

The influence of hypoxia on bioluminescence in luciferase-transfected gliosarcoma tumor cells *in vitro*

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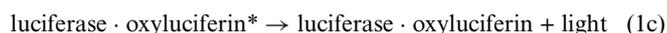
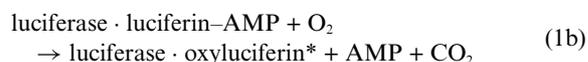
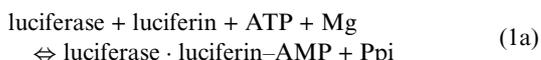
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Firefly luciferase catalyzes the emission of light from luciferin in the presence of oxygen and adenosine triphosphate. This bioluminescence is commonly employed in imaging mode to monitor tumor growth and treatment responses *in vivo*. A potential concern is that, since solid tumors are often hypoxic, either constitutively and/or as a result of treatment, the oxygen available for the bioluminescence reaction could be reduced to limiting levels, leading to underestimation of the actual number of luciferase-labeled cells during *in vivo* experiments. We present studies of the oxygen dependence of bioluminescence *in vitro* in rat 9 L gliosarcoma cells tagged with the firefly luciferase gene (9L^{luc}). We demonstrate that the bioluminescence signal decreases at pO₂ ≤ 5%, falling by approximately 50% at 0.2% pO₂. Further experiments showed that the critical threshold for the initiation of metabolic depression in these cells was around 5%. Below this level, the decrease of oxygen saturation was followed by a decrease in intracellular ATP due to the reduction of mitochondrial membrane potential. Hence, the data suggest that the decrease of intracellular ATP level *in vitro* is the limiting factor for bioluminescence reaction and so is responsible for the reduction of bioluminescence signal in 9L^{luc} cells in acute hypoxia, rather than luciferase expression or oxygen itself.

Introduction

Bioluminescence imaging (BLI) is an *in vivo* optical imaging technique, based on the use of cells of interest tagged with the firefly luciferase (*luc*) gene, the introduction of the labeled cells to a host animal and subsequent administration of luciferin that, in the presence of oxygen, results in an excited oxyluciferin through the adenosine triphosphate, ATP-dependent sequence:



where AMP = adenosine monophosphate and Ppi = pyrophosphate.

When this system relaxes to its ground state, a photon is emitted in the visible wavelength range that can be detected externally even through several millimeters of tissue.¹⁻³ This technique is now widely used for a range of applications, including monitoring of tumor growth and treatment response^{4,5} and monitoring of gene activation.⁶

During *in vivo* imaging, the bioluminescence signal from a given volume of tissue depends on several factors,⁷ including: the number of metabolically-active luciferase-transfected cells, which is usually the measure of interest and that defines the luciferase enzyme concentration, the luciferin concentration, which is generally not rate-limiting at typical administered doses and the ATP and O₂ levels and biophysical factors, such as the spectral emission range of the bioluminescence probe, the tumor depth and tissue optical properties, which affect the attenuation of the light by the tissue and, hence, the detected signal for a given tumor-cell mass. In this work, we focus on the effect of the oxygen and ATP levels on the bioluminescence signal. This could be of particular importance, since solid tumors frequently outstrip their oxygen supply, becoming transiently or chronically hypoxic. Hence, for example, tumor growth determined by BLI may be underestimated as the tumor becomes larger and more hypoxic. Likewise, the cytotoxicity of therapeutic interventions, as assessed by a drop in the constitutive BLI signal post treatment, may be overestimated if the treatment also increases the degree of tumor hypoxia. We previously reported the use of BLI to monitor the response of intracranially-transplanted 9L^{luc} gliosarcoma tumors after photodynamic therapy (PDT).⁵ Since PDT can induce tumor hypoxia, either directly through oxygen consumption during the photochemical reaction⁸ and/or indirectly by damaging tumor vasculature,^{9,10} it is important to determine the effects of oxygenation levels on the bioluminescence signal from 9L^{luc} cells in order to interpret the PDT bioluminescence response data reliably.

