Oxygen Dependence of Cellular Respiration in Endothelial Cells: A Sensitive Toxicological Test

Rosmarie Steinlechner, Thomas Eberl, Raimund Margreiter, Erich Gnaiger

Department of Transplant Surgery, Clinical and Interdisciplinary Bioenergetics, University Hospital of Innsbruck, Anichstr. 35, A-6020 Innsbruck, Fax: +43 5125042605

Introduction

Cellular respiration is an integral indicator of metabolism. Oxygen consumption of the cells is nearly exclusively due to mitochondrial oxidative phosphorylation. Therefore, changes in respiration rate as a result of experimental treatments indicates some alteration of mitochondrial activity and function. Due to the complexity of the intact cellular system, a sublethal toxicological insult may induce a number of overlapping and partially counteracting processes, such as stimulation and inhibition of ATP turnover or uncoupling of oxygen consumption and ATP production. In summary, this may result in a change of oxygen flux in either direction, or even in an insignificant net change of the respiratory rate.

Analysis of the oxygen dependence of cellular respiration may yield additional information on the functional state of the mitochondrial system. Routine measurements of cellular respiration below critical oxygen pressures have been limited in the past due to the insensitivity of standard methods. By high-resolution respirometry, measurement of the oxygen kinetics of isolated mitochondria [1] and cells is possible on a routine basis. We chose endothelial cells as a test system, since *(1)* endothelial cells are most directly exposed to the compounds transported through the blood; *(2)* under aerobic conditions endothelial metabolism is almost exclusively oxidative and mitochondrial (unpublished observations); *(3)* primary cell cultures retain a maximum of the metabolic complexity compared to *in vivo* systems; and *(4)* cultured human umbilical vein endothelium is a clinically relevant model [2].

Materials and Methods

High-resolution respirometry. Respiration of endothelial cell suspensions was measured in a two channel titration-injection respirometer (OROBOROS® *Oxygraph*, Anton Paar, Graz, Austria) at 37 °C. The 2 ml measuring chambers were stirred at 350 rpm, using PEEK coated magnetic stirring bars. The decline of oxygen concentration was recorded digitally at 1 s time intervals. Flux was calculated as the time derivative, corrected for blank controls and for the exponential time constant ($\tau = 3$ s; OROBOROS® DatGraf Analysis software). Anoxic states were usually kept for about 5 minutes to ensure a sufficiently long zero-baseline for the corrections in subsequent data analysis [1]. Internal calibration of the zero signal is necessary for the evaluation of p_{50}-values, *i.e.* p_{O_2} at half-maximal oxygen flux in a hyperbolic O_2-flux/O_2-pressure plot. Depending on the stability of the cells, one or several reaeration cycles were performed. In experiments with low cell concentrations control respiration and uncoupled respiration were measured in separate experiments.

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Fig. 1. Mass of endothelial cells (EGM) [mg·10^{-6} cells] as a function of cell concentration [10^6·cm^{-3}]. Individual cell mass tends to decline with increasing cell density in the culture flasks. Each symbol represents the cell yield of one culture flask (75 cm^2) used in an individual experiment, expressed as cell density after resuspension. Samples for cell counts were taken from the *Oxygraph* measuring chamber at the beginning of an experiment.
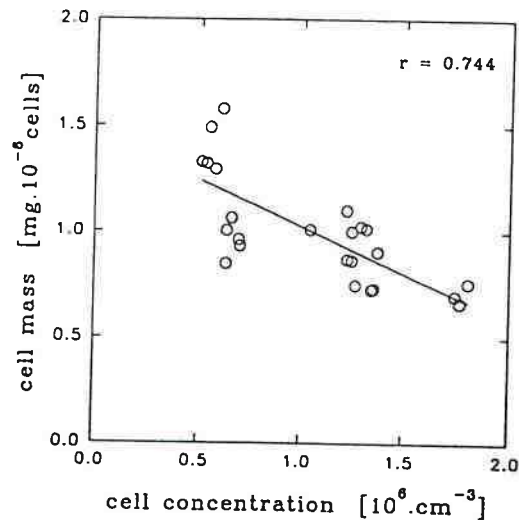
Cell culture: Endothelial cells were grown in primary culture in 75 cm^2 culture flasks on Endothelial Growth Medium (EGM) as described [2]. In addition, 'M199-cells' were grown as follows: M199 medium was supplemented with 15% heat inactivated fetal calf serum (2% for EGM cells), L-glutamine (2.8 mM), Heparin (25 U/ml), Gentamycin (50 μg/ml) and Endothelial Cell Growth Supplement (50 μg/ml) according to standard methods [3,4]. Cells were enzymatically removed from the culture flasks by addition of 0.05% trypsin/EDTA (50 to 70 s incubation during a rapid transition from room temperature to 37 °C). Trypsin activity was inhibited by addition of cold fetal calf serum. Cells were spinned down at 150 g for 8 min at 4 °C and resuspended in culture medium, brought to experimental temperature, and immediately incubated in the *Oxygraph*. 100 μl samples were taken for the determination of cell concentration and trypan blue exclusion. Viability was >95%. After a 1-min equilibration period with a 95% air/5% CO_2 gas phase, the respiration chambers were closed. For dry weight determination, cell suspensions were removed from the respirometer at the end of experiments, and washed in 50% phosphate buffered saline solution.

