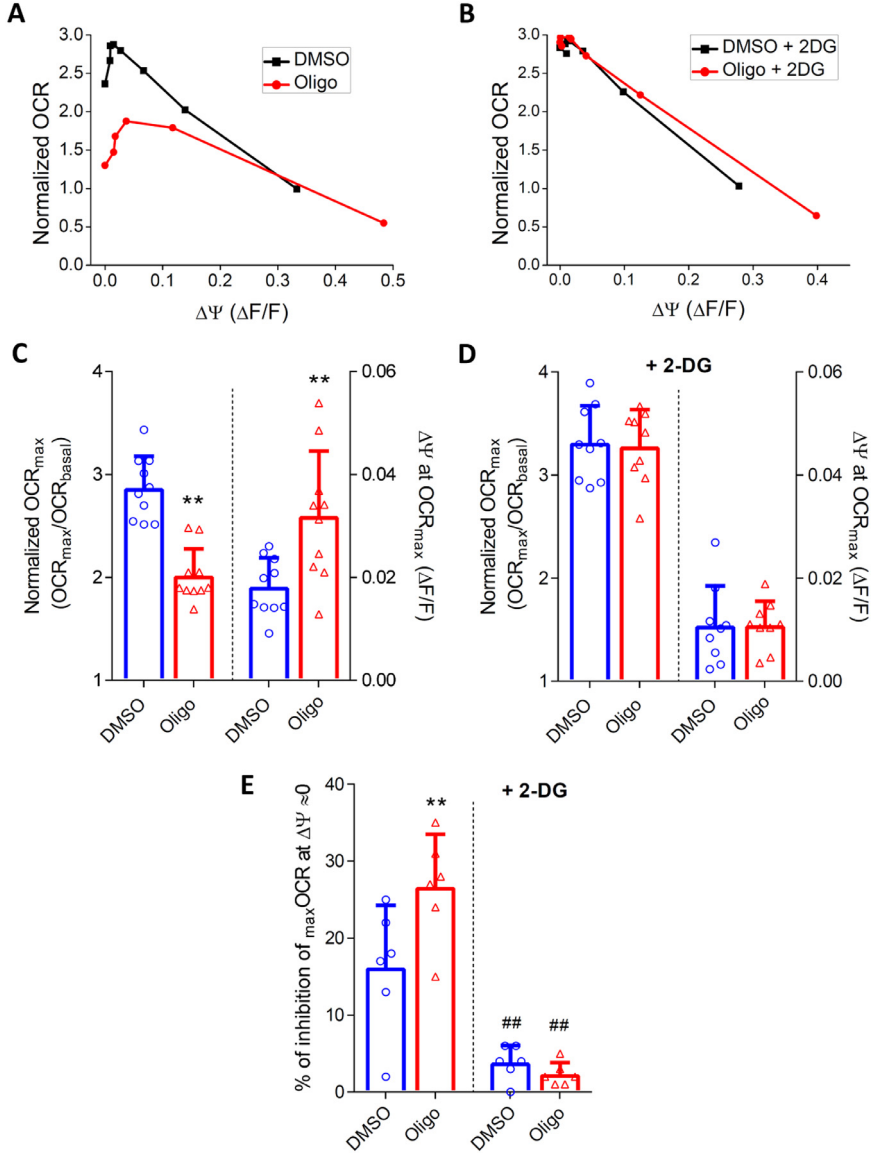

Fig. 6. Simultaneous determination of OCR and mitochondrial membrane potential ($\Delta\Psi$) in T98G cells: 2-DG and oligomycin effects. T98G cells (1.5×10^6/mL) were resuspended in standard incubation medium containing 500 nM TMRM and 1 μM TPB$^-$. DMSO (0.025%, v/v), 1 μg/mL oligomycin (Oligo) or 40 mM 2-DG were added to the experiments as indicated. **A** and **B**: Graphic correlation of OCR and $\Delta\Psi$ values obtained in the absence and presence of 2-DG, respectively. The OCR data were normalized to the respective basal OCR with DMSO ($OCR_{basal} = 1.0$). $\Delta\Psi$ is expressed as $\Delta F/F$, where F is the fluorescent intensity after the last CCCP-addition (i.e., completely dissipated $\Delta\Psi$) and ΔF is F minus any given fluorescent intensity. **C** and **D**: The left ordinate axes represent the effects of DMSO and Oligo on normalized OCR_{max} (OCR_{max}/OCR_{basal}). The right ordinate axes represent $\Delta\Psi$ ($\Delta F/F$) values when OCR_{max} was reached in the presence of DMSO or Oligo. Cells were incubated in the absence (**A** and **C**) or presence (**B** and **D**) of 2-DG. **E**: Inhibition percentage of OCR_{max} when $\Delta\Psi$ was dissipated (≈ 0). $^{**}P < 0.01$, statistically significant difference versus respective DMSO group, $^{\#\#}P < 0.01$, statistically significant difference versus respective group without 2-DG; unpaired Student's *t*-test (**C** and **D**) or two-way ANOVA/Bonferroni post-hoc test (**E**).

Ethics Statement

Our data did not require animals or patient samples, and approvals from the respective ethics committees were not necessary.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships which have or could be perceived to have influenced the work reported in this article.

CRediT Author Statement

Juliana S. Ruas: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft; **Edilene S. Siqueira-Santos:** Investigation, Data curation; **Claudia D.C. Navarro:** Investigation; **Roger F. Castilho:** Conceptualization, Formal analysis, Supervision, Writing – review & editing, Project administration, Funding acquisition.

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