Although the influence of oxygen has been studied in luciferase solution¹¹ and in other luciferase systems such as Renilla luciferase¹² and in bacteria (*lux* operon) as an oxygen biosensor in studies of gas exchange in tissue,¹³ to our knowledge there are

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no published studies correlating the levels of hypoxia with the bioluminescence emission from firefly luciferase-bearing tumor cells. We report here the reduction in bioluminescence observed *in vitro* after exposing firefly luciferase-transfected cancer cells to low oxygen tensions, and attribute this to changes in energy status of the cells, with the reduction in intracellular ATP due to hypoxia being the rate-limiting factor in the bioluminescence reaction.

Materials and methods

Stable cell transfection

The luciferase transfection protocol, using the 9 L rat gliosarcoma cell line, has been described in detail previously.⁵ Briefly, the modified firefly luciferase gene plasmid (pGL3 Enhancer Vector: Promega Corp., Madison, WI, USA) was co-transfected into 9 L cells with a vector containing the neomycin selection marker (pCI mammalian expression vector: Promega) at a 10:1 ratio, using Lipofectamin (Promega). At 18 h after transfection the culture dishes were washed twice with phosphate buffered saline (PBS), and full media containing 700 $\mu\text{g ml}^{-1}$ of G418 sulfate was added and subsequently replaced every 2 d. After 14 d, the resulting colonies were harvested and cultured independently. The cell line with the highest luciferase activity was selected for further experiments. These 9L^{luc} cells were cultured as monolayers in Dulbecco's Modified Eagle Media with a reduced concentration of antibiotic G418 sulfate (350 $\mu\text{g ml}^{-1}$).

Bioluminescence measurements

For bioluminescence detection, a commercial BLI system (IVIS System: Xenogen, Alameda, CA, USA) was used, comprising a cryogenically-cooled, high-sensitivity CCD camera mounted in a light-tight chamber. D-luciferin solution (Xenogen) at 10 mg ml^{-1} in 5 ml vials was also gassed simultaneously under the same conditions so that the cell oxygenation would not be altered when luciferin was added to the suspension. For the bioluminescence measurements, 1.5 ml of oxygen-equilibrated luciferin solution was added to the glass vials containing the 9L^{luc} cells, which were then returned to the water bath and stirred for 10 s at $\sim 200 \text{ rev min}^{-1}$. The final luciferin concentration in the system ($\sim 0.90 \text{ mg ml}^{-1}$) was tested previously and found not to be rate limiting in this system (data not shown). The stir bar was then removed using tweezers and the total bioluminescence signal was measured over an integration time of 1 s.

Induction of hypoxia

9L^{luc} cells were harvested using trypsin for 5 min, spun at 1000 rev min^{-1} for 5 min and resuspended at $10^6 \text{ cells ml}^{-1}$ in PBS containing 5% of fetal bovine serum (FBS). 15 ml of 9L^{luc} cell suspension were dispensed in a 50 ml clear glass tube with a tight rubber stopper and exposed to controlled mixtures of pre-humidified gas containing O₂ at 0.2, 1, 5, 10 or 21% (air) with 5% CO₂ and balanced with N₂ (Praxair, Toronto, ON, Canada). A magnetic stir bar (200 rev min^{-1}) was used to ensure homogeneity of the solution. The gases were delivered through a flexible 4 mm diameter rubber tube, terminating in an 18G syringe, for 4 h at 25 °C using a water bath. To determine the oxygen dependence of firefly luciferase reaction in solution, 5 ml luciferin solution

(1 mM) in PBS was similarly gassed for 4 h under different oxygen concentrations and a 0.25 ml sample was removed and placed into 96 well-plates containing 10 μl of PBS containing 0.1 $\mu\text{g ml}^{-1}$ of ATP, 5 mg ml^{-1} luciferase and 5 mg ml^{-1} of MgCl. The luminescence signal from cell lysate was measured as described above using an integration time of 1 s.

Oxygen measurements

Measurements of pO₂ in the cell suspensions were made with an Oxylite 4000 system (Oxford Optronix Ltd., Oxford, UK). This uses a fiberoptic probe with a fluorescent dye incorporated in the tip, the lifetime of which is inversely proportional to oxygen concentration. The pO₂ measurements were recorded every 30 min for 2.5 h. The metabolic oxygen consumption of the 9L^{luc} cells was measured with the Oxylite probe every 5 min for 2 h in an air-tight quartz cuvette containing $5 \times 10^6 \text{ cells ml}^{-1}$ (4 ml) in PBS with 5% FBS. The measurements were automatically corrected to the temperature of the cell suspensions. Measurements were performed by inserting the oxygen probe through a small aperture in the glass lid, which was closed immediately after the data were recorded, thus minimizing gas exchange between the cell suspension and room air.

