Food derived respiratory complex I inhibitors modify the effect of Leber hereditary optic neuropathy mutations

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Abstract

Mitochondrial DNA mutations in genes encoding respiratory complex I polypeptides can cause Leber hereditary optic neuropathy. Toxics affecting oxidative phosphorylation system can also cause mitochondrial optic neuropathy. Some complex I inhibitors found in edible plants might differentially interact with these pathologic mutations and modify their penetrance. To analyze this interaction, we have compared the effect of rotenone, capsaicin and rolliniastatin-1 on cybrids harboring the most frequent Leber hereditary optic neuropathy mutations and found that m.3460G > A mutation increases rotenone resistance but capsaicin and rolliniastatin-1 susceptibility. Thus, to explain the pathogenicity of mitochondrial diseases due to mitochondrial DNA mutations, their potential interactions with environment factors will have to be considered.

Keywords: Respiratory complex I; Xenobiotic; Mitochondrial DNA; Leber hereditary optic neuropathy; Gene-environment interaction

1 Introduction

Leber hereditary optic neuropathy (LHON) is a kind of blindness mostly due to mitochondrial DNA (mtDNA) mutations in genes coding for respiratory complex I (CI) polypeptides. Some individuals, despite harboring an mtDNA pathologic mutation, fail to express the phenotype (Maresca et al., 2014). Sometimes, incomplete penetrance can be explained by differences in the mutation load (Jacobi et al., 2001). However, LHON patients are usually homoplasmic, i.e. 100% of their mtDNA molecules are mutated. Then, other factors must be considered to justify phenotypic differences. Thus, it has been shown that mtDNA genetic backgrounds, such as mtDNA haplogroups J or K, can alter the

LHON susceptibility (Hudson et al., 2007). Maternal relatives share the mtDNA genotype but more males than females develop LHON (Franks and Sanders, 1990). The difference in sex susceptibility can be attributed to the effect of estrogens that ameliorate LHON mitochondrial dysfunction (Giordano et al., 2011). LHON peak age of onset is between 15 and 30 years (Dimitriadis et al., 2014). Maybe, healthy individuals differ from maternally related patients because they have not reached a particular age. However, reports of healthy individuals, older than patients, are not rare in LHON homoplasmic mutant pedigrees (Vilkki et al., 1989).

Previous observations suggested that other factors should be invoked to explain phenotypic differences between LHON homoplasmic mutant healthy individuals and patients with similar ages and the same mitochondrial genetic background and gender. It was found that mutant (m.3460G > A) osteosarcoma 143B cybrids showed severe CI deficiency. However, a biochemical defect was not apparent in adenocarcinoma A549 cybrids harboring the same mutation (Cock et al., 1998). These results suggested that the nuclear genetic (nDNA) background influences the expression of the biochemical defect in LHON patients. In fact, it has been recently found that a genetic variant of the nDNA-encoded mitochondrial enzyme tyrosyl-tRNA synthetase, which decreases OXPHOS capacity, is a nuclear modifier for LHON (Jiang et al., 2016a).

The exposure to environmental factors might also explain phenotypic differences among homoplasmic individuals from the same maternal pedigree, same gender and similar age. For example, two brothers carrying the m.11778G > A homoplasmic mutation showed a different phenotype. The index case developed LHON at the age of 60 years after a long history of occupational exposure to polycyclic aromatic hydrocarbons (PAHs). However, his 59 years old brother was unaffected (Rufa et al., 2005). A 34 years old individual harboring the m.11778G > A homoplasmic mutation developed LHON after his exposure to erythromycin but three homoplasmic mutant brothers (33, 31 and 13 years old) did not have vision problems (Luca et al., 2004). As LHON mutations mainly affect CI polypeptides, the exposure to CI inhibitors may imitate the phenotype. In fact, after the infusion of rotenone microspheres into the rat's optical layer of the superior colliculus, animals exhibited several signs comparable to those observed in human LHON patients (Zhang et al., 2015). The number of known CI inhibitors is increasing and the exposure to natural origin CI inhibitors is difficult to avoid (Degli Esposti, 1998). These compounds may differentially modify the phenotype due to LHON mutations. Therefore, we decided to check the interaction between CI inhibitors found in edible plants, such as capsaicin and rolliniastatin-1 (Fig. 1), and the most frequent LHON mutations. As a control, we used rotenone, a frequently used CI inhibitor.