RESULTS AND DISCUSSION

Cell density and size

In most cases all cells harvested from one culture flask were used for individual respirometric experiments. Therefore, different cell concentrations in the respirometer (0.9 to 1.8·10^6 cells·cm^{-3}) reflect different monolayer densities (c. 40 to 80·10^3 cells·cm^{-2}). Individual cell mass declines with increasing density and concentration (Fig. 1). This may indicate differences in physiological state and contribute to the variability observed between different primary EGM cultures.

Endogenous oxygen flow, I_{O_2} [pmol·s^{-1}·10^{-6} cells], i.e. respiration expressed per unit cell, tended to decline with increasing cell density in the culture, but was independent of cell concentration in the respirometric chamber. Similarly, mass-specific oxygen flux, $J_{O_2,m}$ [pmol·s^{-1}·mg^{-1}] decreased with an increase in cell size, as might be expected from the allometric rate/size relation observed in transformed cells [5]. The range of cell size was comparatively small within identical culture conditions. Therefore, the effects of cell size on respiration could not significantly explain experimental variation within EGM or M199 cells.

Fig. 2. Oxygen flow of EGM cells, I_{O_2} [pmol·s^{-1}·10^{-6} cells], as a function of oxygen pressure, p_{O_2} [kPa], in the microxic region below 1 kPa of coupled and uncoupled cells. The uncoupler FCCP was added after re-aeration of the cell suspension, which had been anoxic for 3 min. Each dot represents a measured data point at 1 s acquisition intervals. The solid lines show the hyperbolic fit. Coupled: p_{50} = 0.074 kPa; I_{O_2}(max) = 44.5 pmol·s^{-1}·10^{-6} cells. Uncoupled: p_{50} = 0.110 kPa; I_{O_2}(max) = 109.7 pmol·s^{-1}·10^{-6} cells.
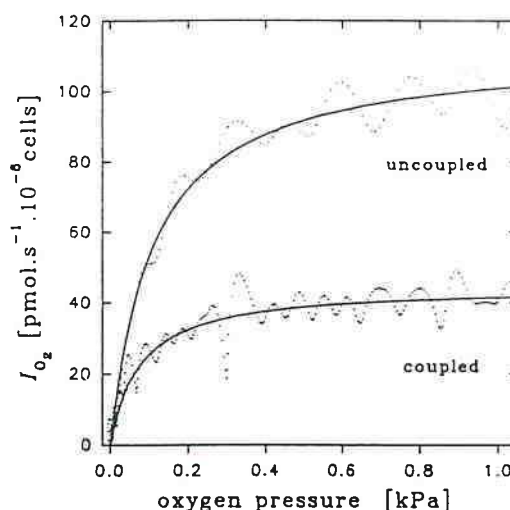
Response of cellular respiration to FCCP

