



## Short Communication

## ANT2-defective fibroblasts exhibit normal mitochondrial bioenergetics



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## ARTICLE INFO

## Article history:

Received 25 February 2015

Received in revised form 22 March 2015

Accepted 22 March 2015

Available online 2 April 2015

## Keywords:

Adenine nucleotide translocase 2

ANT2

SLC25A5

Mitochondria

## ABSTRACT

Adenine nucleotide translocase 2 (ANT2) transports glycolytic ATP across the inner mitochondrial membrane. Patients with ANT2 deletion were recently reported. We aimed at characterizing mitochondrial functions in ANT2-defective fibroblasts. In spite of ANT2 expression in fibroblasts, we observed no difference between ANT2-defective and control fibroblasts for mitochondrial respiration, respiratory chain activities, mitochondrial membrane potential and intracellular ATP levels.

This indicates that ANT2 insufficiency does not alter fibroblast basal mitochondrial bioenergetics.

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## 1. Introduction

Adenine nucleotide translocase 2 ANT2, encoded by *SLC25A5* (chromosome Xq24), is a protein expressed mainly in proliferative and undifferentiated cells whose function is to transport cytosolic ATP produced by glycolysis across the inner mitochondrial membrane into the mitochondrial matrix [1,2]. Recently, ANT2 whole gene deletion has been reported in 3 unrelated males with nonsyndromic intellectual disability (ID) [3] and ANT2 therefore was proposed as a new candidate gene for nonsyndromic ID. Our aim was to characterize potential abnormalities in mitochondrial bioenergetics in one of these patients' fibroblasts.

Based on the role of ANT2 in maintaining mitochondrial membrane potential in cancer cells [1,2], we hypothesized that cells lacking ANT2 would, as recently reported in *Ant2* knockout mouse [4], exhibit a respiratory chain deficiency, decreased mitochondrial membrane potential and low intracellular ATP levels. We also hypothesized that the absence of ANT2 would increase their sensitivity to mitochondrial oxidative phosphorylation (OXPHOS) inhibitors, because with mitochondrial ATP synthesis being inhibited, cells would rely on the transport of cytosolic ATP into the mitochondrial matrix (ANT2) to maintain mitochondrial homeostasis and eventually survive.

## 2. Methods

## 2.1. Patient

The patient (reported elsewhere as patient C [3]) was a boy born from non-consanguineous healthy parents. He had a congenital cataract. At the age of 2 years, he was diagnosed with global developmental delay and myoclonic epilepsy. At the current age of 4 years, he was diagnosed with nonsyndromic ID and had a normal brain MRI. His mother also suffers from questionable seizures versus non-epileptic events.

Oligo-array analysis on his blood DNA identified a 209 kb deletion on X chromosome (position 118,377–118,586 Mb). Further mapping by PCR showed that this deletion harbored the genes *PGRMC1* (118,370,211–118,378,429), *SLC25A43* (118,533,258–118,588,437) and *SLC25A5* (118,602,363–118,605,359). Using the same oligo-array, the patient's mother was found to carry this deletion too. As reported, *SLC25A5* was shown to be the candidate gene at the origin of this neurodevelopmental phenotype [3].

## 2.2. RT-PCR

RNA was isolated from fibroblasts using the miRNeasy miRNA isolation kit (Qiagen, Valencia, CA). Reverse transcription reactions were performed using the SuperScript III First-Strand Synthesis System for

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RT-PCR (Invitrogen), following the manufacturer's instructions and using primers for *ANT2* and *GAPDH* that are available on request.

### 2.3. Respiratory chain activity measurement

Patient's fibroblasts and fibroblasts from an age-matched normal individual (control fibroblasts) were cultured in DMEM containing 4 mM glutamine, 4.5 g/L glucose and 2 mM pyruvate. Then, the cells were trypsinized and centrifuged at 1,500xg for 5 minutes. The supernatant was discarded and the pellet washed (at 1,500xg for 5 minutes) with 1 mL PBS. Polarographic and spectrophotometric assays were performed as already reported [5].