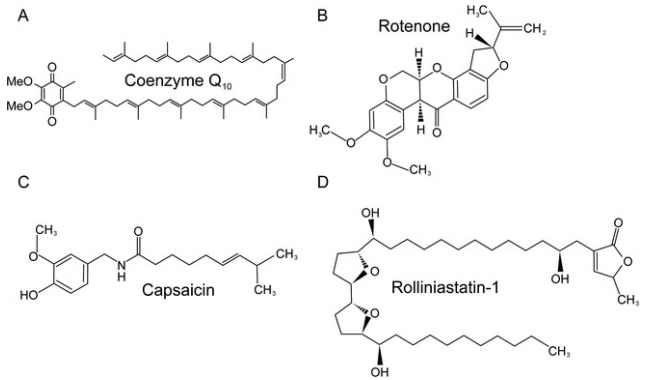

Fig. 1 Respiratory complex I (CI) inhibitors. The structural formula of CI substrate (A) and inhibitors (B-D) are represented.

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2 Material and methods

To homogenize nuclear and environmental factors, we built cybrids with the osteosarcoma 143B rho^0 nuclear background using patient and control platelets (Chomyn et al., 1994). All samples were collected with written informed consent and the Ethics Review Committees of the involved hospitals and the Government of Aragón approved the study (Comité Ético de Investigación Clínica de Aragón-CEICA-12/2014). These cybrids were maintained in Dulbecco's modified eagle medium with no antibiotics and containing glucose (4.5 g/l), pyruvate (0.11 g/l) and fetal bovine serum (5%). To perform the experiments, glucose concentration was reduced to 1.0 g/l.

Screening for the three primary LHON mutations was done by polymerase chain reaction/restriction fragment length polymorphism (Emperador et al., 2018). The complete mtDNA was amplified and sequenced according to previously described protocols (Emperador et al., 2018). The conservation index was used to predict detrimental effects of mtDNA missense mutations (Martin-Navarro et al., 2017). The AmpFLSTR® Identifiler® PCR Amplification Kit (Life Technologies) was used to determine the cybrids nDNA genetic fingerprints. The molecular cytogenetic analysis was done according to already published procedures (Lopez-Gallardo et al., 2016).

Oxygen consumption and caspase-3 activation analyses were performed according to previously described protocols (De Miguel et al., 2013; Gomez-Duran et al., 2010). CI inhibitors were added during the oxygen consumption, or two days before the caspase-3, studies.

The three-dimensional structure of the bovine p. MT-ND1 subunit (PDB 5LNK), ortholog of human p. MT-ND1, was obtained with the RasMol 2.6 program (http://www.rasmol.org).

3 Results

3.1 Characterization of cybrid cell lines

Transmitochondrial cell lines (cytoplasmic hybrids or cybrids) allow an important homogenization of physiologic factors and nuclear genetic backgrounds (Iglesias et al., 2012). Therefore, phenotypic differences between LHON and wild-type cybrids should be mostly due to their mtDNA genotypes.

To analyze the effects of different LHON mutations on mitochondrial bioenergetics, we first built 4 osteosarcoma 143B cybrid cell lines harboring wild-type mtDNA (Owt) or LHON (m.3460G > A, O3460; m.11778G > A, O11778; m.14484T > C, O14484) mutations and 3 adenocarcinoma A549 cybrid cell lines with (m.3460G > A, A3460; m.11778G > A, A11778) or without (Awt) LHON mutations (Fig. 2A). To confirm that they shared the nuclear background, we performed a nuclear genetic fingerprint of 16 short tandem repeats (STRs). Osteosarcoma 143B cybrids showed the same nuclear markers and coincided with those reported in American Type Culture Collection (ATCC) osteosarcoma 143B parental cells (Table 1A). There were some minor differences between adenocarcinoma A549 cybrids and only one difference with those reported in ATCC adenocarcinoma A549 parental cells (Table 1A). Karyotyping was used to verify that nuclear backgrounds were equivalent (Fig. 2B). To rule out the presence of other mtDNA mutations that could affect cybrid bioenergetics phenotypes, we sequenced the whole mtDNAs. Non-synonymous mutations changed evolutionary poorly conserved amino acids and, probably, with no functional importance (Table 1B).

