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Control of cell respiration by nitric oxide in Ataxia Telangiectasia lymphoblastoid cells

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Abstract

Ataxia Telangiectasia (AT) patients are particularly sensitive to oxidative–nitrosative stress. Nitric oxide (NO) controls mitochondrial respiration *via* the reversible inhibition of complex IV. The mitochondrial response to NO of AT lymphoblastoid cells was investigated. Cells isolated from three patients and three intrafamilial healthy controls were selected showing within each group a normal diploid karyotype and homogeneous telomere length. Different complex IV NO-inhibition patterns were induced by varying the electron flux through the respiratory chain, using exogenous cell membrane permeable electron donors. Under conditions of high electron flux the mitochondrial NO inhibition of respiration was greater in AT than in control cells ($P \leq 0.05$). This property appears peculiar to AT, and correlates well to the higher concentration of cytochrome *c* detected in the AT cells. This finding is discussed on the basis of the proposed mechanism of reaction of NO with complex IV. It is suggested that the peculiar response of AT mitochondria to NO stress may be relevant to the mitochondrial metabolism of AT patients.
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Keywords: Nitrosative stress; Bioenergetics; Cytochrome *c* oxidase; Genetic disease; Reaction mechanism; Respiratory chain

1. Introduction

AT is an autosomal recessive multisystemic disorder characterised by conjunctival telangiectasia and by degeneration of the cerebellar Purkinje and granule neurons, leading to progressive ataxia [1,2]. The disease is caused by mutations of the *AT-Mutated* gene (*ATM*), encoding for a nuclear 350 kDa protein [3]. The ATM protein plays a fundamental role in controlling cell cycle and in responding to DNA damage [4,5]. AT patients as well as *ATM*-deficient mice are characterised by a high degree of genetic instability and susceptibility to spontaneous and ionising radiation-induced oxidative stress, with high levels of oxidative DNA damage [6–10]. Compared to wt cells, AT cells are characterised by a defective reactive oxygen species (ROS) scavenging capacity [11,12]. The intracellular concentration of reduced glutathione is decreased in AT cells [12], and the dietary supplementation of the antioxidant and NO scavenger *N*-acetyl cysteine to *ATM*-deficient mice improves their genetic stability and off-spring fitness [7].

ROS as well as reactive nitrogen species (RNS), and particularly NO and the superoxide ion O_2^- play important roles in cell signalling and pathophysiology [13–15]. Bioavailability of ROS and RNS depends on their relative rate of production and degradation/scavenging [16] as well as on the reciprocal O_2^- and NO self-quenching rate; these species almost instantaneously react to form peroxynitrite $ONOO^-$, another highly reactive compound [17,18]. At sub-micromolar concentrations, NO displays a variety of physiological actions [19–21], including the rapid and reversible inhibition of mitochondrial respiration [22–25].

Cytochrome *c* oxidase (CcOX), i.e. mitochondrial complex IV, has been identified as the primary fast-reacting (milliseconds) and high affinity target for NO ($K_d \approx$ nM) [24, and references therein cited], although other mitochondrial complexes also react

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and are inhibited by NO on the time scale of several minutes to hours [26]. The reaction of NO with CcOX is responsible for the fast inhibition of respiration that occurs with the addition of NO to respiring systems *in vitro* [22–24]. This reaction has been shown to follow two different pathways [24,27], leading to formation of either a nitrite- (CcOX–NO_2^-) or a nitrosyl-derivative (CcOX–NO) of CcOX [28]. The rate of electron flow through the respiratory chain, particularly at the cytochrome *c*/cytochrome *c* oxidase site is crucial to the formation of the two derivatives [28,29]. CcOX–NO is formed in competition with O_2 and predominates at high electron fluxes through the respiratory chain [24]. Both nitrite- and the nitrosyl-adducts are reversibly inhibited and regenerate fully active CcOX, although with markedly different kinetics. CcOX–NO_2^- recovers function more rapidly than CcOX–NO and its formation is not competitive with O_2 [24,30]. CcOX–NO is more stable in the dark (as in our body) than in light [28,29,31] and in its presence the respiratory chain remains inhibited for longer times than in the presence of CcOX–NO_2^- [28,29]. Last but not least, CcOX–NO releases NO since the nitrosylated mitochondrial enzyme is unable to further oxidise or reduce NO, at least *in vitro* [24,32].

In this paper we report that mitochondrial respiration of peripheral lymphoblastoid (Epstein–Barr virus transformed) cells from AT patients, in the presence of atmospheric O_2, micromolar NO (0.5–2 μM) and cell permeable reductants, remains inhibited for longer times than *wt* cells. The results suggest that under conditions favouring electron transfer to cytochrome *c* oxidase and in the presence of NO, CcOX–NO is accumulated in AT cells significantly more than in wt cells.