### 2.4. Oligomycin treatment

Both the control and patient fibroblasts were treated with increasing concentrations of oligomycin (Sigma-Aldrich, Saint-Louis, MO), a mitochondrial ATP synthase inhibitor. Cell survival was assessed every 24 h as follows. Cells were trypsinized and collected. The cells were then incubated with an equal volume of trypan blue, counted using an automated cell counter (Countess, Invitrogen, Grand Island, NY) and plated at similar initial density of 15,000 cells per cm<sup>2</sup>. The experiments were performed in triplicates. Oligomycin was diluted in 100% ethanol and tested at 0.25, 0.5, and 0.75 ng/mL.

### 2.5. Enzyme assays

Fibroblasts were trypsinized and centrifuged at 1,500xg for 5 minutes. The supernatant was discarded and the pellet washed (at 1,500xg for 5 minutes) with 1 mL PBS. The majority of the fresh pellet was used for polarographic assay [5] (see below). A small aliquot of the pellet was deep-frozen in 20–40  $\mu$ L PBS solution and subsequently thawed using 1 mL of ice-cold solution consisting of 0.25 M sucrose, 20 mM Tris (pH 7.2), 2 mM EGTA, 40 mM KCl and 1 mg/mL BSA, 0.004% digitonin (w/v), and 10% Percoll (v/v) (medium A). After 7 min incubation at ice temperature, cells were centrifuged (at 2,300xg for 5 minutes), the supernatant discarded, and the pellet washed (at 2,300xg for 5 minutes) with 1 mL of medium A devoid of digitonin and Percoll. The final pellet was re-suspended in 20–30  $\mu$ L of this medium and used for spectrophotometrical enzyme assays.

Respiratory chain enzyme activities were spectrophotometrically measured using a Cary 50 UV–visible spectrophotometer (Varian Inc., Les Ulis, France) [5,6].

Intact cell respiration and mitochondrial substrate oxidation (using 0.006% digitonin-permeabilized cells) were polarographically estimated [5] in a magnetically-stirred 250  $\mu$ L-cell thermostated at 37 °C (DW1 Clark oxygen electrode; Hansatech Instruments; Norfolk, United Kingdom). All chemicals were of the purest grade available from Sigma-Aldrich (St Quentin Fallavier, France). Protein concentration was measured according to Bradford.

### 2.6. Testing mitochondrial membrane potential

To measure mitochondrial membrane polarization,  $1 \times 10^6$  cells were suspended in 1 mL PBS and incubated with JC-1 dye (Invitrogen, Grand Island, NY) at 0.2  $\mu$ M for 30 min. Cells were pelleted by centrifugation and re-suspended in 500  $\mu$ L PBS. The cells were analyzed by flow cytometry measuring both green and red fluorescence. Relative degrees of mitochondrial polarization were quantified by measuring the ratio of red-shifted JC-1 aggregates, which are favored under conditions of high membrane potential, and green-shifted monomers, which tend to predominate under conditions of low membrane potential [7].

### 2.7. Cellular ATP levels

ATP levels were measured using the ATPlite™ Luminescence Assay System (Perkin Elmer, Waltham, MA) according to the manufacturer's instructions. The reaction of ATP with added Luciferase results in emitted light. The amount of light emitted should be proportional to the ATP concentration. Luminescence was measured using a microplate reader.

## 3. Results

First we verified that *ANT2* was normally expressed in control fibroblasts and absent in the patient's fibroblasts (Fig. 1A). Then, we measured mitochondrial respiratory chain activities and mitochondrial respiration with mitochondrial substrate oxidation by spectrophotometric and polarographic assays respectively. These were comparable between the patients' fibroblasts and control fibroblasts (Fig. 1B and C). Similarly, there was no difference in mitochondrial membrane potential (Fig. 1D) or intracellular ATP levels (Fig. 1E).

To evaluate whether *ANT2*-defective fibroblasts would be more sensitive to cell death induced by OXPHOS inhibitors, we analyzed the effects of increasing concentrations of oligomycin on cell growth. The obtained results showed no significant difference between patient's and control fibroblasts where 100% of death occurred from 0.75 ng/mL 48 h after oligomycin treatment.

## 4. Discussion

These data show that while *ANT2* is expressed in human fibroblasts, its absence does not affect basal mitochondrial bioenergetics. Mitochondrial respiration and OXPHOS, membrane potential, oligomycin sensitivity and intracellular ATP levels are comparable in both control and *ANT2*-defective fibroblasts.