Calculation of cellular oxygen consumption

The oxygen requirements for the bioluminescence reaction observed *in vitro* were estimated as follows. The cell density in the sample vial was $10^6 \text{ cells ml}^{-1}$. The total system detection efficiency (including geometric factors and the quantum efficiency of the CCD camera) was $\sim 10^{-5}$. Hence, assuming that the generation of a bioluminescence photon requires one molecule of oxygen,² sustaining the measured bioluminescence signal of $1.05\text{--}1.97 \times 10^7 \text{ counts s}^{-1}$ from the sample in the cuvette requires $0.12\text{--}0.22 \times 10^{-12} \text{ mol ml}^{-1} \text{ s}^{-1}$ of molecular oxygen: for example, for 1.5×10^7 observed counts per second: $1.5 \times 10^7 / 15 \text{ ml (suspension vol.)} / 10^{-5}$ (efficiency/ 6.02×10^{23}). The measured metabolic oxygen consumption rate was $\sim 2 \times 10^{-12} \text{ mol ml}^{-1} \text{ s}^{-1}$, assuming a respiratory rate of $1 \times 10^{-17} \text{ mol cell}^{-1} \text{ s}^{-1}$ (based on Fig. 1C, as discussed below). Hence, the rate of oxygen consumption necessary for bioluminescence was about one order of magnitude lower than the metabolic consumption rate for this particular transfected cell line. At the two lowest pO₂ values *in vitro* (0.2 and 1% = 2.3×10^{-9} and $11.4 \times 10^{-9} \text{ mol ml}^{-1}$, respectively, using the standard conversion factor of 21% pO₂ = 240 μM), this metabolic consumption rate was significant: e.g. for 2.3 nmol ml^{-1} the oxygen would be depleted in less than 100 s at a consumption rate of 2 pmol $\text{ml}^{-1} \text{ s}^{-1}$.

The effect of this metabolic consumption during the gassing phase of the experiments was estimated as follows. According to the diffusion model of Whillans and Rauth,¹⁴ for an initial oxygen concentration in the cell suspension of C_o , an oxygen concentration of the overlying gas of C_g and continuous mixing of the suspension, the oxygen concentration as a function of time is given by:

$$C(t) = C_\infty - (C_o - C_\infty) \exp(-k_1 t) \quad (2)$$

The asymptotic concentration is $C_\infty = C_g - R/k_1$, where R is the rate of oxygen consumption inside the media and k_1 is given

by $k_1 = Ad/(VD)$, where A is the top surface area of the cell suspension, d is the thickness of the non-turbulent layer that facilitates exchange of the overlying gas,¹⁴ V is the volume of the suspension and D is the oxygen diffusion coefficient. The following values were used for the calculation: $A = 3.14 \text{ cm}^2$, $d = 50 \text{ }\mu\text{m}$,¹⁴ $V = 15 \text{ ml}$, $D = 2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$,¹⁵ and $C_o = 2.4 \times 10^{-7} \text{ mol ml}^{-1}$ (21%).

ATP measurements

In order to detect the possible changes in intracellular ATP resulting from cellular hypoxia, the total intracellular ATP was measured in lysed cells using a separate assay that is also luciferase-based (CellTiter-Glo®, Promega). Briefly, cells were harvested and exposed to one of the different oxygen tensions for 4 h, as described above. Then, 100 μl of cell solution ($\sim 10^5$ cells) was removed from the vial and immediately added to 96-well plates containing 150 μl of luciferase solution. The solution was mixed manually for 2 min and the luminescent signal allowed to stabilize at room temperature for 10 min. The ATP content was measured by recording the luminescent signal from cell lysates using the IVIS system with an integration time of 1 s. A standard curve of known ATP concentration dissolved in PBS (Sigma-Aldrich, Oakville, Canada) was used to correlate the intracellular ATP per cell and the luminescent signal (data not shown).