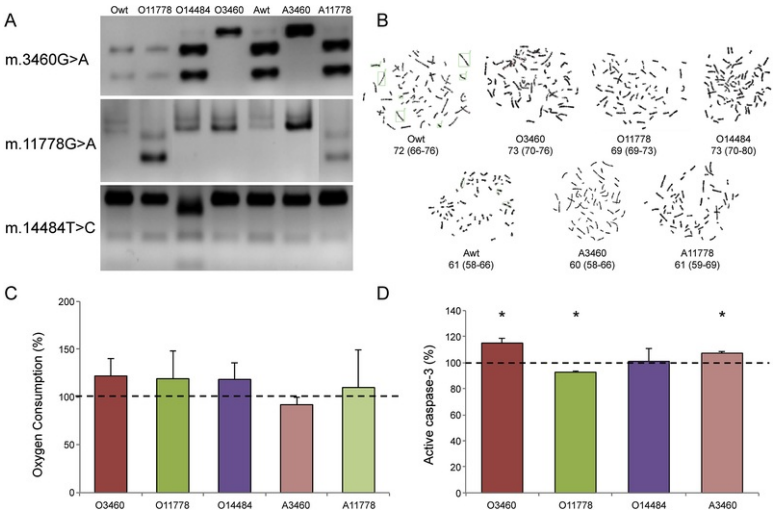

Fig. 2 Characterization of cybrid cell lines. A) Confirmation of LHON mtDNA mutations. Gels showing the results of restriction fragment length polymorphism analyses. B) Karyotypes. Representative images of metaphase preparations. Chromosome modal number and range (in brackets) are indicated. C) Basal oxygen consumption. The value for each wild-type (Owt or Awt) cybrid is considered 100% (dashed line). D) Active caspase-3 levels. The value for each wild-type (Owt or Awt) cybrid is considered 100% (dashed line). * (vs Owt or Awt), P ≤ 0.0284.

alt-text: Fig. 2

Table 1 Cybrid genetic characterization.

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A. Nuclear genetic fingerprint.										
Chr	Marker	O143B (ATCC)	Owt	O3460	O11778	O14484	A549 (ATCC)	Awt	A3460	A11778
2	D2S1338		24,25	24,25	24,25	24,25		24,25	24,25	24,25
2	TPOX	11	11	11	11	11	8,11	8,11	8,11	8,11
3	D3S1358		15	15	15	15		16	16	16,17

4	FGA		24	24	24	24		23	23	23
5	D5S818	13	13	13	13	13	11	11,*12*	11,*12*	11
5	CSF1PO	12	12	12	12	12	10,12	10,12	10,12	10,12
7	D7S820	11,12	11,12	11,12	11,12	11,12	8,11	8,11	8,11	8,11
8	D8S1179		11,14	11,14	11,14	11,14		13,14	13,14	13,14
11	TH01	6	6	6	6	6	8,9.3	8,9.3	8,9.3	8,9.3
12	vWA	18	18	18	18	18	14	14	14	14
13	D13S317	12	12	12	12	12	11	11	11	11
16	D16S539	10,13	10,13	10,13	10,13	10,13	11,12	11,12	11,12	11,12
18	D18S51		17	17	17	17		14,17	14,17	14,17
19	D19S433		13	13	13	13		13	13	13
21	D21S11		31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2		29	29	29,*30*
X	AMEL	X	X	X	X	X	X,Y	X,Y	X,Y	X,Y

Chr, chromosome. ATCC, American type culture collection. Italic numbers indicate differences between A549 cybrids.

B. mtDNA sequence

Cybrid	Owt	O3460	O11778	O14484	Awt	A3460	A11778
GenBank	JN635299	MH080305	MH080306	MH080307	KT002149	MH080308	MH080309
Haplogroup	J1c8a	J1c2e2	J1c1b	J2a2c	U5a1a2b	T2b	T2a1b1a1b
Pathologic		3460	11778	14484		3460	11778
Mutation		(*MT-ND1*)	(*MT-ND4*)	(*MT-ND6*)		(*MT-ND1*)	(*MT-ND4*)
Private Mutations	185 (*MT-DLOOP*) **3387A (*MT-ND1*)** **14189 (*MT-ND6*)** 16241 (*MT-DLOOP*)	**10398 (*MT-ND3*)** **12131 (*MT-ND4*)** **14198 (*MT-ND6*)**	188 (*MT-DLOOP*) 482 (*MT-DLOOP*) 501 (*MT-DLOOP*) 5231 (*MT-ND2*) 6275 (*MT-CO1*)	**7269 (*MT-CO1*)** 11377 (*MT-ND4*) 14569 (*MT-ND6*)	15148 (*MT-CYB*) 15530 (*MT-CYB*)	12040 (*MT-ND4*)	1547Ti (*MT-RNR1*) 12121 (*MT-ND4*) 16051 (*MT-DLOOP*) 16086 (*MT-DLOOP*) 16218 (*MT-DLOOP*)

Non-synonymous private mutations are indicated in bold font. mtDNA position, affected polypeptide, amino acid substitution and conservation index (CI) of wild-type amino acid are shown next: 3387A (p.MT-ND1:I27M, CI = 23.5%); 7269 (p.MT-CO1:V456M, CI = 8.4%); 10398 (p.MT-ND3:A114T, backmutation); 12131 (p.MT-ND4:S458P, CI = 1.2%); 14189 (p.MT-ND6:V162A, CI = 13.9%); 14198 (p.MT-ND6:T159M, CI = 2.6%). Haplogroup assignation according to (van Oven and Kayser, 2009).