2. Materials and methods

2.1. Cell culture

Lymphoblastoid cells were isolated and stabilized from 3 Ataxia Telangiectasia patients genetically characterised by RT-PCR, and showing in-frame deletions or premature truncation throughout the *ATM* gene, fully supporting the classical AT phenotype [33,34]. Cells were grown (incubator) at 37 °C and 5% CO_2 in RPMI 1640, 15% foetal calf serum, containing 100 IU–100 μg/ml penicillin–streptomycin and 2 mM L-glutamine. All patients and healthy controls signed an informed consensus, according to the guidelines approved by the Italian Society of Human Genetics (SIGU), and in full compliance with the principles enunciated in the Declaration of Helsinki. Three healthy relatives were selected as controls, sharing with the patients common genetic background. Cell ploidy was monitored on 200 mitoses for each cell line, to assess putative cytogenetic differences related to cell aging and time of culture. All measurements were carried out using cells at comparable growing stages, monitored by analysis of telomeres carried out using quantitative fluorescence *in situ* hybridization (Q-FISH). The integrated fluorescence intensity value proportional to the number of hybridized probes was calculated for densitometry using the IAS2000 software (Delta Sistemi, Roma, Italy). The fluorescence shown by AT was slightly (20%) lower than that of wild type cells, consistently with previous reports [35].

2.2. Reduction of mitochondrial tetramethyl-p-phenylenediamine (TMPD)

Cells, 1×10^7 ml^{-1}, were incubated for ~10 min with 3 mM TMPD, carefully washed and suspended in dye-free medium. In the absence of exogenous ascorbate the TMPD reaching mitochondria is oxidised to $TMPD^+$ by cytochrome *c* oxidase and O_2, and the cell suspension turns to bluish within minutes. To determine the plasma membrane permeability of ascorbate, TMPD-loaded cells, 0.5×10^6 cells ml^{-1}, were mixed (by hand) under gentle stirring with ascorbate at 25 °C; the reduction of TMPD was measured spectrophotometrically in the presence of 100 μM cyanide, to avoid TMPD re-oxidation by CcOX. The use of rapid mixing devices (stopped flow) operating at relatively high pressure (≥2.5 atm) was not compatible with cell integrity.

Regardless to cell type, oxidised TMPD was found accumulated in the cells, likely in the mitochondria [36,37] at approximately 1 μM concentration, as determined spectrophotometrically using the extinction coefficient at 610 nm $\Delta E_{(ox-red)}=10$ mM^{-1} cm^{-1} independently calculated in PBS, 10 mM glucose and 1 mM $CaCl_2$ (not shown). Cell viability determined after the experiment was >95%, by trypan blue exclusion. $TMPD^+$ was reduced by ascorbate under conditions compatible with a bimolecular reaction lasting a few tenths of seconds.

The apparent second order rate constant *k* was calculated by fitting the experimental time courses to the equation:

$$\ln\left(\frac{B_o(A_o-x)}{A_o(B_o-x)}\right)=kt(A_o-B_o)$$

where A_o and B_o are the initial concentrations of *A* (ascorbate) and *B* ($TMPD^+$) and *x* is the amount reacted at time *t*.

2.3. Immunodetection analysis

AT and wt cell lysates were obtained under rigorously comparable conditions. In the immunoblotting assay, 30 μg proteins were resolved on 12% SDS-PAGE, transferred to nitrocellulose membranes (Whatman) and incubated with the primary antibody anti-cytochrome *c* ((7H8):SC-13560, Santa Cruz) diluted 1:200, and anti-β-actin ((clone AC15:A-1978) Sigma Chemical Co.), diluted 1:800, overnight at 4 °C. After incubation with the appropriate species-specific IgG conjugates, the membranes were exposed to chemiluminescence reagents (Amersham). Quantification of band intensities was carried out by the KODAK 1D Image Analysis Software (Eastman Kodak Company, Rochester NY). The blot shown is representative of three independent experiments of both AT and wt cells.

In the ELISA assay, 1.5×10^6 cells were suspended in 1 ml lysis buffer (ELISA kit Bender Med Systems, Vienna, A) and after a 1:50 dilution in the same buffer, an aliquot was processed by commercially available enzyme-linked immunosorbent assay for quantitative detection of human cytochrome *c*, according to the manufacturer's instructions. The data shown represent the average of eight replicates of both AT and wt cells.

2.4. Citrate synthase

The total cell mitochondrial mass was evaluated by measuring the citrate synthase (CS) activity. Cells were lysed using CelLytic ™ M Cell-Lysis reagent from Sigma. Extracts of 10^6 cells were centrifuged at 20,000 ×*g* and supernatants assayed for the total protein content [38] and for citrate synthase [39]. The citrate synthase activity of AT and wt cells was 0.105±0.0030 and 0.107±0.0084 μmol/min/mg protein, respectively, indicating that the mitochondrial mass of these cell lines is very similar.