To assess whether the reduction of ATP due to hypoxia could have an influence on the BLI signal, we reduced the cellular ATP content by exposing the cells to oligomycin (Sigma, ON, Canada) diluted in dimethyl sulfoxide (DMSO, 5 $\mu\text{g } \mu\text{l}^{-1}$) for 30 min (3.5 h after starting exposure to either 0.2% pO_2 or air). The final DMSO concentration in the cell suspension was less than 1%. Oligomycin is a potent inhibitor of $\text{F}_0\text{F}_1\text{ATPase}$ and so would be expected to reduce ATP formation, thereby reducing the global intracellular ATP concentration to limiting levels for bioluminescence under normoxia.¹⁶ The oxygen levels in the solution contained in the 96-well plate and the cuvette containing oligomycin remained above the detection range of the Oxylite system ($\sim 100 \text{ mmHg}$) during the signal collection, demonstrating that oxygen was not limiting during the ATP measurements (data not shown).

Measurements of mitochondrial membrane potential ($\Delta\Psi_m$)

The mitochondrial membrane potential ($\Delta\Psi_m$) of 9L^{luc} cells after exposure to hypoxia was assessed using JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide/chloride; Molecular Probes, CA, USA). This dye localizes in mitochondria according to the membrane potential ($\Delta\Psi_m$). Mitochondria membrane depolarization with decrease of mitochondrial function induces a rapid shift from red to green fluorescence as the dye accumulates in the cytosol. Following gassing of the cells with the different oxygen concentrations, a 1 ml cell suspension was removed from the vial, seeded in coverslips containing 10 μl of 200 μM JC-1 and incubated at room temperature for 10 min. The dye fluorescence was measured using a confocal microscope (LSM510; Zeiss, Jena, Germany) with an excitation wavelength of 488 nm, detecting the green (510–560 nm) and red ($> 590 \text{ nm}$) fluorescence within 15 min. The signals were measured from the extranuclear regions of ≥ 10 cells per group, in triplicate for each experimental condition.

Immunoblot assay

Immediately after hypoxia exposure, cells were transferred into a 15 ml tube and spun at 3 000 g for 5 min and boiled in 1 ml of SDS-sample buffer (2% SDS w/v, 10% glycerol, 50 mM dithiothreitol, and 0.01% w/v bromophenol blue) for 5 min. Samples were then centrifuged for 1 min at $14 \times 10^3 g$ to pellet any debris. 20 μl of the supernatant was loaded and run at 180 V until the dye front reached $\frac{3}{4}$ down the gel, which was then transferred to a nitrocellulose membrane using transfer buffer solution and a semi-dry transfer apparatus at 15 V for 45 min. The membrane was then blocked with 5% milk, 0.1% Tween-20 PBS solution (PBST + 5% milk) for 30 min. Immunoblotting consisted of 3 washes of PBS + 0.1% Tween-20 for 10 min each, followed by incubation with HRP-conjugated anti-luciferase antibody at 1:20,000 dilution in 5% milk + PBST, and washing 3 times with PBST. 2 ml of chemiluminescence reagent (Santa Cruz, CA, USA) was added and the gel was exposed to film for 10 s.

Results and discussion

The generally-accepted hypothesis for the physiology of bioluminescence in fireflies is that light flashing is controlled by gating oxygen in specialized photocytes, *i.e.* that the dark state represents repression of bioluminescence by limiting oxygen¹⁷ However, there is no such mechanism to control oxygen diffusion in mammalian cells, so that constitutive bioluminescence proceeds in an uncontrolled manner upon luciferin becoming available, until one of the reagents is consumed. Given the occurrence of significant levels of hypoxia in solid tumors, including malignant brain tumors,¹⁸ the obvious limiting factor would be oxygen itself.

Fig. 1A shows the oxygenation levels in the cell suspension as a function of time, measured with the Oxylite probe: the equilibrium values for 10%, 5%, 1% and 0.2% oxygen gassing were $8 \pm 0.15\%$, $3.28 \pm 0.43\%$, $0.5 \pm 0.01\%$ and $0.06 \pm 0.01\%$, respectively. Since, at the lowest two oxygen concentrations, the pO_2 levels are below the sensitivity range of the probe ($\sim 0.7 \text{ mmHg}$), we also calculated the oxygen consumption for BLI and the metabolic oxygen rate. Fig. 1B shows the calculated oxygen concentration in the sample as a function of time after the start of gassing for each applied O_2 concentration. According to this model, the oxygen concentration inside the cell suspension for either 1% or 0.2% gas mixture fell to zero within approximately 1 h, based on the metabolic oxygen consumption rate of 9L^{luc} cells (Fig. 1C).