C. Amino acid variation at p.MT.ND1,3 subunits

Cybrid	p.MT-ND1	p.MT-ND3
Owt	15I,**27M**,30Y,52A,304H	**9T**,114T
O3460	15I,27I,30Y,**52T**,304H	9I,114T

O11778	15I,27I,**30H**,52A,304H	9I,**114A**
O14484	15I,27I,30Y,52A,304H	9I,**114A**
Awt	15I,27I,30Y,52A,**304Y**	9I,114T
A3460	15I,27I,30Y,**52T**,304H	9I,114T
A11778	**15T**,27I,30Y,52A,304H	9I,114T
Cybrid differences in p.MT-ND1,3 amino acids are shown in bold font.		

To check the phenotypic effect of these LHON mutations in cybrids, we analyzed endogenous oxygen consumption, which provides clues on cell respiration (Hofhaus et al., 1996), and active caspase-3 levels, a marker of apoptosis (Danielson et al., 2002). Endogenous oxygen consumption in mutant cybrids was no significantly different from that in wild-type cybrids (Fig. 2C). Active caspase-3 levels were significantly higher in both cybrids with the m.3460G > A mutation (O3460 and A3460) and significantly lower in O11778. There was no difference between O14484 and Owt (Fig. 2D).

All these results suggested that, besides LHON mutation, other factors were required for phenotype expression in patients. Below, we explore environmental exposures.

3.2 Oxygen consumption

3.2.1 Rotenone

First, we analyzed the rotenone effect on oxygen consumption in 4 osteosarcoma 143B cybrids. The blood rotenone concentrations from dead persons by insecticide ingestion were between 10.3 nM and 6.1 μM (De Wilde et al., 1986; Patel, 2011; Rhee et al., 2016). Rotenone is also present in plants. Yam bean is a common food, but its seeds are rarely consumed because contain rotenone and are poisonous (Hung et al., 2007). Blood from a dead person by Yam bean seeds ingestion showed a rotenone concentration of 182 nM (Narongchai et al., 2005). After these reports, we selected rotenone concentrations ≤6.25 nM.

Compared with untreated cybrids, rotenone 0.5 nM decreased oxygen consumption in Owt and O3460 cybrids (Fig. 3A). This concentration had apparently a larger effect on O11778 and O14484 cybrids, but a higher rotenone concentration (2.5 nM) was required to significantly diminish oxygen consumption in these cybrids. Similar to other studies on osteosarcoma 143B cybrids (Carelli et al., 2002; Datta et al., 2016), rotenone effect was dose-dependent. Thus, there were significant negative correlations between rotenone concentrations and oxygen consumption (Table 2). We did not observe significant differences to rotenone sensitivity between mutant and wild-type cybrids (Fig. 3A). However, at rotenone 2.5 nM O3460 were more resistant than O11778 cybrids. At rotenone 3.75 nM, O3460 were more resistant than O11778 and O14484 cybrids.

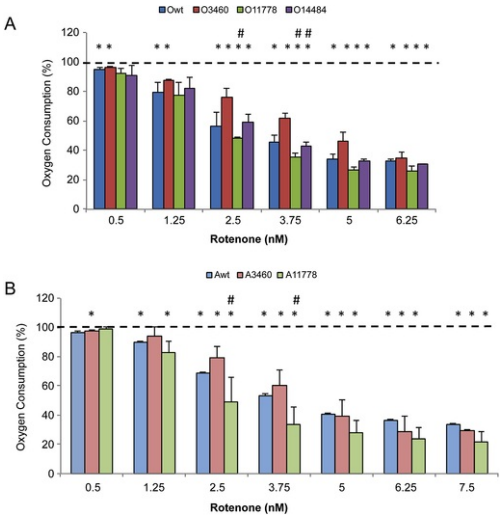

Fig. 3 Rotenone effect on endogenous oxygen consumption. A) Osteosarcoma 143B cybrids. B) Adenocarcinoma A549 cybrids. The value for each untreated cybrid is considered 100% (dashed line). * (vs untreated cybrid), P ≤ 0.0450. # (vs O3460 or A3460 at the same rotenone concentration), P ≤ 0.0496.

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Table 2 Statistical correlations between xenobiotic concentrations and basal oxygen consumption.