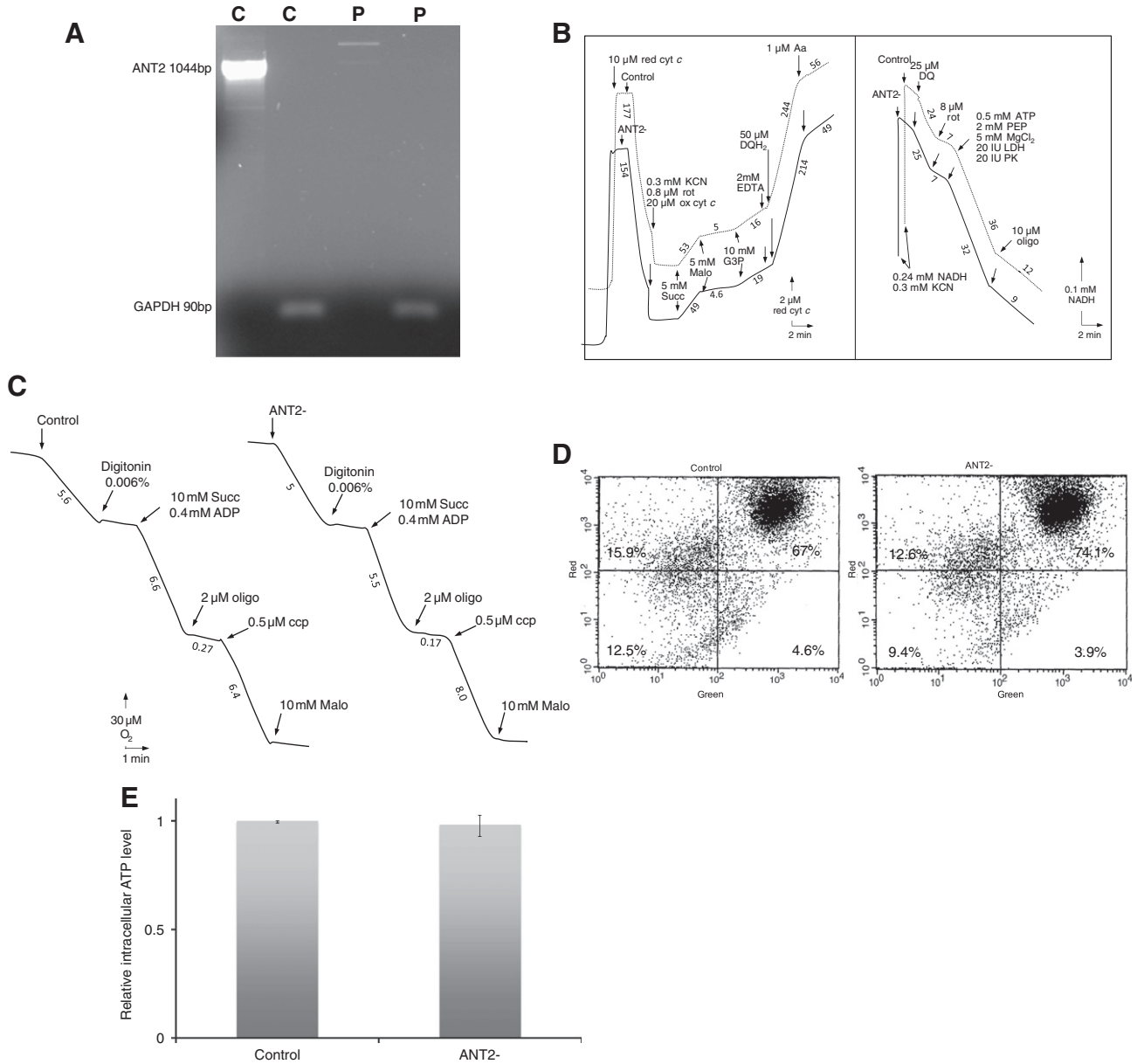
We had hypothesized that, in conditions of OXPHOS inhibition induced by oligomycin, glycolytic ATP, which remains the only source of cellular ATP, cannot be transported into mitochondria in *ANT2*-defective cells. Subsequently, *ANT2*-defective cells would be more sensitive to oligomycin treatment than control fibroblasts. However, no difference could be observed upon oligomycin treatment. This is difficult to interpret but might be due to a drastic interruption of ATP production with subsequent high cellular toxicity without difference between *ANT2*-defective cells and control fibroblasts. Further tests under varied conditions should be conducted to more broadly characterize the impact of *ANT2* insufficiency. For example, mitochondrial membrane permeabilization induction by lonidamine was shown to be facilitated by *ANT2* silencing in several hormone-dependent cancer cell lines [8].