Fig. 2 shows that the constitutive bioluminescence signal decreases under hypoxia, by about 50% when 9L^{luc} cells are exposed to $\text{pO}_2 \leq 1\%$ (statistically different from the 21% value, $p < 0.05$), but is not significantly reduced at 5% or higher. The average bioluminescence signals corresponding to 21%, 10%, 5%, 1% and 0.2% oxygen were 1.97 ± 0.2 , 1.85 ± 0.2 , 1.45 ± 0.3 , 1.34 ± 0.3 and $1.04 \pm 2 \times 10^7 \text{ counts s}^{-1}$, respectively, after 4 h of incubation at each oxygen level. No significant cell death was observed from the 4 h of hypoxia at any level (data not shown).

While some cells have the ability to adapt to hypoxic environments by reversibly suppressing oxygen consumption under oxygen limiting conditions while maintaining intracellular ATP levels,¹⁹ 9L^{luc} cells continue to consume oxygen continuously, even after reaching low oxygen gradients (Fig. 1). As a result,

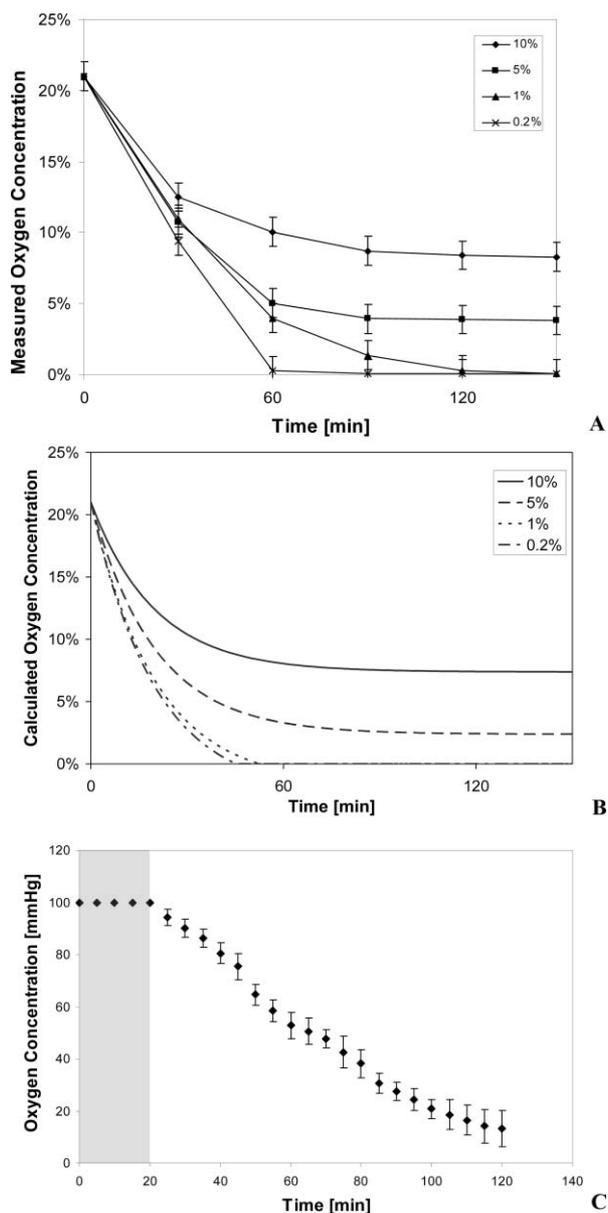


Fig. 1 (A) Oxygen tension in cell suspension as a function of time using different oxygen concentrations. Oxygen levels were measured at 30 min intervals over 2.5 h using a fiberoptic-based probe. Error bars represent the standard deviation (s.d.) from three independent experiments. (B) Calculated oxygen concentration inside the cell suspension as a function of time after the start of gassing, for each oxygen concentration used. Both graphs suggest that, at the lowest pO₂, the oxygen levels in the cell suspension fall essentially to zero after about 50–60 min. (C) Oxygen consumption in 9L^{luc} cells (5 × 10⁶ cells ml⁻¹, 4 ml) from three independent experiments. The error bars represent ±1 s.d. The shaded area represents oxygen levels above the detection range of the Oxylite system.