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Cybrid	Rotenone	Capsaicin	Rolliniastatin-1
Osteosarcoma 143B			
Owt	y = - 11.0 x + 92.8	y = - 2.0 x + 115.0	y = - 0.12 x + 114.0
	R^2 = 0.9215	R^2 = 0.9964	R^2 = 0.9582
O3460	y = - 10.8 x + 101.7	y = - 2.0 x + 91.9	y = - 0.15 x + 109.3
	R^2 = 0.9982	R^2 = 0.9535	R^2 = 0.9582
O11778	y = - 11.9 x + 89.1	y = - 1.6 x + 103.7	y = - 0.08 x + 109.1
	R^2 = 0.8867	R^2 = 0.9983	R^2 = 0.9802
O14484	y = - 11.2 x + 92.4	y = - 1.9 x + 105.8	y = - 0.09 x + 107.8
	R^2 = 0.9404	R^2 = 0.9990	R^2 = 0.9823
Adenocarcinoma A549			
Awt	y = - 11.2 x + 100.2	y = - 1.9 x + 114.1	y = - 0.07 x + 105.6
	R^2 = 0.9674	R^2 = 0.9742	R^2 = 0.9876
A3460	y = - 13.0 x + 108.2	y = - 2.2 x + 85.8	y = - 0.11 x + 101.7
	R^2 = 0.9870	R^2 = 0.9282	R^2 = 0.9990
A11778	y = - 13.3 x + 95.4	y = - 1.5 x + 101.7	y = - 0.11 x + 113.9
	R^2 = 0.8861	R^2 = 0.9803	R^2 = 0.9819

All correlations were statistically significant ($P < 0.0001$).

In adenocarcinoma A549 cybrids, rotenone 1.25 nM was required to diminish oxygen consumption in Awt and A11778 cybrids. Rotenone 2.5 nM decreased oxygen consumption in A3460 cybrids (although, 0.5 nM also decreased oxygen consumption in this cybrid) (Fig. 3B). There were also significant negative correlations between rotenone concentrations and oxygen consumption (Table 2). We did not observe significant differences to rotenone sensitivity between mutant and wildtype cybrids (Fig. 3B), but again, at rotenone 2.5 and 3.75 nM, A3460 were more resistant than A11778 cybrids. No significant differences were found between cybrids with different nuclear and mitochondrial genetic backgrounds but with the same LHON mutation and drug concentration. These results suggested that m.3460G > A mutation modified the rotenone phenotypic effect but other nDNA and mtDNA factors had less effect.

3.2.2 Capsaicin

The genus Capsicum (pepper) includes a large number of cultivated species. The plants are grown all over the world. Pepper fruits have been used for fresh and cooked consumption, as well as for medicinal purposes. These fruits are enriched in CI inhibitor capsaicin (Anonymous, 2007; Barceloux, 2009; Wahyuni et al., 2013). Capsaicin concentrations ranged from 8 to 6639 mg kg^{-1} in dried fruit (Garces-Claver et al., 2006). At least 220 mg of capsaicin have to be consumed for reaching a plasma concentration of 100 μM (Hochkogler et al., 2018). Capsaicin 240 μM decreased oxygen consumption in human promyelocytic leukemia HL60 cells (Herst et al., 2004). In human squamous cell carcinoma COLO-16 and SRB-12 cells, capsaicin 100 μM inhibited oxygen consumption (Hail and Lotan, 2002). It has been reported that capsaicin ≥10 μM caused a concentration dependent decrease in oxygen consumption of isolated rat heart mitochondria (Athanasiou et al., 2007). In human hepatocellular carcinoma HepG2 cells, ≥ 0.1 μM capsaicin reduced oxygen consumption (Hochkogler et al., 2018). We checked capsaicin concentrations below 30 μM and found immediate reductions of oxygen consumption. Compared with untreated cybrids, capsaicin 10 μM decreases oxygen consumption in O3460 and O14484 cybrids (Fig. 4A). A higher capsaicin concentration (20 μM) is required to diminish oxygen consumption in O11778 and Owt cybrids. Capsaicin effect is dose-dependent. Thus, there are significant negative correlations between capsaicin concentrations and oxygen consumption (Table 2). Between capsaicin 10–20 μM, O3460 cybrid is more susceptible to capsaicin than Owt (Fig. 4A). Between capsaicin 5 and 15 μM, O3460 cybrid is more susceptible

to capsaicin than O14484 (Fig. 4A).

