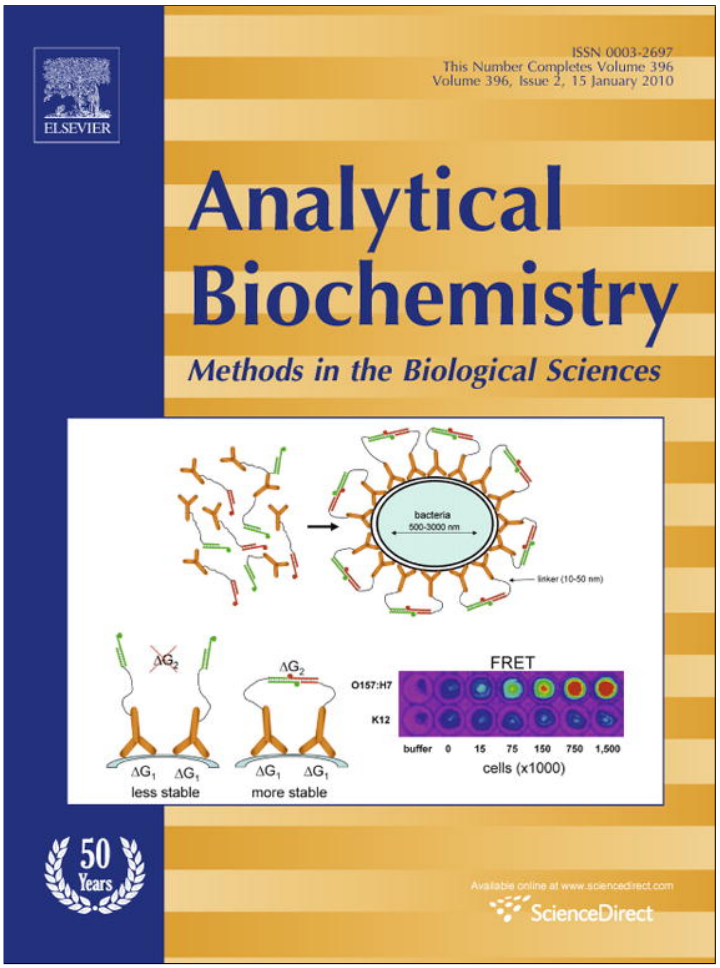

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Comparison of methods for measuring oxygen consumption in tumor cells in vitro

Caroline Diepart [a], Julien Verrax [b], Pedro Buc Calderon [b], Olivier Feron [c], Bénédicte F. Jordan [a], Bernard Gallez [a,*]

[a] Laboratory of Biomedical Magnetic Resonance, Louvain Drug Research Institute, Université catholique de Louvain, B-1200 Brussels, Belgium
[b] Laboratory of Pharmacokinetics, Metabolism, Nutrition, and Toxicology, Louvain Drug Research Institute, Université catholique de Louvain, B-1200 Brussels, Belgium
[c] Laboratory of Pharmacotherapy, Université catholique de Louvain, B-1200 Brussels, Belgium

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ABSTRACT

The oxygen consumption rate of tumor cells affects tumor oxygenation and response to therapies. Highly sensitive methods for determining cellular oxygen consumption are, therefore, needed to identify treatments that can modulate this parameter. We compared the performances of three different methods for measuring cellular oxygen consumption: electron paramagnetic resonance (EPR) oximetry, the Clark electrode, and the MitoXpress fluorescent assay. To compare the assays, we used K562 cells in the presence of rotenone and hydrocortisone, compounds that are known to inhibit the mitochondrial electron transport chain to different extents. The EPR method was the only one that could identify both rotenone and hydrocortisone as inhibitors of tumor cell oxygen consumption. The Clark electrode and the fluorescence assay demonstrated a significant decrease in cellular oxygen consumption after administration of the most potent inhibitor (rotenone) but failed to show any significant effect of hydrocortisone. EPR oximetry is, therefore, the most sensitive method for identifying inhibitors of oxygen consumption on cell assays, whereas the Clark electrode offers the unique opportunity to add external compounds during experiments and still shows great sensitivity in studying enzyme and chemical reactions that consume oxygen (non-cell assays). Finally, the MitoXpress fluorescent assay has the advantage of a high-sample throughput and low bulk requirements but at the cost of a lower sensitivity.