Endogenous oxygen flux of suspension of endothelial cells was constant with time (typically 60 to 100 min, depending on cell concentration and I_{O_2}) down to very low oxygen levels when oxygen flux became kinetically dependent on oxygen pressure. After a period of 3-5 min of anoxia, the medium was reoxygenated by lifting up the stopper for a short time and resealing the chamber. Oxygen consumption returned to the same levels as observed initially, emphasizing the stability of aerobic respiration (see Fig. 5A in [2]). After this control period, the uncoupler FCCP was titrated into the chamber, using FCCP as a toxicological test substance with well known mechanism of action, i.e. the cyclic protonophoric effect and accompanying dissipation of ΔpH and mitochondrial membrane potential [6]. The critical concentration of FCCP for maximum uncoupled respiration was c. 10 μM.

Endogenous respiration, calculated as the maximum rate in the hyperbolic flux/pressure fit, was higher in EGM than in M199 cells, reflecting the smaller size in the latter (Tab. 1). Oxygen flow increased 2.4-fold after uncoupling in EGM cells and 3.1-fold in M199 cells (Table 1). With increasing concentrations of FCCP, inhibition of oxygen consumption was obtained, such that respiration was depressed to control levels of coupled cells. Under this condition, respiratory measurements cannot distinguish between normal and highly toxic conditions. Little is known about the inhibitory mechanism of uncouplers. The simultaneous analysis of normoxic respiratory levels and the oxygen dependence of respiration yields a higher resolution for the detection of toxicological effects.

TABLE 1

Maximum oxygen flow per 10^6 cells, and p_{50} for oxygen in two types of endothelial cell cultures, comparing coupled and uncoupled conditions

Values are means ± SE (n).

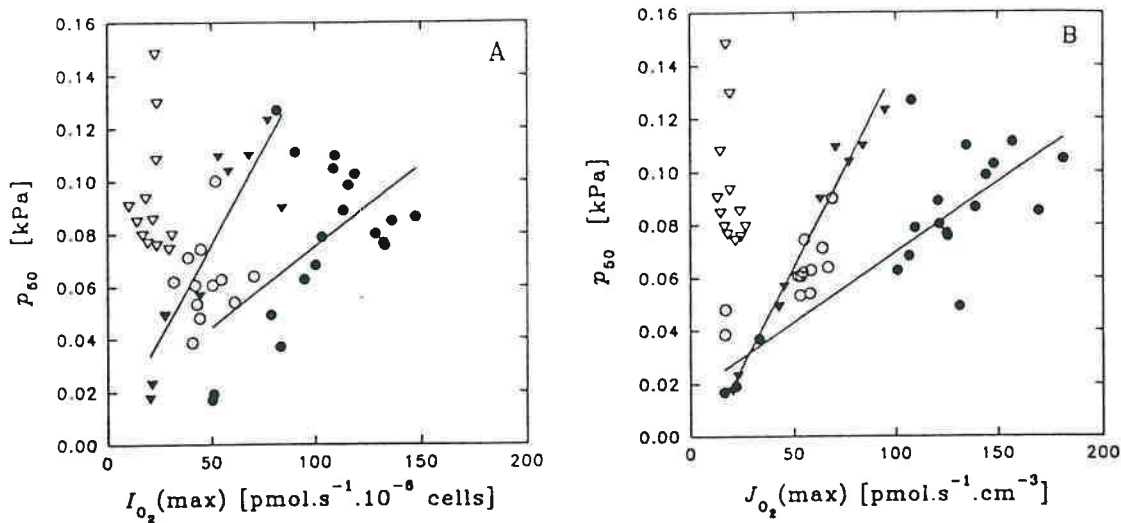| Cell | I_{O_2} [pmol·s^{-1}·10^{-6} cells] | | p_{50} [kPa] | |
	coupled	uncoupled	coupled	uncoupled
EGM	46.1 ±3.4 (10)	111.0 ±7.0 (8)	0.063 ±0.005 (10)	0.103 ±0.005 (8)
M199	21.1 ±1.7 (12)	64.3 ±6.2 (6)	0.094 ±0.007 (12)	0.099 ±0.009 (6)