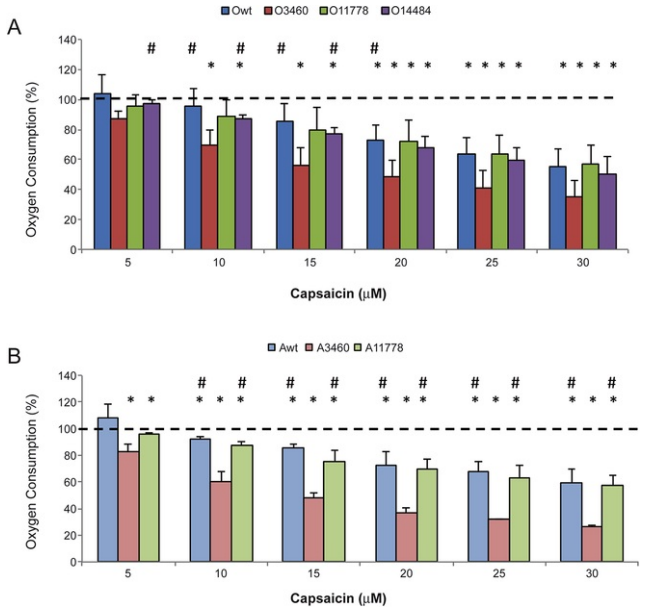

Fig. 4 Capsaicin effect on endogenous oxygen consumption. A) Osteosarcoma 143B cybrids. B) Adenocarcinoma A549 cybrids. The value for each untreated cybrid is considered 100% (dashed line). * (vs untreated cybrid), $P \leq 0.0277$. # (vs O3460 or A3460 at the same capsaicin concentration), $P \leq 0.0458$.

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In adenocarcinoma A549 cybrids, capsaicin 5 µM decreases oxygen consumption in A3460 and A11778 cybrids but 10 µM is required to diminish oxygen consumption in Awt cybrids (Fig. 4B). There are also significant negative correlations between capsaicin concentrations and oxygen consumption (Table 2). The same pattern of capsaicin sensitivity is repeated in these cybrids. Between capsaicin 10–30 µM, A3460 cybrid is more susceptible than Awt or A11778 (Fig. 4B). No significant differences are found between cybrids from different nuclear and mitochondrial genetic backgrounds but with the same LHON mutation and drug concentration. These results suggested that m.3460G > A mutation modified the capsaicin phenotypic effect but other nDNA and mtDNA factors had less effect.

3.2.3 Rolliniastatin-1

Tropical and subtropical annonaceous plants produce acetogenins. The quantities of acetogenins in annonaceous fruits are such that a cumulative dose sufficient to cause neurodegeneration in rats can be attained in humans by regular consumption within a year (Hollerhage et al., 2009). In Guadeloupe (French West Indies) there is an unusually high incidence of atypical parkinsonism, which has been linked to regular consumption of these plants, in particular soursop (Lannuzel et al., 2003, 2007, 2008). The thiophene-3-carboxamide analog of annonaceous acetogenins JCI-20679 inhibited the oxygen consumption of human non-small cell lung carcinoma NCI-H23 cells with a half maximal inhibitory concentration (IC_{50}) of 170 nM (Akatsuka et al., 2016). The acetogenin bullatacin, or rolliniastatin-2, inhibits respiration in insect-derived Sf9 cells. The IC_{50} was 1.2 nM (Ahammadsahib et al., 1993). We checked a more potent acetogenin, rolliniastatin-1, at concentrations below 500 pM in osteosarcoma 143B and adenocarcinoma A549 cybrids.

Compared with untreated cybrids, rolliniastatin-1 ≥ 200 pM decreases oxygen consumption in all osteosarcoma cybrids (Fig. 5A). Rolliniastatin-1 effect is dose-dependent. Thus, there are significant negative correlations between rolliniastatin-1 concentrations and oxygen consumption (Table 2). At concentrations ≥200 pM, O3460 cybrid is more susceptible to rolliniastatin-1 than the other cybrids (Fig. 5A). At concentrations ≥400 pM, Owt cybrid is more susceptible than O11778 or O14484 cybrids (Fig. 5A).