The importance of oxygen in cancer biology is now well established. Hypoxia is a cause of tumor resistance to radiation and other cytotoxic treatments. Multiple clinical studies using the Eppendorf pO_2 (partial pressure of oxygen)[1] histograph have demonstrated a direct relationship between tumor hypoxia (low oxygen concentration, $pO_2 < 10$ mmHg) and disease progression in a wide variety of human cancers. Hypoxia is also involved in the development of a more aggressive phenotype and contributes to metastasis [1,2].

Tumor oxygenation depends on the balance between oxygen supply and consumption, and both factors should be considered in developing strategies to reduce tumor hypoxia. A number of strategies have been considered in an effort to improve tumor oxygenation during radiation treatment, with the goal of increasing tumor sensitivity to irradiation. Theoretical simulation of oxygen handling in tumors [3] suggests that decreasing the oxygen consumption rate of tumor tissue could be a particularly effective strategy to reduce the fraction of hypoxic tissue in solid tumors. Indeed, several pharmacological drugs that inhibit cellular oxygen consumption have been characterized in vivo for their potential to increase tumor oxygenation and, thereby, to enhance radiosensitivity. Meta-iodobenzylguanidine [4], insulin [5], anti-inflammatory drugs [6], corticoids [7], inhibitors of vascular endothelial growth factor (VEGF) receptor tyrosine kinase (SU5416) [8], and thyroid hormones [9] all play a major role in the metabolism of tumor cells by modifying the rate of oxygen consumption. Other means of reducing oxygen consumption have also been proposed, including administration of glucose, through the Crabtree effect [10], and decreasing local tumor temperature to less than 25 °C [11].

In view of its clinical importance, several techniques have been developed to measure oxygenation status in tissues and tumors in living animals and humans [12,13], but methods to measure rates of oxygen consumption are more limited [14,15]. Three common methods are currently available to measure oxygen consumption in vitro: electron paramagnetic resonance (EPR) oximetry, the Clark oxygen electrode, and the MitoXpress fluorescent assay. EPR is a powerful technology that permits continuous monitoring of oxygenation in tissues (Fig. 1A). The method is based on the variation of the linewidth of a paramagnetic material in the presence of oxygen-consuming cells in a closed system. The effect of treatments on the tumor cell oxygen consumption rate can be

* Corresponding author. Fax: +32 2 764 73 90.
E-mail address: bernard.gallez@uclouvain.be (B. Gallez).

[1] *Abbreviations used:* pO_2, partial pressure of oxygen; VEGF, vascular endothelial growth factor; EPR, electron paramagnetic resonance; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; LiPc, lithium phthalocyanine.

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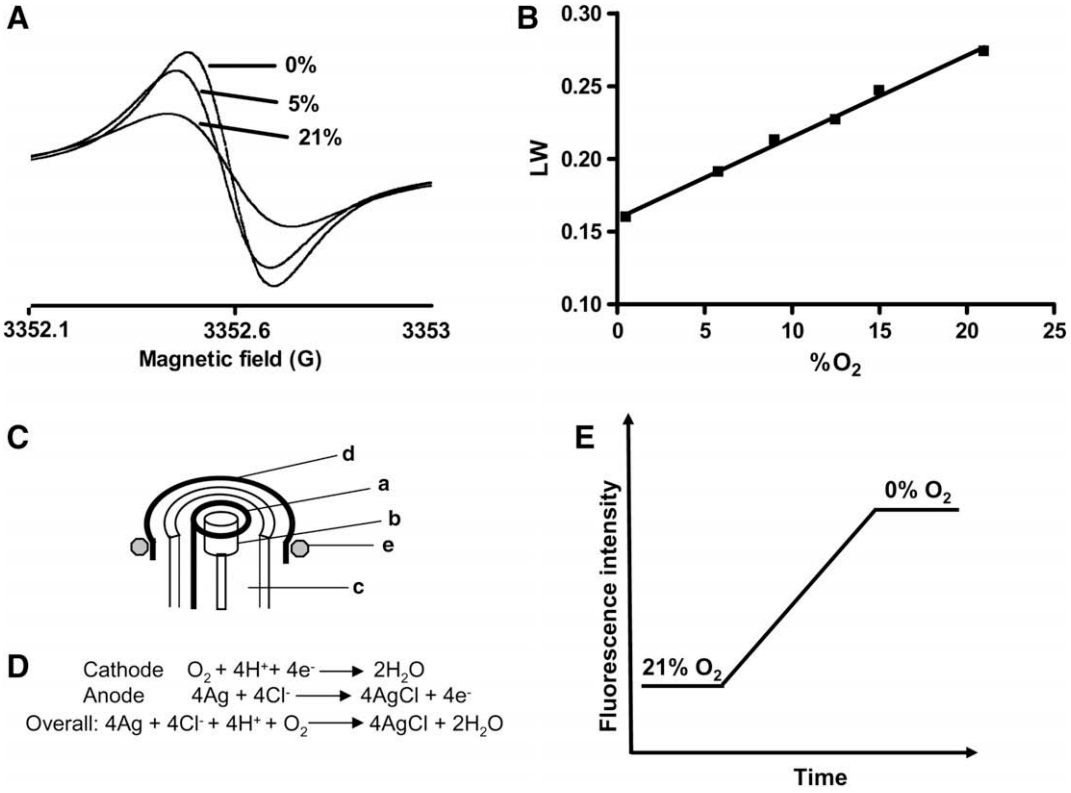