2.5. Polarographic measurements (O_2, NO)

Oxygen and NO (authentic gas solution) consumption was measured amperometrically in a gas proof vessel containing NO- and O_2-sensitive electrodes, either from WPI (Duo18 or Apollo 4000-Model, World Precision Instruments, Inc., Sarasota, FL-USA) or from OROBOROS Instruments (Innsbruck, A) allowing high resolution respirometry; in this case data points were collected using the built in software DatLab 4, and kinetic flux analysis performed with DatLab 2 (integration time ≤2 s, and minimizing smoothing). The NO-inhibition experiment was designed according to [28,29] with minor modifications to probe the mitochondrial accumulation of the CcOX–NO adduct. Cells (1×10^7 ml^{-1}) were allowed to respire at 25 °C in air-equilibrated medium (~270 μM O_2) as such or in the presence of exogenous ascorbate, up to 30 mM and TMPD, reductants able to maintain cytochrome *c* (virtually) fully reduced [29,37]. After stabilization, NO (2 μM, typically) was added to the reaction chamber and inhibition of respiration occurred immediately, confirming that the observed O_2 consumption was due to CcOX; care was taken to keep the

system in the dark to stabilize the photosensitive CcOX–NO adduct putatively formed [28,29]. Afterwards, residual free NO was removed with human oxy-haemoglobin (HbO_2 2.0 μM, heme basis) and the time course of respiration recovery in the absence of free NO followed. Rates of O_2 consumption were measured under stationary conditions before addition of NO (V_b) and compared to the rate measured 45 s after HbO_2 addition (V_a), i.e. at a time suitable for complete removal of NO. The V_a/V_b ratio has been taken as indicative of the fraction of CcOX–NO still existing 45 s after free NO removal, and at 25 °C. The V_a/V_b ratio is proportional to the fraction of active enzyme which is limited by the mono-exponential thermal dissociation of NO from the enzyme. When a significant amount of CcOX–NO is formed, the V_a/V_b ratio decreases. As previously reported [28,29] illumination of the sample with a bright (heat-filtered) white light (150 W tungsten lamp) removes inhibition. The estimated rate of the NO dissociation is consistent with $k_{off} \approx 10^{-2}$ s^{-1} (T=25 °C) [28]. In the presence of CcOX–NO, 45 s after free NO removal ~40% residual CcOX inhibition is expected ($A_t = A_0 e^{-kt}$).

2.6. ATP measurements

Cells (4–8×10^5 cells/ml) were cultured overnight using an antibiotic-free RPMI 1640 medium; thereafter, cells were further incubated 4 h in the absence or presence of 11 mM glucose and 2 mM L-glutamine. When required, 2.5 μg/ml oligomycin was added to the samples during the last 1.5 h. A 100 μl aliquot of each sample (5×10^4 cells, containing similar amounts of total proteins) was used for the chemiluminometric determination of ATP (ATPlite detection kit, Perkin Elmer). Measurements of the luminometric signal were carried out with the plate reader VICTOR™ Multilabel Counter (Perkin Elmer) in white 96 well plates.

2.7. Lactate measurements

Cells (4–8×10^5 cells/ml) were cultured overnight in antibiotic-free RPMI medium and then starved for 1 h in PBS free of glucose. After starvation, cells (1×10^6 cells/ml) were incubated in PBS containing glucose, 1 mM, and in the presence or absence of myxothiazol and antimycin A (10 μM each); after 3 h, cells were treated with $HClO_4$, 80 mM [40]. After centrifugation (800–1000 ×g, at 4 °C, 10 min), supernatant was collected and the lactate concentration determined spectrophotometrically. The difference between the basal glycolytic lactate and the lactate produced in the presence of myxothiazol and antimycin was defined as Δlactate [41]. The Δlactate divided by the basal lactate value has been taken as indicative of the ratio between oxidative phosphorylation (OXPHOS) and glycolytic ATP.

2.8. Statistics

Data points represent the average of repeats (figure legends) carried out using different cell lines from patients (n=3) and from healthy controls (n=3). Significance was determined using the Student t-test, run by Excel (Microsoft Windows platform). The error bars correspond to the standard error of the mean (SEM); all P values correspond to 2-sample t-test assuming unequal variances.

2.9. Chemicals

Sodium ascorbate, tetramethyl-p-phenylenediamine (TMPD) and cell culture medium were from Sigma Chemical Co. (St. Louis, MO). Stock solutions of NO (Air Liquide, Paris, France) were prepared by equilibrating degassed water with the pure gas at 1 atm and at 20 °C; the NO concentration was [NO]=2.1±0.1 mM. Concentration of human oxy-haemoglobin (HbO_2) is expressed on the heme basis, ($\in_{555}$=12.5 mM^{-1} cm^{-1} in the deoxygenated state).