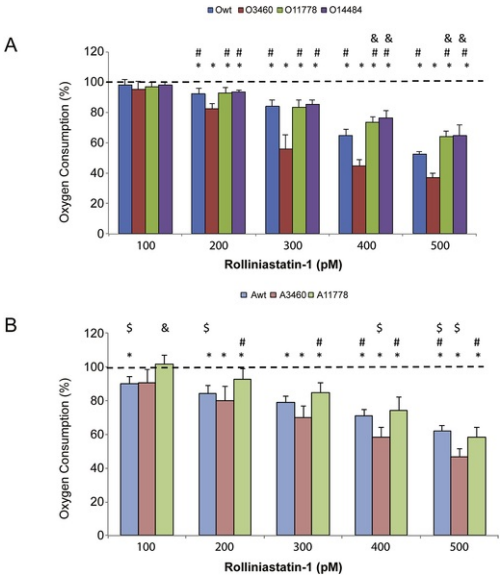

Fig. 5 Rolliniastatin-1 effect on endogenous oxygen consumption. A) Osteosarcoma 143B cybrids. B) Adenocarcinoma A549 cybrids. The value for each untreated cybrid is considered 100% (dashed line). * (vs untreated cybrid), $P \leq 0.0464$. # (vs O3460 or A3460 at the same rolliniastatin-1 concentration), $P \leq 0.0448$. & (vs Owt or Awt at the same rolliniastatin-1 concentration), $P \leq 0.0448$. $ (vs Owt or O3460 at the same rolliniastatin-1 concentration), $P \leq 0.0348$.

alt-text: Fig. 5

In adenocarcinoma A549 cybrids, rolliniastatin-1 ≥ 100 pM decreases oxygen consumption in Awt cybrids but ≥200 pM are required to diminish oxygen consumption in A3460 and A11778 cybrids (Fig. 5B). There are also significant negative correlations between rolliniastatin-1 concentrations and oxygen consumption (Table 2). The same pattern of rolliniastatin-1 sensitivity is repeated in these cybrids. At concentrations ≥200 pM, A3460 cybrid is more susceptible to rolliniastatin-1 than A11778 cybrid. At concentrations ≥400 pM, A3460 cybrid is more susceptible than Awt cybrid. At 100 pM, Awt cybrid is more susceptible than A11778 cybrid (Fig. 5B). A3460 cybrid is more resistant than O3460 cybrid to rolliniastatin-1400 and 500 pM concentrations. Awt cybrid is more resistant than Owt cybrid to rolliniastatin-1500 pM. However, it is more sensitive to rolliniastatin-1 ≤ 200 pM (Fig. 5B). These results suggest that other nDNA or mtDNA factors beside the LHON mutation are also involved in the rolliniastatin-1 susceptibility.

3.3 Apoptosis

The previous results suggested that m.3460G > A/p.MT-ND1:A52T mutation modifies CI interaction with these compounds, making these cybrids more resistant to rotenone, but more susceptible to capsaicin and rolliniastatin-1. To confirm these observations, we determined active caspase-3 levels in Owt and O3460 after exposure to rotenone (2.5 and 5 nM), capsaicin (15 and 30 μM) and rolliniastatin-1 (200 and 400 pM). A reduction on ATP levels and an increase in reactive oxygen species (ROS) production are potential factors to trigger apoptosis. Similar rotenone (1 nM), capsaicin (20 μM), and rolliniastatin-2 (2500 pM) concentrations had already shown to increase active caspase-3 levels in other cell lines, such as human cervical cancer HeLa, osteosarcoma U2OS and MG63, and epidermoid carcinoma KBv200 cell lines, respectively (Agarwal et al., 2016; Liang et al., 2009; Ying et al., 2013). Both rotenone concentrations and the rolliniastatin-1 highest one significantly increased active caspase-3 levels in Owt (Fig. 6). Both capsaicin and rolliniastatin-1 concentrations significantly increased active caspase-3 levels in O3460 cybrids. Interestingly, highest toxic concentrations (and the lowest rotenone concentration) differentially affected Owt and O3460. Similar to oxygen consumption results, Owt cybrids were more susceptible to rotenone than O3460 cybrids and O3460 cybrids were more susceptible to capsaicin and rolliniastatin-1 than Owt cybrids. To analyze a different nuclear genetic background, we also checked the rolliniastatin-1 effect on Awt and A3460 active caspase-3 levels. Both toxic concentrations increased these levels in A3460 cybrid and there was a difference between A3460 and Awt for the highest concentration (Fig. 6). The active caspase-3 level was higher in osteosarcoma cybrids exposed to 400 pM rolliniastatin-1 than in adecarcinoma ones. Therefore, they were more susceptible to this drug, confirming the result obtained for endogenous oxygen consumption.

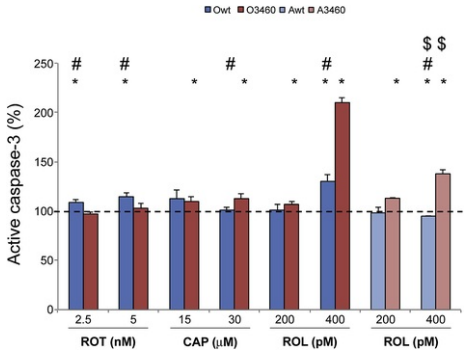

Fig. 6 Effect of CI inhibitors on active caspase-3 levels. The value for each untreated cybrid is considered 100% (dashed line). * (vs untreated cybrid), $P \leq 0.0394$. # (vs O3460 or A3460 at the same drug concentration), $P \leq 0.0241$. $ (vs Owt or O3460 at the same rolliniastatin-1 concentration), $P \leq 0.0006$.