Fig. 1. Principles of the measurement of oxygen using EPR oximetry, the Clark electrode, and the MitoXpress fluorescent assay. (A) EPR spectra recorded in nitrogen or in air. (B) Calibration curve (EPR linewidth [LW] as a function of the pO_2). (C) The Clark-type electrode consists of a platinum electrode (a) and a reference Ag/AgCl electrode (b) covered by a film of half-saturated KCl electrolyte (c) enclosed within a Teflon membrane (d) that is held in place by a rubber ring (e). (D) Current flows from the silver electrode to the platinum electrode as electrons are released into solution from the latter. Removal of electrons from solid silver produces silver ions. The silver ions combine with chloride ions in solution to precipitate silver chloride on the surface of the silver electrode. This leaves potassium ions behind; however, because hydrogen ions are taken out of solution by the consumption of oxygen, the charge remains balanced. (E) Schematic fluorescence intensity profile of MitoXpress during the oxygen consumption assay.

measured because of the relationship between EPR linewidth and pO_2 (Fig. 1B) [16]. The Clark oxygen electrode consists of an anode and a cathode in contact with an electrolyte solution and covered by a semipermeable membrane. Oxygen diffuses through the membrane to the cathode, where it is reduced. The current produced by the electrode is proportional to the oxygen tension in the solution [17] (Fig. 1C and D). Finally, the MitoXpress assay is based on a phosphorescent oxygen-sensitive probe developed by Luxcel. The assay is based on the ability of oxygen to quench the excited state of the MitoXpress probe. Depletion of oxygen in the surrounding solution is perceived as an increase in probe phosphorescence signal. Therefore, changes in oxygen consumption, reflecting changes in mitochondrial activity, are measured as changes in MitoXpress probe signal over time [18] (Fig. 1E).

Oxygen consumption by tumor cells is increasingly recognized as a key parameter for evaluating the efficacy of chemo- and radiotherapy. It is, therefore, timely to compare the sensitivity of the available methods for determining oxygen consumption. In the current study, we compared three methods for measurement of cellular oxygen consumption. We applied the three methods described above to tumor cells exposed to rotenone or hydrocortisone, treatments known to inhibit the mitochondrial electron transport chain to different extents [7,19], so as to provide insight into the advantages and disadvantages of each technique.

Materials and methods

Cell lines

K562 human chronic myelogenous leukemia cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were routinely cultured in RPMI-1640 containing 10% fetal bovine serum (FBS), 1.2% glutamine, and 1% penicillin–streptomycin. The cultures were kept at a density of 1 to 2×10^5 cells/ml. The medium was changed at 48- to 72-h intervals. All cultures were kept at 37 °C in a 95% air/5% CO_2 atmosphere with 100% humidity.

Chemicals

Hydrocortisone was purchased from Pfizer (Solu-Cortef, Pharmacia, Pfizer, Belgium) and diluted in saline. Rotenone was purchased from Sigma–Aldrich (Bornem, Belgium) and diluted in dimethyl sulfoxide (DMSO) to 0.5 mg/ml (Sigma–Aldrich). Cells were treated with hydrocortisone (140 μM for 60 min) or rotenone (20 μM for 30 min) in all of the experiments. Menadione sodium bisulfite (vitamin K_3) and sodium ascorbate (vitamin C) were purchased from Sigma (St. Louis, MO, USA) and diluted in distilled water to 20 μM and 2 mM, respectively.