3. Results

3.1. Cell respiration

Mitochondrial respiration was evaluated using intact cells maintained in culture under basal metabolic conditions. The experiments were carried out using the cells as such or in the presence of excess ascorbate and increasing amounts of TMPD, to directly reduce cytochrome c and thereby CcOX [37]. The rate of O_2 consumption in the absence of exogenous reductants was $V_{O_2} \approx 6 \pm 0.5$ nmol min^{-1} 10^7 $cells^{-1}$, a value similar for both AT and wt cells (Fig. 1). Upon addition of exogenous reductants, respiration increases with TMPD in both cell lines but to a greater extent (approximately twice as much) in AT than in wt. The intracellular reduction of oxidised TMPD by ascorbate was evaluated. Both the amplitude and rate of TMPD reduction were similar in wt and AT cells, thus excluding a significant difference in the cell membrane permeability to ascorbate (Fig. 2). The calculated bimolecular rate constant values were k_b=3.5 and 4.5×10^4 M^{-1} s^{-1}, in AT and wt respectively. The rate constant value measured in solution, k_b=8.5×10^4 M^{-1} s^{-1} (inset to Fig. 2) was faster by a factor of two, suggesting that the membrane does exert some degree of control of ascorbate permeation.

The concentration of cytochrome c was measured by immunoblot and ELISA. As shown in Fig. 3, the relative densitometric intensity of the cytochrome c band detected in AT cells is ~70% greater than in wt cells. The amount of cytochrome c detected by ELISA was 0.48±0.013 μg and 0.67±0.012 μg/mg total proteins for wt and AT, respectively, accounting for an increment of ~40%.

3.2. Cytochrome c oxidase response to NO

Cytochrome c oxidase purified, in mitochondria or in intact cells, undergoes rapid inhibition by NO, reacting with the metals in the active site of the enzyme (see Introduction). The degree of CcOX inhibition depends on the electron flux through the respiratory chain appearing higher in AT cells; thus, we have measured the response of AT and control cells to NO. In order to vary the rate of electron transfer at the cytochrome c/CcOX site, the cells suspended in air-equilibrated medium were allowed to respire in the presence of excess ascorbate and increasing amounts of TMPD. Inhibition of respiration was induced by

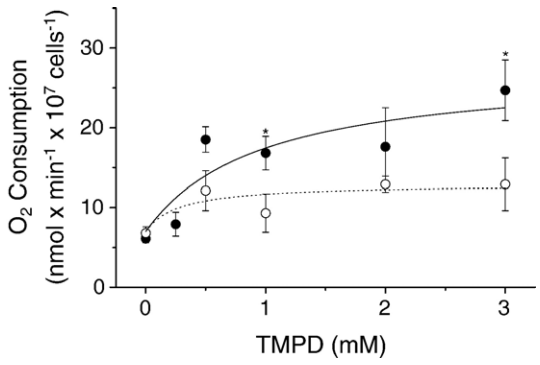

Fig. 1. Cell respiration. Cells (1×10^7 ml^{-1}) suspended in PBS, glucose 10 mM and $CaCl_2$ 1 mM were transferred into the polarographic vessel. Excess ascorbate, and TMPD were added from concentrated stock solutions to minimize dilution. Each data point represents the average of independent measurements carried out on every cell line. Wild type cells (○), Ataxia Telangiectasia cells (●). T=25 °C. Values represent the mean±SEM (n=6–9). *, P<0.05 vs wt cells (details in Materials and methods).

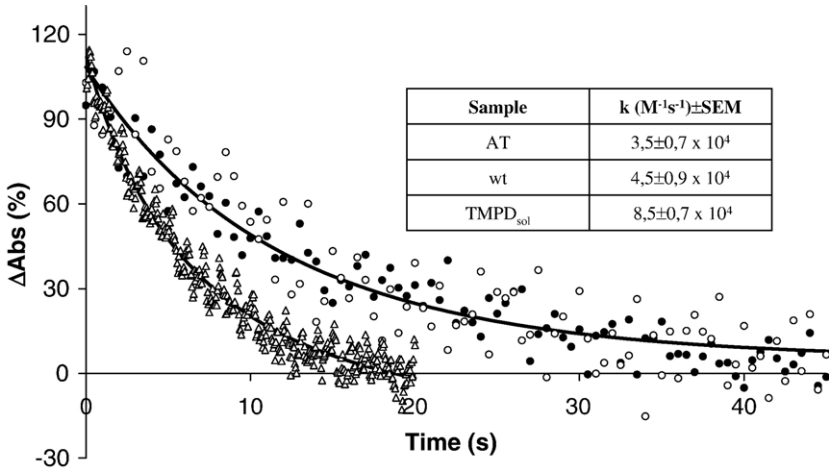

Fig. 2. Ascorbate reduction of TMPD in solution (△) and in cells, AT (●) and wt (○) Time courses of reduction of TMPD, ~1 μM, by ascorbate, 2 μM. The solid lines represent the best fit of the experimental data to a second order kinetic equation; Inset: second order rate constant values of reduction of oxidised TMPD by ascorbate. Values represent the mean±SEM (n=3). T=25 °C.