intracellular concentration of ATP is reduced when cells are exposed to pO₂ levels below 5% (Fig. 3) by about 30% at 0.2% pO₂ compared to normoxic conditions. Note that the ATP curve (Fig. 3) is very similar in shape to Fig. 2, suggesting that the cell metabolism is also highly influenced by the oxygen levels in this cell line.

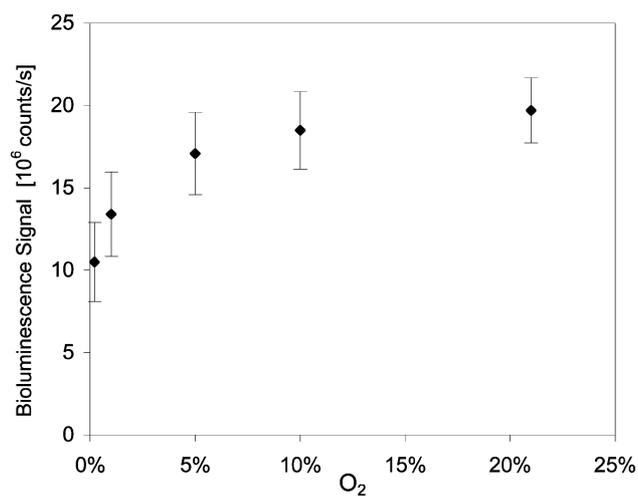


Fig. 2 Hypoxia-induced reduction of the bioluminescence signal in 9L^{luc} cells *in vitro* after exposure to different oxygen levels over 4 h. Data points represent mean ±1 s.d. over at least 3 independent experiments.

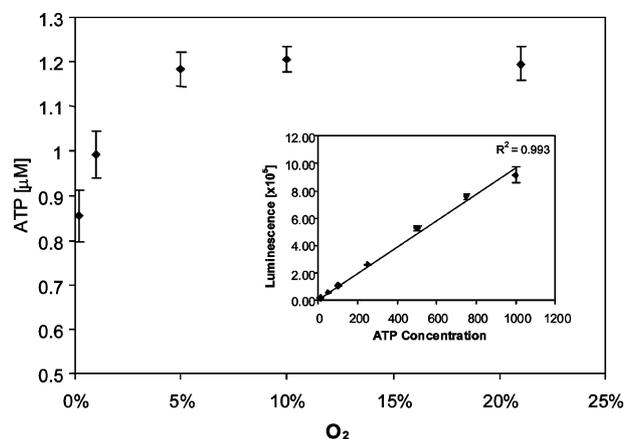


Fig. 3 Corresponding total intracellular ATP levels from 10⁵ 9L^{luc} cells exposed to different oxygen tensions for 4 h. The insert shows the linear correlation between the bioluminescence signal and the intracellular ATP concentration, measured using a luciferase-based assay.

The present study suggests that, under reduced oxygen tension, 9L^{luc} cells undergo changes in the mitochondrial membrane potential and changes in cellular energy metabolism, as shown by the reduction of intracellular ATP at low oxygen levels. The assessment of mitochondrial function under various oxygen tensions using the 9L^{luc} cells labeled with the fluorescent dye JC-1 demonstrated that hypoxia below 5% lowers ΔΨ_m, as shown in Fig. 4A, with the mean JC-1 red/green ratio after 4 h for 10%, 5%, 1% and 0.2% oxygen gassing being 0.84 ± 0.3, 0.97 ± 0.12, 0.73 ± 0.15 and 0.62 ± 0.075, respectively. The values of ΔΨ_m in 9L^{luc} cells for 1% and 0.2% oxygenation were significantly lower than controls (*p* < 0.05). This demonstrates that 9L^{luc} cells are capable of modulating ATP production in response to hypoxia. Hence, differences in intracellular ATP can be related to variation in mitochondrial function (Fig. 3 and 4A).