Measurement of oxygen consumption rate by EPR

EPR spectra were recorded on a Bruker EMX EPR spectrometer operating at 9.5 GHz. Cells were suspended in 10% dextran (to maintain cells in suspension) in complete medium. A neutral nitroxide, ^{15}N 4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-^{15}N-1-oxyl at 0.2 mM (CDN Isotopes, Pointe-Claire, QC, Canada), was added to 100-μl aliquots of tumor cells (2×10^7 cells/ml and 5×10^6 cells/ml), which were then drawn into glass capillary tubes. The capillary tube was visually checked to avoid air bubbles and was disregarded if it did not conform. The probe (0.2 mM in 20% dextran in complete medium) was calibrated at various pO_2 values between

100% nitrogen and air so that the linewidth measurements could be related to pO_2 at any value. Nitrogen and air were mixed in an Aalborg gas mixer, and the oxygen content was analyzed using a Servomex oxygen analyzer OA540. The sealed tubes were placed into quartz EPR tubes, and samples were kept at 37 °C. The resulting linewidth reports on pO_2, and so oxygen consumption rates were obtained by measuring the pO_2 in the closed tube over time and determining the slope of the resulting linear plot [5].

Fluorescence-based assay of cell respiration

Cultured cells were diluted with prewarmed air-saturated (37 °C) medium (without phenol red) to the desired concentration (2×10^7 cells/ml), and 150 μl/well was dispensed into a 96-well plate (black body, clear bottom). An A65 N-1 oxygen probe (Luxcel Biosciences, Cork, Ireland), supplied as dry powder in a vial, was reconstituted in 1 ml of water [20]. Next, 10 μl of this solution was transferred into each well, and the wells were then covered with 100 μl of prewarmed (37 °C) mineral oil. Time-resolved measurements were carried out at 37 °C on a Victor X4 plate reader (PerkinElmer Life Sciences, Waltham, MA, USA) using standard 340-nm excitation and 642-nm emission filters, reading every minute for 90 min. Oxygen consumption rates were assessed by determining the rate of increase in the probe fluorescent signal for each sample (i.e., $\Delta I/\Delta t$) by linear regression using instrument software. These values were then corrected with respect to blanks (baseline slopes in the absence of cells) and normalized with respect to initial intensity (with 21% of oxygen) (I_{21} at 37 °C) to give normalized intensities [20].

Measurement of oxygen consumption rate by Clark-type electrode

The YSI 5300 Clark-type electrode (Yellow Springs Instrument, Yellow Springs, OH, USA) consists of a platinum cathode and a silver anode, both in contact with an electrolyte solution (50% saturated KCl solution). It is covered at the tip by a semipermeable membrane that is permeable to gases. The electrode was placed and fixed on the top of the measuring chamber. A magnetic stirrer was positioned on the bottom of the measuring chamber. To maintain a stable chamber temperature, an additional surrounding chamber was filled with water and connected with a water circulation system. When a current is applied to the electrodes, the platinum electrode is negatively charged, whereas the silver electrode is positively charged. Oxygen diffuses through the Teflon membrane to the platinum electrode, where it is reduced. The current produced by the electrode is proportional to the oxygen tension in the solution [17]. Air-saturated medium (4 ml) was introduced into the electrode chamber (capacity of 10 ml) and incubated until a steady baseline was obtained on the chart. Cells were suspended in 100 μl (5×10^6 cells/ml) of medium and introduced into the incubation chamber using a Hamilton syringe. The Clark electrode was also used to measure the oxygen uptake from a mixture of ascorbate (2 mM) and menadione (20 μM) dissolved in well-oxygenated phosphate buffer (0.05 M). All of the measurements were carried out at 37 °C.

Statistical analysis

Results are presented as means ± standard errors. Comparisons between groups were analyzed by Student's t test or analysis of variance (Dunnett's multiple comparison test) for experiments with more than two groups. P values less than 0.05 were considered as statistically significant.

Results

We first compared the oxygen consumption rate using EPR oximetry and the Clark electrode. Using EPR, we measured the decrease in pO_2 over time and observed a significant difference in oxygen consumption between control cells and cells treated with hydrocortisone or rotenone (5×10^6 cells/ml) (Fig. 2). The graphs present the variations in the percentage of oxygen as a function of time. The mean slope was –0.55 ± 0.01 μM/min/5×10^6 cells for control cells ($n = 4$) (Fig. 2A). The slopes were statistically different for rotenone-treated cells (−0.12 ± 0.009 μM/min/5×10^6 cells, $P < 0.001$, $n = 4$) and for hydrocortisone-treated cells (−0.367 ± 0.007 μM/min/5×10^6 cells, $P < 0.001$, $n = 4$). However, using the Clark electrode (Fig. 2B), there was a significant difference between control and rotenone-treated cells ($P < 0.05$, $n = 4$), but cells treated with hydrocortisone had a nonsignificant trend to slower oxygen consumption ($P > 0.05$, $n = 6$). The mean slopes were −0.4 ± 0.07 μM/min/5×10^6 cells for control cells, −0.19 ± 0.008 μmol/L/min/5×10^6 cells for rotenone-treated cells, and −0.32 ± 0.05 μM/min/5×10^6 cells for hydrocortisone-treated cells.