adding a single bolus of pure authentic NO gas solution to the respiring cells, and in the dark to stabilize the nitrosyl CcOX–NO derivative (Fig. 4). It is worth recalling that the detection of a delay in the recovery of respiration has been shown to be related to the existence of CcOX–NO [28]. As previously reported with neuroblastoma cells [29], when respiration of both wt and AT cells is sustained only by endogenous substrates, no detectable residual inhibition of cell respiration is observed after (free) NO exhaustion (not shown). Upon addition of TMPD and ascorbate, compared to wt, AT cells displayed a substantial residual inhibition after disappearance of bulk NO even in the presence of HbO_2 used to trap any free NO (putative, and below the electrode sensitivity). This residual inhibition, observed at 25 °C and in the absence of bulk NO, was abolished by exposing cells to an intense heat-filtered white light, confirming the existence of the light-sensitive $Fe^{2+}NO$ bond (Fig. 5A) [28]. The percentage of residual inhibition measured 45 s after addition of HbO_2 increases with the concentration of TMPD and is significantly higher in AT compared to wt cells (Fig. 5A). The correlation between the inhibition of respiration observed at the time fixed and the rate of O_2 consumption at any TMPD concentration is reported in Fig. 5B. Regardless of the cell type, all data points lie on the same curve and extrapolate to the same limiting value corresponding to approximately ~40% residual inhibition, compatible with the previously reported dissociation kinetics of NO from the CcOX active site [28] (see Materials and methods). This finding suggests that the mechanism of CcOX inhibition *via* formation of CcOX–NO and its dependence on the electron transfer rate, i.e. respiration rate, are similar in all cell lines investigated. Thus, the different NO inhibition of AT and control cells is likely related to properties of the respiratory chain downhill with respect to TMPD, well correlating to the higher concentration of cytochrome *c* observed in AT.

3.3. Mitochondria metabolism

The steady state concentration of ATP in AT and wt cells has been evaluated under standard growing conditions. The overall amount of ATP detectable at steady state in wt and AT cells is 21.0±2.6 and 23.0±2.8 nmol/10^7 cells, in the presence of glucose; in its absence, ATP concentrations are 22.0±1.4 and 26.0±2.3 nmol/10^7 cells, respectively (see Fig. 6A, top). Thus the absolute concentration of ATP appears only slightly higher in AT than in wt, and more clearly in the absence of glucose. In

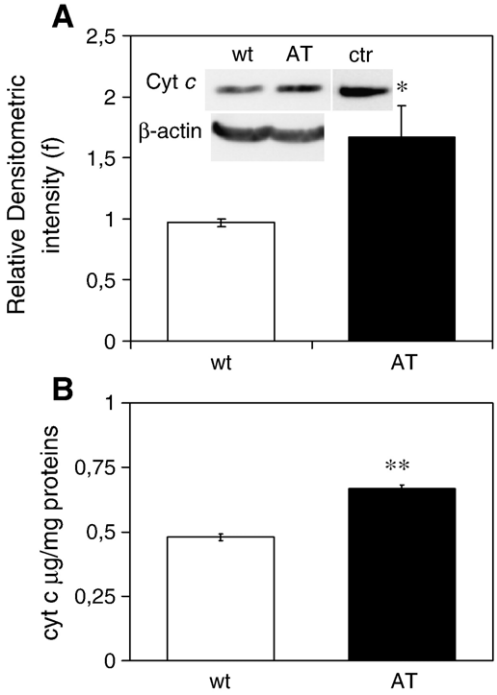

Fig. 3. Cytochrome *c* immunodetection. Panel A: Immunoblot. Cell lysates, 30 μg of protein per well, ctr = cytochrome *c* control. The relative densitometric intensity (RDI) value of wild type cells (wt), and Ataxia Telangiectasia cells (AT) represents the average of 3 independent experiments, *, P<0.05 *vs* wild type cells. Panel B: ELISA. Lysates (3×10^4 cells/ml) were assayed according to the manufacturer's instructions (Bender Med Systems, Vienna, A) and values obtained were expressed as μg cytochrome *c* per mg of total proteins, determined according to Bradford [38]. n=8, **, P<0.01 *vs* wild type cells.