Fig. 3. p_{50} [kPa] of coupled (open symbols) and uncoupled (closed symbols) human endothelial cells cultured in M199 (triangles) and in EGM (circles), (A) plotted against maximum oxygen flow, I_{O_2}(max) [pmol·s^{-1}·10^{-6} cells], and (B) against maximum oxygen flux, J_{O_2}(max) [pmol·s^{-1}·cm^{-3}]. A: The means of both I_{O_2}(max) and p_{50} were significantly different between coupled M199-cells and EGM-cells. The correlation of p_{50} and oxygen flow was significant in uncoupled cells but not in coupled cells. B: The p_{50} of coupled cells was independent of the maximal oxygen flux in the measuring system [pmol·s^{-1}·cm^{-3}] when both cell types are considered. In the uncoupled cells the correlation between p_{50} and maximum oxygen flux was significant and distinct for the two cell types. The slope of the regression line was 0.00149 and 0.00053 kPa per unit of J_{O_2}(max) for M199-cells and EGM-cells, respectively.

Oxygen dependence of cellular respiration

The oxygen flow/oxygen pressure plot of a representative experiment (Fig. 2) shows *(1)* the large number of sampling points in the oxygen-dependent region; *(2)* the good fit obtained by a hyperbolic function in the range <5% air saturation (<1 kPa); *(3)* the high affinity of endogenous respiration for oxygen, with a p_{50} of 0.07 kPa; *(4)* the effect of the uncoupler FCCP (10 μM) on I_{O_2}(max) (a 2.5-fold increase); and *(5)* the 1.5-fold increased p_{50} (0.11 kPa) of uncoupled respiration.

The p_{50} for oxygen in the controls (0.06 to 0.09 kPa) compares well with p_{50} values reported for endogenous respiration of neuroblastoma cells (0.075 kPa [7]). Surprisingly, the p_{50} was significantly lower in the EGM compared to M199 cells (Tab. 1). The contrary might have been expected taking into account the larger cell volume of EGM cells. We conclude, therefore, that the observed difference in the oxygen sensitivity between the two cell types reflects variations in physiological state rather than a difference in oxygen diffusion limitations.

Uncoupling significantly increased the p_{50} for oxygen in EGM cells (Tab. 1). After successive FCCP titrations and consequent inhibition of O$_2$ flows, the p_{50} declined proportionally with decreasing I_{O_2}(max). A linear O$_2$ flow/Δp_{O_2} relation is suggestive of diffusion limitation [7-9], with different proportionalities in the two cell types (Fig. 3A). The lowest p_{50} values are in the same range as that of isolated mitochondria respiring at volume-specific fluxes of 50 to 100 pmol·s^{-1}·cm^{-3}

in state 4 (maximum ATP/ADP ratio [1]). A similar pattern is reported for uncoupled neuroblastoma and cardiac cells inhibited with amytal [7,8].

The p_{50} of coupled cells was independent of maximal oxygen flux in the measuring system [pmol·s^{-1}·cm^{-3}] when both cell types were considered. However, the lowest p_{50} values were obtained in experiments with low volume-specific oxygen fluxes (c. 20 pmol·s^{-1}·cm^{-3}]. There are no comparably low p_{50} values at high oxygen fluxes (Fig. 3B). This might indicate an instrumental limitation of identifying low p_{50} values at high flux. But identical analyses of similar experiments with mitochondrial suspensions at high volume-specific oxygen fluxes have shown, that also at high fluxes (c. 100 pmol·s^{-1}·cm^{-3}) p_{50} values as low as 0.02 kPa could be measured [1]. The decrease of p_{50} in diluted EGM cells (hence low volume-specific oxygen flux) might be interpreted as a crowding effect. This point requires further study.

CONCLUSION

When O_2 flows in controls and FCCP treated cells were identical, the different p_{50} values provided nevertheless an indicator of the toxicological effect (Fig. 3A). A one-dimensional analysis, based on either oxygen flows or p_{50} values alone, provides an insufficient diagnostic tool. This demonstrates the importance of investigating the response to toxicological or pharmacological agents under a range of defined physiological and experimental conditions.

ACKNOWLEDGEMENTS

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