In a second experiment, we measured oxygen uptake in the presence of a cocktail of menadione (vitamin K_3) and ascorbate in a cell-free system using the Clark electrode system. This type of experiment is feasible only with the Clark system, in which extra compounds can be added during the experiment by an access slot in the plunger of the electrode. This type of experiment is not possible with EPR oximetry, which requires a closed and sealed setup. Compared with the rates observed with ascorbate alone, the addition of a solution of menadione was associated with an enhanced

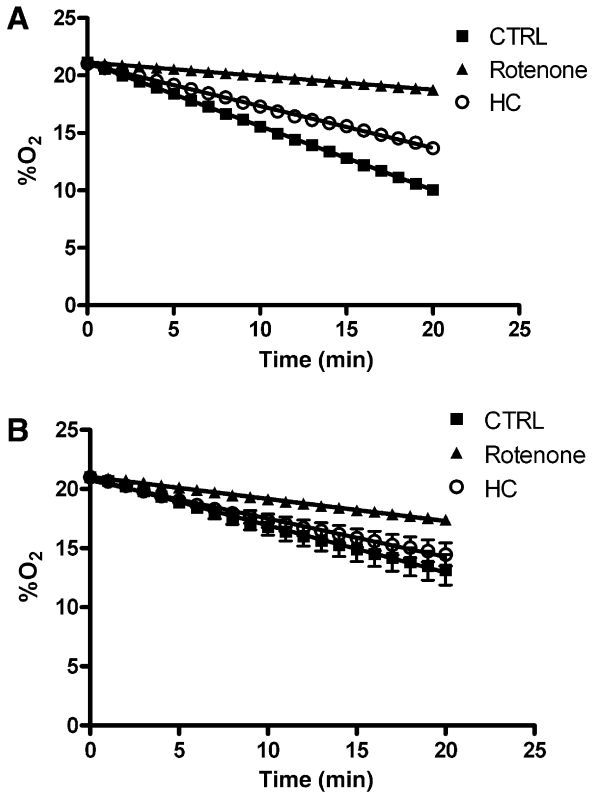

Fig. 2. Oxygen consumption rate measured by EPR oximetry and Clark electrode of K562 cells (5×10^6 cells/ml). (A) When using EPR oximetry, rotenone- and hydrocortisone-treated cells consumed oxygen significantly more slowly than control cells ($P < 0.01$, $n = 4$). (B) When using the Clark electrode, only rotenone induced a significant decrease in oxygen consumption ($P < 0.01$, $n = 4$ for rotenone-treated cells and $n = 6$ for control and hydrocortisone-treated cells). CTRL, control; HC, hydrocortisone.

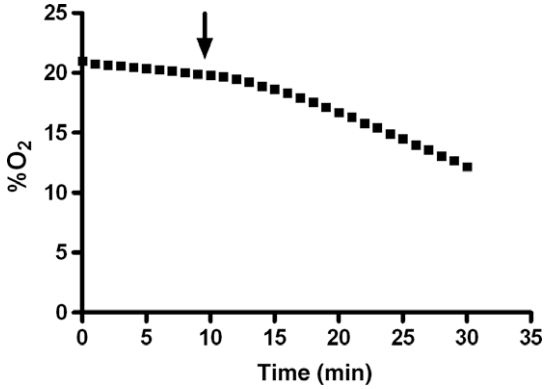

Fig. 3. Effect of 20 μM menadione (vitamin K_3) on oxygen uptake in the presence of 2 mM ascorbate (vitamin C). The arrow corresponds to the injection of menadione. The results are mean values of three separate experiments ± standard errors.

oxygen uptake, as shown in Fig. 3. These results are consistent with those obtained in a previous study [21].