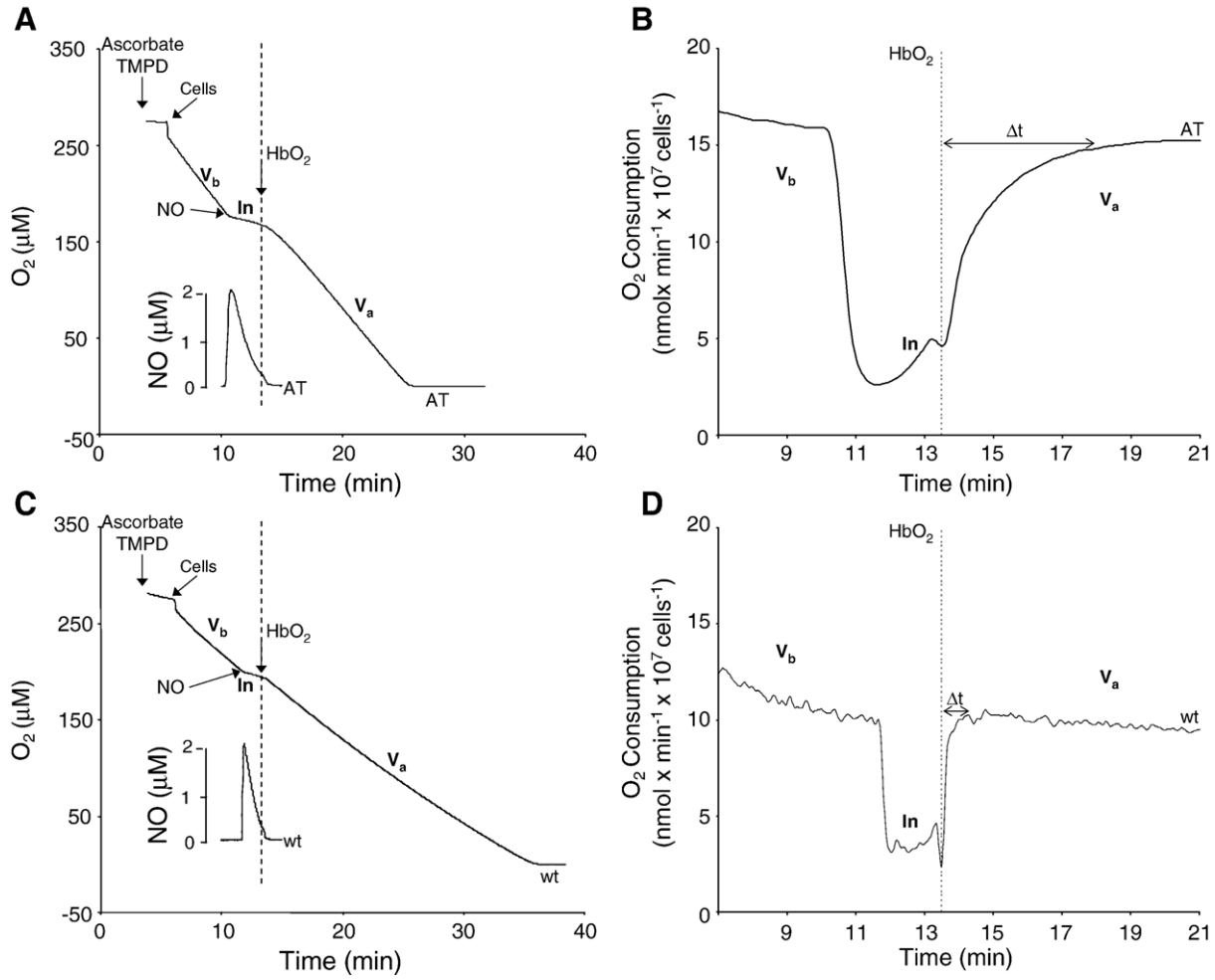

Fig. 4. Inhibition of wild type and AT cells respiration by NO. (Panels A, C) Typical amperometric NO and O_2 profiles, recorded in the dark in the presence of excess ascorbate and TMPD, 1 mM. V_b = rate of O_2 consumption before NO addition; V_a = rate of O_2 consumption after addition of HbO_2, i.e. in the absence of free NO (see lower profiles). In = inhibited state (in the presence of free NO). In order to assess the fraction of residual inhibited CcOX–NO, the instantaneous rate was measured 45 s after HbO_2 addition. Under these conditions, AT is inhibited by 32.38±3.8%, whereas wt by 16.89±3.2%. (Panels B, D) 1st derivative plots (integration time t=2 s), where the $\triangle t$ values evaluated from addition of HbO_2 and the full recovery of activity are also shown (details in Materials and methods). T=25 °C.

the presence of oligomycin and glucose favouring glycolysis [42] the steady state concentration of ATP decreased, by 27% and 32% in wt and AT cells (Fig. 6A, bottom). As expected, in the absence of glucose the sensitivity to oligomycin increased in both cell lines, and the concentration of ATP was decreased by 48% and 70% in wt and AT respectively. This finding suggests that OXPHOS and glycolysis contribute differently to mitochondrial metabolism in AT and wt cells.

The relative contribution of OXPHOS and glycolysis to the overall ATP production was further evaluated by comparing the lactate production under standard culture conditions and in the presence of antimycin and myxothiazole. As shown in Fig. 6B, OXPHOS ATP was significantly higher in AT than in wt cells, suggesting that AT cells rely on the oxidative production of ATP more than controls.

4. Discussion

The results of the experiments herein reported suggest that upon increasing the electron flux through the respiratory chain the NO inhibition of CcOX is significantly greater in AT cells than in controls. As expected, the respiration of both AT and wt cells was promptly inhibited by μM NO added as a single bolus [28]. Under basal metabolic conditions the rapid removal of free NO from both AT and wt cells (e.g. by HbO_2) resulted in the rapid, not time resolved in the O_2 electrode, recovery of respiration [28], attributed to oxidation of NO to nitrite in the active site [28,29,43]. The stable formation of the nitrosyl CcOX–NO derivative, was obtained in the presence of excess ascorbate and TMPD in the dark [28,29]; following inhibition, and after exhaustion of bulk NO, AT cells recovered activity with slower kinetics than wt cells. Owing to the presence of HbO_2 trapping any (putative) trace of NO the slow recovery of respiration cannot be attributed to re-binding to CcOX of free NO (not detected by the electrode). As also shown previously [28,29], the slow recovery rather suggests that even in unlimited O_2 (air-equilibrated media) with high electron flux through the respiratory chain, CcOX in AT cells reacts with NO forming the more stable CcOX–NO inhibited derivative. This latter condition is compatible with mitochondrial impairment since