The clinical phenotype of the patient is purely neurologic (epileptic encephalopathy). Therefore, the cellular impact of an *ANT2* defect in the brain may be different than in skin fibroblasts. Indeed, additional evidence of the role of *ANT2* in the central nervous system exists:

- i) Insertional mutagenesis of *ANT2* in zebrafish has severe neurodevelopmental consequences [9].
- ii) *ANT2* duplication in humans is associated with severe neurological impairment [10].

Recently, the *Ant2*  $-/-$  mouse was characterized with severe postnatal growth retardation, macrocytic anemia, B lymphocytopenia, lactic acidosis, bloated stomach, and death within 4 weeks. Interestingly, unlike *ANT2*-defective fibroblasts, *Ant2*  $-/-$  mouse splenocytes exhibited decreased mitochondrial respiration as well as decreased mitochondrial potential and decreased relative intracellular ATP levels [4].

In conclusion, *ANT2* insufficiency does not affect mitochondrial bioenergetics in fibroblasts grown under basal conditions. This might be due to tissue-specificity of *ANT2* function as *ANT2* plays a role in the transport of cytosolic ATP into mitochondria mainly in glycolytic tissues such as cancer cells. Accordingly, the encephalopathic phenotype



**Fig. 1.** (A) *ANT2* and *GAPDH* mRNA expression levels were assessed by standard RT-PCR in control (C) and *ANT2*-defective patient's (P) fibroblasts. (B) Respiratory chain activities in control and *ANT2*-defective patient's fibroblasts (*ANT2*<sup>-</sup>). Left panel: sequential measurements of complex IV, complex II + III, glycerol-3-phosphate dehydrogenase (G3Pdh) + complex III, and complex III activities. Complex IV activity is triggered by the addition of reduced cytochrome c. Malonate-sensitive complex II + III activities and G3Pdh + complex III activities are subsequently measured before assaying for antimycin-sensitive complex III activity triggered by reduced decylubiquinone. Right panel: sequential measurement of rotenone-sensitive complex I and oligomycin-sensitive ATPase (complex V) activities. Aa, antimycin A; ATP, adenosine triphosphate; Cyt, cytochrome; DQ, DQH<sub>2</sub>, duroquinone (oxidized), duroquinol (reduced), respectively; EDTA, ethylene diamine tetraacetic acid; G3P, glycerol-3-phosphate; KCN, potassium cyanide; LDH, lactate dehydrogenase; malo, malonate; MgCl<sub>2</sub>, magnesium chloride; NADH, reduced nicotinamide adenine dinucleotide; oligo, oligomycin; ox, oxidized; PEP, phosphoenol pyruvate; PK, pyruvate kinase; red, reduced; rot, rotenone; succ, succinate. Numbers along the traces are nmol/min/mg protein. Values are means ± SEM from triplicate experiments. Experimental procedures as described under *Methods* section. (C) Respiration and mitochondrial substrate oxidations in control and *ANT2*-defective patient's fibroblasts (*ANT2*<sup>-</sup>). For details see [5]. Numbers along the traces are nmol/min/mg protein. Values are means ± SEM from triplicate experiments. Experimental procedures as described under *Methods* section. Ccp, carbonyl cyanide m-chlorophenylhydrazone; malo, malonate; oligo, oligomycin; Succ, succinate. (D) Mitochondrial membrane potential in control and *ANT2*-defective patient's fibroblasts as determined by FACS analysis with JC-1 staining. See experimental procedures. (E) Relative intracellular ATP levels in control and *ANT2*-defective patient's fibroblasts. Values are means ± SEM from triplicate experiments.

observed in *ANT2*-deficient patients might be attributable to the effects of *ANT2* insufficiency on neurodevelopment where aerobic glycolysis is the major source of ATP [11], thus strengthening the role of *ANT2* insufficiency as a cause of X-linked nonsyndromic ID.