We then compared the ability of the MitoXpress fluorescence assay to detect changes in the oxygen consumption rates of K562 cells treated with rotenone and hydrocortisone. It should be emphasized that no oxygen consumption was visible in the conditions described in the first assay comparing EPR oximetry and the Clark electrode. Indeed, it was necessary to use a concentration of 20×10^6 K562 cells/ml to observe significant oxygen consumption with the MitoXpress assay. It is impossible to inject this quantity of cells using a Hamilton syringe, so the results could be compared only with the EPR method and not with the Clark electrode (see above). Fig. 4A shows the results of the evolution of the normalized fluorescence intensity over the time period. The initial portion of the profile, reflecting temperature equilibration, was disregarded. The graph with the initial portion of the profile show that the starting intensities are the same for the various procedures. The slopes were calculated from the 20th minutes. Initially, the probe emission is quenched by dissolved oxygen present at air-saturated concentrations. When the level of oxygen consumption due to cellular respiration exceeds the rate of back diffusion, a depletion in dissolved oxygen is observed. This is achieved by using a sufficient concentration of cells and adding a layer of mineral oil to impede back diffusion of oxygen. Oxygen depletion reduces the quenching effect, resulting in an increase in probe emission intensity. To make it easier to compare the kinetics, the relative evolution of the fluorescence intensity compared with the initial value is shown in Fig. 4B. There was a marked decrease in respiratory activity of K562 cells treated with rotenone ($P < 0.01$) (Fig. 4B and C), although the small decrease observed in the presence of hydrocortisone was not significant. We used EPR oximetry to measure oxygen consumption using the same concentration of tumor cells