respiration of AT cells exposed to NO remains inhibited for longer times [28,29]. Based on the ELISA quantification of the cell cytochrome *c* content, and the immunoblotting results, we suggest that the higher O_2 consumption rate induced by TMPD in AT, under otherwise identical conditions of O_2 concentration (270 μM) and mitochondrial mass (virtually identical citrate synthase activity in the two cell lines) is due to the higher (~50%) concentration of cytochrome *c* detected in AT cells. This finding is fully consistent with the hypothesis linking CcOX nitrosylation to the level of electron flux through the respiratory chain [28–30] providing a feasible molecular explanation for the greater degree of CcOX nitrosylation observed in AT compared to wt in the presence of ascorbate and TMPD. Alternative possibilities such as more favoured reaction of electron donation by cytochrome *c* at the binding site on CcOX or intrinsically different reactivity of CcOX towards NO in AT cells, cannot be excluded but definitely appear less likely. Under standard culture conditions, the expected NO concentration in the cell cytoplasm is in the few 10th of nM range [44,45], and

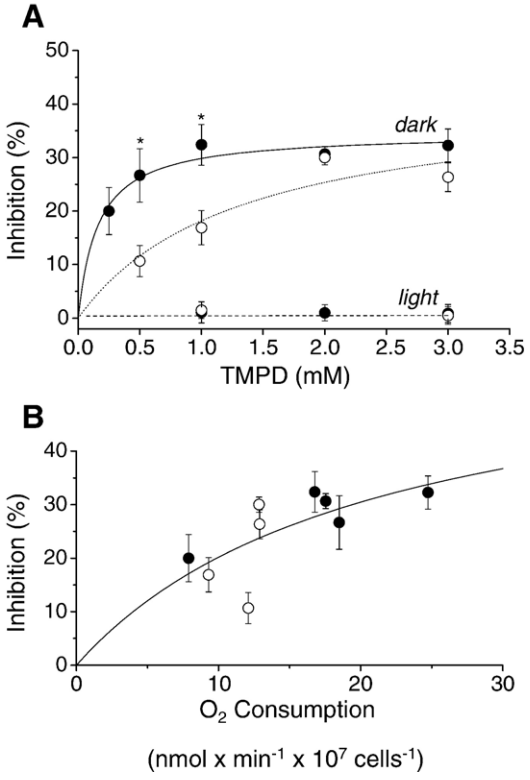

Fig. 5. Residual inhibition of cell respiration by NO. (Panel A) Dependence of inhibition on the concentration of TMPD added to wild type (○) and Ataxia Telangiectasia (●) cells, 1×10^7 cells ml^{-1}. Inhibition (%) is defined as $[1-(V_a/V_b)]\times100$ and is reported in *y* axis. The ratio $V_a/V_b=1$ when residual inhibition=0, whereas it decreases when inhibition increases ($V_a/V_b<1$). Measurements were carried out in the presence of excess ascorbate, and in the dark to stabilize the $Fe^{2+}NO$ adduct of CcOX formed in the presence of 2 μM NO; light fully abolished the inhibition detectable after removal of free NO by HbO_2. Notice that when AT cells are close to the maximal inhibition predictable wt is inhibited only by 50% of this value. Data points represent the mean±SEM (n=6–9). *, $P<0.05$ *vs* wild type cells. (Panel B) Extent of inhibition as a function of the rate of O_2 consumption by cytochrome *c* oxidase. The percentages of residual inhibition are reported for any cell line as a function of the rate of respiration measured at any given TMPD concentration before addition of NO (symbols as in panel A). The curve is the best fit to a hyperbola obtained using Origin 7.0 Global Best Fitting (Northampton, MA 01060 — USA) (details in Materials and methods).