#### Acknowledgments

This study was partly supported by PHS grants NIH R01 DK78775 (JV), Children's Hospital of Pittsburgh of UPMC Research Advisory Committee (MS) and the Philippe Foundation (MS).

#### References

- [1] A. Chevrollier, D. Loiseau, B. Chabi, G. Renier, O. Douay, Y. Malthiery, G. Stepien, *ANT2* isoform required for cancer cell glycolysis, *J. Bioenerg. Biomembr.* 37 (2005) 307–316.
- [2] A. Chevrollier, D. Loiseau, P. Reynier, G. Stepien, Adenine nucleotide translocase 2 is a key mitochondrial protein in cancer metabolism, *Biochim. Biophys. Acta* 1807 (2011) 562–567.
- [3] J. Vandewalle, M. Bauters, H. Van Esch, S. Belet, J. Verbeeck, N. Fieremans, M. Holvoet, J. Vento, A. Spreiz, D. Kotzot, E. Haberlandt, J. Rosenfeld, J. Andrieux, B. Delobel, M.B. Dehouck, K. Devriendt, J.P. Fryns, P. Marynen, A. Goldstein, G. Froyen, The mitochondrial solute carrier SLC25A5 at Xq24 is a novel candidate gene for non-syndromic intellectual disability, *Hum. Genet.* 132 (2013) 1177–1185.

- [4] J. Cho, J. Seo, C.H. Lim, L. Yang, T. Shiratsuchi, M.H. Lee, R.R. Chowdhury, H. Kasahara, J.S. Kim, S.P. Oh, Y.J. Lee, N. Terada, Mitochondrial ATP transporter Ant2 depletion impairs erythropoiesis and B lymphopoiesis, *Cell Death Differ.* (2015) in press.
- [5] P. Rustin, D. Chretien, T. Bourgeron, B. Gerard, A. Rotig, J.M. Saudubray, A. Munnich, Biochemical and molecular investigations in respiratory chain deficiencies, *Clin. Chim. Acta* 228 (1994) 35–51.
- [6] P. Benit, S. Goncalves, E. Philippe Dassa, J.J. Briere, G. Martin, P. Rustin, Three spectrophotometric assays for the measurement of the five respiratory chain complexes in minuscule biological samples, *Clin. Chim. Acta* 374 (2006) 81–86.
- [7] S.T. Smiley, M. Reers, C. Mottola-Hartshorn, M. Lin, A. Chen, T.W. Smith, G.D. Steele Jr., L.B. Chen, Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1, *Proc. Natl. Acad. Sci. U. S. A.* 88 (1991) 3671–3675.
- [8] M. Le Bras, A. Borgne-Sanchez, Z. Touat, O.S. El Dein, A. Deniaud, E. Maillier, G. Lecellier, D. Rebouillat, C. Lemaire, G. Kroemer, E. Jacotot, C. Brenner, Chemosensitization by knockdown of adenine nucleotide translocase-2, *Cancer Res.* 66 (2006) 9143–9152.
- [9] G. Golling, A. Amsterdam, Z. Sun, M. Antonelli, E. Maldonado, W. Chen, S. Burgess, M. Haldi, K. Artzt, S. Farrington, S.Y. Lin, R.M. Nissen, N. Hopkins, Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development, *Nat. Genet.* 31 (2002) 135–140.
- [10] T. Takenouchi, H. Okuno, R. Kosaki, D. Ariyasu, C. Torii, S. Momoshima, N. Harada, H. Yoshihashi, T. Takahashi, M. Awazu, K. Kosaki, Microduplication of Xq24 and Hartsfield syndrome with holoprosencephaly, ectrodactyly, and clefting, *Am. J. Med. Genet. A* 158A (2012) 2537–2541.
- [11] A.L. Bauernfeind, C.C. Babbitt, The appropriation of glucose through primate neurodevelopment, *J. Hum. Evol.* 77 (2014) 132–140.