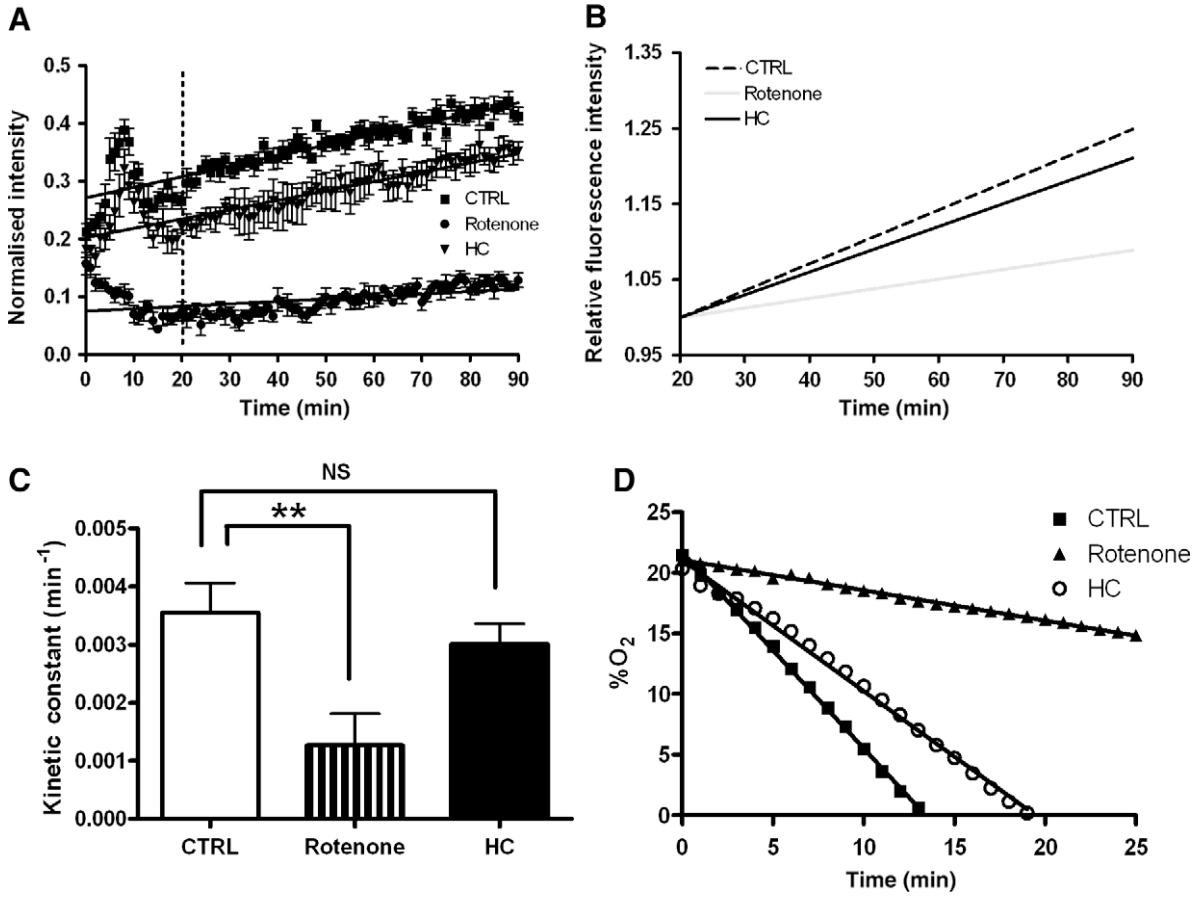

Fig. 4. Oxygen consumption rate of K562 cells (20×10^6 cells/ml) measured by EPR oximetry and MitoXpress fluorescence. (A) Normalized intensity of the assay. By MitoXpress, only rotenone induced a significant decrease in oxygen consumption ($P < 0.01$, $n = 6$). (B) Relative fluorescence intensity of control and treated cells. (C) Kinetic constants measured in control and treated cells. The kinetic constants measured in control cells were higher than those in rotenone-treated cells but not higher than those in hydrocortisone-treated cells ($^{**}P < 0.01$). (D) When using EPR, rotenone- and hydrocortisone-treated cells consumed oxygen significantly more slowly than control cells ($P < 0.01$, $n = 4$). CTRL, control; HC, hydrocortisone; NS, nonsignificant.

(20×10^6 cells/ml) (Fig. 4D). Hydrocortisone induced a significant reduction in oxygen consumption (-1.1 ± 0.02 μM/min/20×10^6 cells, $P < 0.001$), as did rotenone (-0.25 ± 0.007 μM/min/20×10^6 cells, $P < 0.001$), compared with control cells (-1.62 ± 0.01 μM/min/20×10^6 cells).

Discussion

The current study is the first to compare three methods for measuring cellular oxygen consumption. This comparison provides insight into which is the best method for demonstrating the effects of a given treatment on the mitochondrial respiratory chain and may, therefore, be helpful for optimizing existing therapies such as chemotherapy and radiotherapy. Indeed, it has been predicted theoretically that modification of oxygen consumption influences oxygen transport much more effectively than modification of oxygen delivery [3]. Our group further validated this approach as an effective way of radiosensitizing experimental tumors in vivo [5–9]. In the current study, the action of two different treatments acting on the mitochondrial respiratory chain to decrease cellular oxygen consumption to different degrees was evaluated using three techniques: EPR, Clark electrode, and MitoXpress fluorescence. EPR showed a significant decrease in oxygen consumption by K562 cells treated with rotenone and hydrocortisone. The Clark electrode and MitoXpress systems demonstrated a significant decrease in cellular oxygen consumption after administration of rotenone, but there were no significant effects on cells treated with hydrocortisone.

The known advantages of the EPR method include the fact that the cells and oxygen sensors are distributed homogeneously throughout the samples and that the spin probe does not consume oxygen, allowing low concentrations of oxygen in solutions to be measured [19]. It is known that the reduction of nitroxides and the oxidation of hydroxylamines by cells are strongly dependent on the concentration of oxygen. However, the kinetic constants are altered only at very low oxygen concentrations [22,23]. So, the oxygen consumption by the system itself does not play a role above 3% of oxygen, as we used in our study. This method has been shown to be a useful tool for accurate repeated measurements of local concentrations of oxygenation and respiration in cell suspensions [5–9,24,25]. In the current study, EPR oximetry was the most sensitive method for demonstrating subtle differences in oxygen consumption rates. Moreover, EPR measurements were not limited by the numbers of cells, in contrast to the other two techniques, although for different reasons. Indeed, with the Clark electrode the number of cells is limited by the volume added to the incubation chamber, whereas with the MitoXpress method a minimal sensitivity threshold needs to be reached to provide reproducible measurements (i.e., 20×10^6 cells/ml). However, a key limitation of the EPR method is that it is not technically possible to add additional compounds during the experiment. In this study, for instance, we could not measure the oxygen consumption of the mixture of ascorbate and menadione. Moreover, ascorbate interferes with the nitroxide used as the oxygen probe by reducing the free radical into the diamagnetic hydroxylamine that is EPR silent [26]. A possible solution to this problem would be to use a particulate probe such as lithium phthalocyanine (LiPc) to measure the oxygen consumption of the mixture [24], but the EPR system still does not have the facility to enable extra compounds to be added during the experiment. Finally, the cost of the equipment required for EPR oximetry may represent a barrier to choosing EPR for the measurement of cellular oxygen consumption.