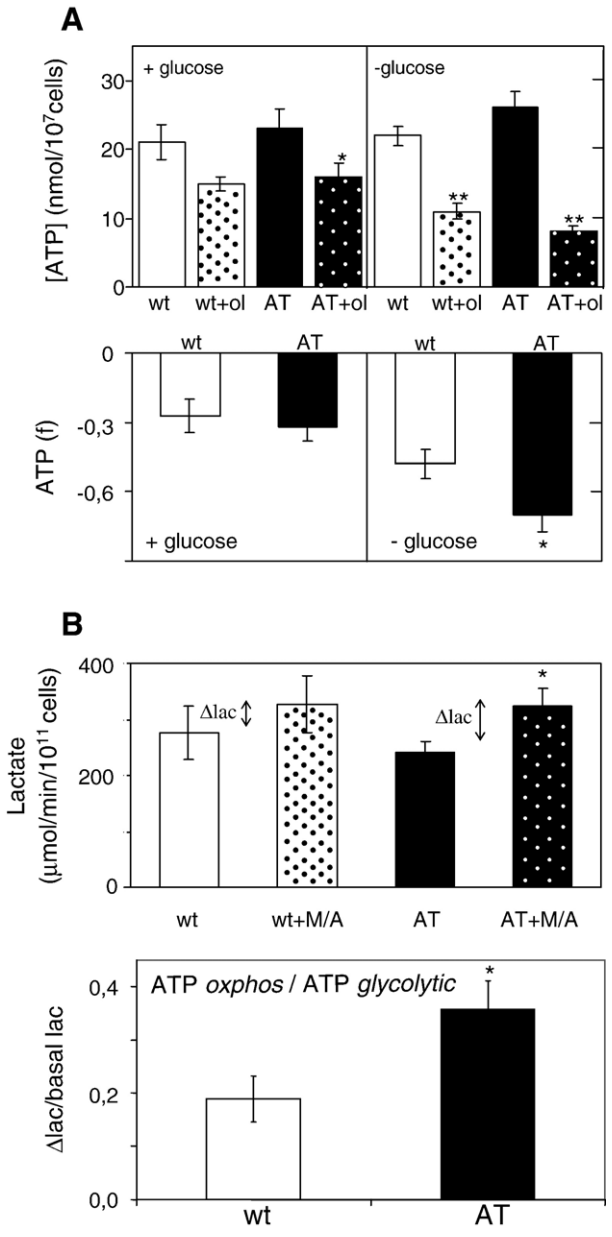

Fig. 6. OXPHOS versus glycolytic ATP in wild type and Ataxia Telangiectasia lymphoblastoid cells. (Panel A) Stationary concentrations of ATP determined in AT and wt cells, before (solid bars) or after (dotted bars) 1.5 h oligomycin treatment. Measurements were carried out in the presence (left) or absence (right) of glucose. Top: Absolute ATP values (*P value<0.05 *vs* untreated AT cells, ** P value<0.001 *vs* untreated AT and wt cells). Bottom: fractional ATP changes. (* P value<0.05 *vs* wt cells). Values represent the mean±SEM (n=6). (Panel B) Stationary concentration of lactate in AT and wt cells. Top: lactate produced in the presence of 1 mM glucose, by cells treated or not with myxothiazol (M), and antimycin A (A) (OXPHOS inhibitors), 10 μM each. *, $P<0.05$ *vs* untreated AT cells. *Bottom*: Difference in lactate (Δlac) was divided by the basal lactate (no inhibitors) and the value reported as indicative of the ratio between OXPHOS and glycolytic ATP (details in Materials and methods). *, $P<0.05$ *vs* wild type cells. Values represent the mean±SEM (n=6).

mitochondrial CcOX appears able to rapidly degrade NO to nitrite [27,46], particularly under conditions of unlimited O_2 supply and basal electron transfer rates [28]. Under these basal metabolic conditions and production of NO by the constitutive NOSs, a limited depression of mitochondrial potential has been reported [24,44], and herein confirmed (not shown). The evaluation of the ATP and lactate produced by intact cells in the presence and absence of respiratory chain inhibitors suggests that AT cells rely on oxidative ATP more than wt, suggesting in turn that AT cells may contain a higher fraction of state 3 mitochondria than wt. This observation further supports the idea that CcOX in AT cells is more reactive towards NO, since state 3 mitochondria were reported to be more susceptible to NO inhibition [29,47,48]. In this respect, however, the correlation between the extent and type of NO inhibition and the mitochondrial functional state (state 3 or state 4) of intact AT and wt cells needs to be directly assessed.

The mitochondrial response to NO particularly at the CcOX level has been proposed to be part of the cell signalling pathways [49], so that the ability for a cell line to efficiently control the mitochondrial NO concentration and traffic appears an important property for cell survival. The CcOX and NO chemistry might become particularly relevant under all conditions of increased half life of NO, particularly when O_2 supply is diminished [30] and eNOS is activated [50], and/or during infections (iNOS activation), with massive NO release. Based on our results, it is tempting to speculate that under similar conditions of electron flux rates (high) and O_2 availability (low), also *in vivo* the respiratory chain of AT cells exposed to NO will be more nitrosylated, i.e. more inhibited, than that of the wt cells. If confirmed, this finding would imply that lymphocytes of AT patients exposed to pulses of NO, for example in hypoxic infected sites (tissues, organs), would be able to synthesize OXPHOS ATP to a lower level than normal lymphocytes. One might speculate that the immunodeficiency characteristic of the AT patients can be attributed, at least to some extent, to severe NO inhibition impairing the mitochondrial bioenergetics of their lymphoid cells. According to our results, the availability in the cell of cytochrome *c* at the CcOX site controls type and extent of mitochondrial NO inhibition. It is tempting to speculate, whether the control of the cytochrome *c* expression may form the basis of a possible therapeutic intervention in this and similar pathologies.

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