The relative bioluminescence signal from cells exposed to 0.2% pO₂, oligomycin and the combination of 0.2% pO₂ plus oligomycin and their respective images is shown in Fig. 5A and B, respectively.

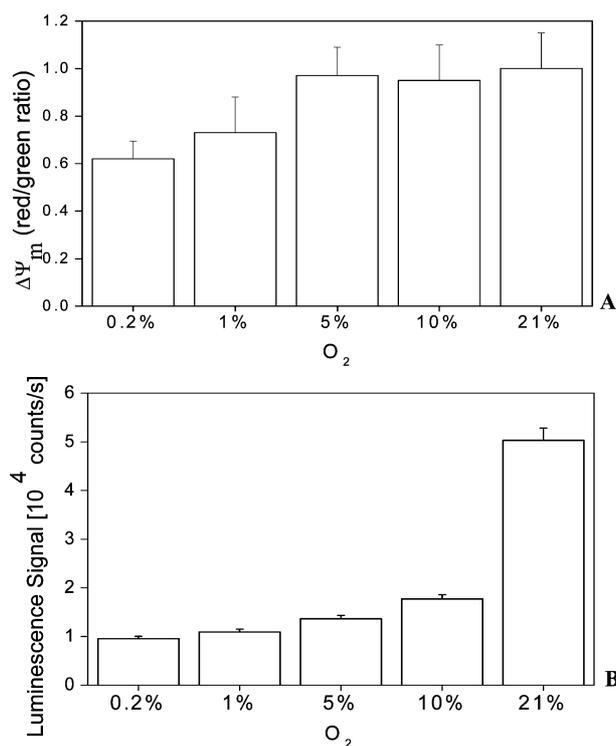


Fig. 4 Changes in mitochondrial membrane potential (JC-1 fluorescence) in $9L^{luc}$ cells exposed for 4 h to different oxygen tensions. The columns represent mean values from at least 3 independent experiments (error bars = s.d.) (A) A significant decrease of $\Delta\Psi_m$ ($p < 0.05$) was observed after exposing $9L^{luc}$ cells to 1% and 0.2% pO_2 for 4 h. (B) Oxygen dependence of the luminescence reaction in solution. The columns represent mean values from three independent experiments (error bars = ± 1 s.d.). A significant decrease in luminescence signal was observed after exposing the solution to $pO_2 < 10\%$.

A decrease in the BLI signal and ATP level can be observed after exposure to either oligomycin or 0.2% pO_2 for 4 h. However, a further decrease in bioluminescence signal, concomitant with the reduction of intracellular ATP was observed after exposing cells to oligomycin combined with 0.2% pO_2 . Since the bioluminescence signal from cells exposed to oligomycin was measured under normoxic conditions (21% pO_2), the relative reduction of the BLI signal can be explained exclusively by the decrease in the global ATP level. When the hypoxic cells were exposed to oligomycin, the reduction of intracellular ATP was more evident than when the cells were only exposed to hypoxia, followed by a marked decrease in the BLI. This points to a complementary effect of oligomycin on cell metabolism, through which intracellular ATP levels can be further decreased, even when the cell energy status is already reduced. As a result, a further decrease in the bioluminescence signal was observed, implying that the overall reduction of bioluminescence signal under hypoxia is associated with reduced cell metabolism that limits the intracellular ATP concentration available for the bioluminescence reaction.

Hence, in general, the relationship between cell oxygenation status, ATP level and the bioluminescence signal is complex. The additional experiments in solution demonstrated that oxygen can be limiting even above 5% (Fig. 4B). The differences in the relative luminescence signal between luciferase solution and cell suspen-