The Clark-type oxygen electrode has long been the main technique for measuring pO_2 and oxygen consumption, and it is still widely used on a laboratory scale. Although this measurement approach has proved to be very useful, the methodology is associated with a number of inherent limitations. These include the oxygen consumption by the electrode, the sensitivity to mass exchange (stirring requirements) that may damage the cells during a long experiment, and the need for a large assay volume compared with other techniques. Here we used a standard system with a sample chamber size of 2 to 10 ml. A micro system with a sample chamber size of 600 μl is also available, but the volume is still larger than in the other two methods (100 μl for EPR and <100 μl for fluorescence). Nevertheless, the method offers the unique advantage of being able to add other compounds during the experiment, and this is particularly important in the measurement of mitochondrial oxygen consumption. Indeed, classical mitochondrial assessments entail analysis of oxygen consumption after the addition of substrates (e.g., glutamate, succinate) (state 2), consumption rate on the addition of ADP (state 3), and final return to the basal state after all ADP has been phosphorylated (state 4). For the EPR and fluorescence methods, sequential additions are difficult, so that state 2 is substituted for state 4, yielding a nontraditional ratiomet-

Table 1
Comparison of main characteristics of the three methods for measuring oxygen consumption.

	EPR	Clark electrode	MitoXpress fluorescence
Equipment	EPR spectrometer, probe (nitroxide)	Clark electrode and chamber	Standard microtiter plate, standard fluorescent reader, and oxygen-sensitive probe
Assay volume	100 μl	2–10 ml/600 μl	<100 μl
Cell concentration	Any	Maximum 10×10^6 cells/ml	Minimum 20×10^6 cells/ml
Stirring requirements	No	Yes	No
System sealed	Yes	Yes	Partially
Probe	Yes	No	Yes
Usability	Easy but some knowledge needed	Easy	Easy
Sampling	1 at a time	1 at a time	>96 at a time
Compound addition	Not possible	Possible (syringe)	Not possible
Reproducibility	High	Poor for cells; high for enzyme and chemical reactions	Poor
Sensitivity	High	Poor for cells; high for enzyme and chemical reactions	Poor
Price of equipment	Expensive	Inexpensive	Medium
Price of consumables	Inexpensive	Inexpensive	Inexpensive
Value of pO_2	Quantitative	Semiquantitative	Qualitative

Table 2
Comparison of sensitivity of methods for evaluating the effects of treatments that inhibit oxygen consumption by tumor cells.

	MitoXpress fluorescence	EPR	EPR	Clark electrode
Rotenone	36% ± 18 ($P < 0.01$)	15% ± 0.64 ($P < 0.001$)	22% ± 2.3 ($P < 0.001$)	47.5% ± 11.3 ($P < 0.05$)
Hydrocortisone	83% ± 17.2 ($P > 0.05$)	68% ± 0.7 ($P < 0.001$)	67% ± 1.1 ($P < 0.001$)	80% ± 21.1 ($P > 0.05$)

Note. Each value is expressed as a percentage of the kinetic constant obtained using untreated tumor cells.

ric index [18,27]. The Clark electrode seems to be an easy, reproducible, and sensitive technique for studying enzyme and chemical reactions that consume oxygen, as shown in the assay using the mixture of menadione and ascorbate [21,28].

The advantages of the fluorescence method are the simplicity of the measurement procedure, the high-sample throughput (up to 384 wells), and the low bulk requirements (standard multiwall plates, standard fluorescent reader, and oxygen-sensitive probe). However, to achieve the convenience of measurement in a standard microplate, a level of sensitivity is sacrificed. Due to the incomplete sample sealing in the microplate formats and the associated back diffusion of atmospheric oxygen, the rate of change of dissolved oxygen measured using this approach is lower than the consumption rates measured using a sealed system [18,20]. The concentration of K562 cells should be at least 20×10^6 cells/ml to be able to measure oxygen consumption in this system. The MitoXpress method, using partially sealed systems, produces the same general trends as the Clark electrode system, with fully sealed systems, but does not provide a real value of the pO_2, so it is not possible to measure the absolute quantity of oxygen consumed. The fluorescence assay detected only the effect of a treatment with a strong action on the mitochondrial respiratory chain. In addition, as with EPR, it is not possible to add other compounds during the experiment. A comparison of the main characteristics of the three techniques is given in Table 1, and a quantitative comparison is provided in Table 2.

In conclusion, EPR oximetry appears to be the most sensitive method for measuring the oxygen consumption rate of cells; in the current study, it was the only method able to identify hydrocortisone as an effective inhibitor of oxygen consumption by tumor cells. However, the Clark electrode remains the most appropriate system if the addition of compounds during the course of the experiment is required and shows a very good sensitivity for non-cell assays. The MitoXpress fluorescent method is a cheap, simple, and direct way of measuring cellular oxygen consumption with a sensitivity that is comparable to that of the Clark electrode for cell assays; however, it does not provide the opportunity of adding additional compounds during the experiment.

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