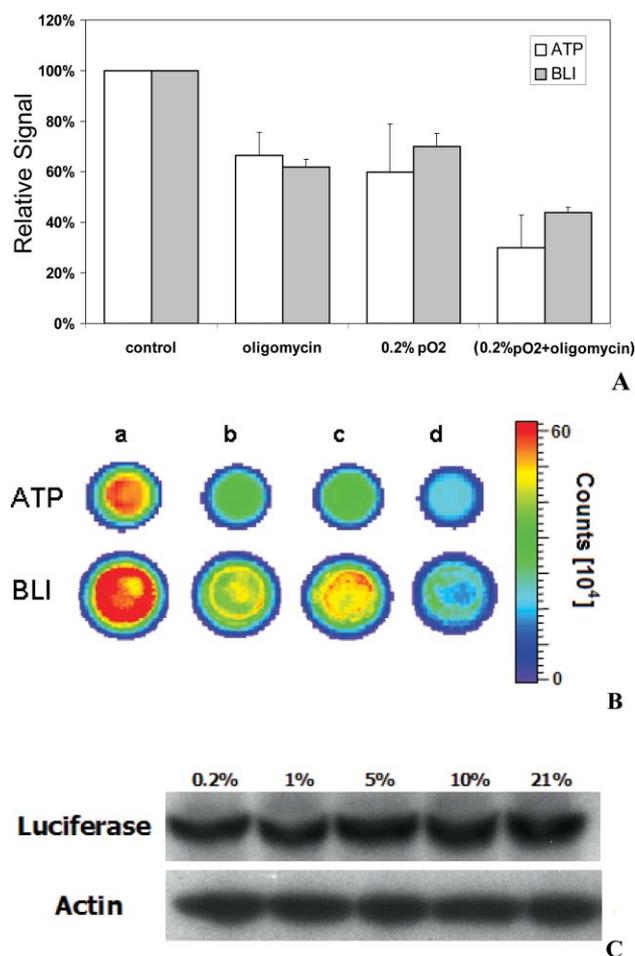


Fig. 5 (A) ATP (white bar) and BLI (gray bar) reduction in response to ATP depletion using $5 \mu\text{g ml}^{-1}$ of oligomycin for 30 min, 4 h exposure to 0.2% pO_2 , or the combination of oligomycin plus 0.2% pO_2 . (B) Example of the changes in ATP (upper) and the bioluminescence signal measured by the IVIS System obtained by mixing $100 \mu\text{l}$ of cell suspension (10^5 cells) and $150 \mu\text{l}$ of CellTiter-Glo[®] solution in 96-well plates: Figures a, b, c and d represent the bioluminescence signal from control cells and cells with oligomycin, 0.2% pO_2 , oligomycin plus hypoxia, respectively. (C) Luciferase protein levels after exposure to different oxygen tensions for 4 h, with actin as reference standard.

sion (bioluminescence) can be explained by the ability of the cells to continue producing ATP during periods of hypoxia, possibly *via* alternatives mitochondrial metabolic pathways,^{20,21} or in cases where the oxygen tensions are above 5% due to the maintenance of $\Delta\Psi_m$ (Fig. 4A), whereas the luminescent reaction (in solution) occurs in a non restricted mode, limited only by oxygen. However, there is also the possibility that normal cellular functions, such as luciferase synthesis, could be restricted by a diminished oxygen and/or ATP supply over a long period. Hence, additional experiments were performed to evaluate the changes in luciferase protein levels in $9L^{luc}$ cells after exposure to 4 h of hypoxia. Fig. 5C shows that exposing cells to severe hypoxia for 4 h does not produce any significant effect on luciferase protein synthesis, although this should be sensitive to change in energy supply.^{20,22} However, it is unclear whether chronic hypoxia, such as those found in tumors, could interfere with luciferase and ATP synthesis.

Conclusions

The *in vitro* results presented here suggest that hypoxia could be a significant factor in the use of BLI for quantitative studies of tumor growth and treatment response, particularly when the pO_2 is less than a few percent. It appears, at least *in vitro*, that the reduced intracellular ATP associated with hypoxia, is the limiting factor rather than directly the lack of oxygen per se in hypoxia-sensitive cell lines such as 9 L cells. Experiments are in progress to determine how substantial are the corresponding effects in tumors *in vivo*, resulting from either constitutive or treatment-induced hypoxia. In particular, experiments are in progress to determine whether hypoxia induced *in vivo* by photodynamic therapy can significantly alter the bioluminescence signal under different PDT treatment conditions. This is particularly critical, since rapid oxygen depletion during PDT has been reported in preclinical models^{8,23} due to direct photochemical oxygen consumption and/or from the acute effects of treatment on the tumor vasculature.^{9,24,25}

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