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4 Discussion

The endogenous oxygen consumption in O3460, O11778 or O14484 cybrids was not different from Owt cybrids. Besides culture conditions and nDNA genetic background, all these cybrids also shared the same mtDNA haplogroup J. However this fact did not happen for A3460, A11778 and Awt. Despite they belong to different mtDNA haplogroups, they did not show differences in endogenous oxygen consumption. It was previously reported that the rates of glutamate-malate-, pyruvate-malate- or succinate-driven respiration were not lower in osteosarcoma 143B cybrids with LHON mutations (Baracca et al., 2005; Porcelli et al., 2009). Moreover, although endogenous oxygen consumption was significantly decreased in some osteosarcoma 143B cybrids carrying LHON mutations (Cruz-Bermudez et al., 2016; Floreani et al., 2005; Jiang et al., 2016b; Vergani et al., 1995), it was not found different in others (Cruz-Bermudez et al., 2016; Floreani et al., 2005; Hofhaus et al., 1996; Martinez-Romero et al., 2014). Similar variability was also previously reported for active caspase 3 levels. The Owt active caspase-3 amount was similar to that of O14484, but was higher and lower than that of O11778 and O3460 (and A3460), respectively. No cleavage of caspase 3 was found in wild-type or mutant (m.3460G > A, m.11778G > A or m.14484T > C) osteosarcoma 143B cybrids in another study (Zanna et al., 2003). Moreover, no differences in active caspase-3 levels were found between wild-type and m.11778G > A osteosarcoma 143B cybrids (Danielson et al., 2002).

Despite LHON individuals are usually homoplasmic mutants, only retinal ganglion cells, highly dependent on OXPHOS energy, are affected. The analysis of other unaffected cells in LHON individuals, such as blood cells or fibroblasts that are less dependent on mitochondrial energy, might be the reason why most of patients do not show respiratory chain defects. The reason for the absence of differences between LHON mutant and wild-type cybrids could be their generation from very glycolytic tumor cell lines and their lower dependence on OXPHOS energy in basal situations. However, stress conditions, increasing OXPHOS energy requirements, might push cells to a limit. In this sense, the optic nerve preceding the lamina cribrosa is characterized by an altered blood-brain barrier with non-specific vesicular permeability (Hofman et al., 2001). Maybe this feature makes optic nerve more susceptible to toxic damage from environmental chemicals. We have shown that low concentrations of natural CI inhibitors, present in fruits frequently used in human feeding, alter mitochondrial bioenergetics parameters, such as oxygen consumption, and apoptotic variables, such as active caspase-3 levels. As an OXPHOS dysfunction appears to be the etiologic factor for LHON, the exposure to these compounds would facilitate developing LHON. Of course, the involvement of these fruits as LHON triggers would require more investigation (see https://scienceofparkinsons.com/2017/12/16/paq/#more-48745).

The differential susceptibility to these compounds would help to explain the incomplete penetrance of LHON mutations. We have shown that A3460 and O3460 cybrids resist better rotenone toxic effects. The amino acid at p. MT-ND1 position 52 is part of the amphipathic helix 1 (AH1) that, along with p. MT-ND3, frames the entrance into the coenzyme Q binding (Q)-site (Baradaran et al., 2013; Hirst, 2013; Martinez-Romero et al., 2014), and rotenone is a Q-site inhibitor (Hirst and Roessler, 2016). This threonine might difficult rotenone access to CI Q-site. Interestingly, it has been recently showed that oxygen consumption of osteosarcoma 143B cybrids from mtDNA haplogroups J1 and K1 is more sensitive to rotenone 10 nM than that of cybrids from haplogroup H1 (Strobbe et al., 2018). These J1 and K1 cybrids share a m.10398A > G mutation, which cause a p. MT-ND3:T114A amino acid substitution. The J1 cybrid also harbors a m.4216T > C mutation, which cause a p. MT-ND1:Y304H amino acid change. This position is also close to p. MT-ND1 residues implicated in the Q-site (Strobbe et al., 2018). All our osteosarcoma 143B cybrids are from mtDNA haplogroup J and harbor the p. MT-ND1:304H amino acid and, similarly to the Strobbe's rotenone susceptible J1 and K1 cybrids, our more rotenone vulnerable cybrids (O11778 and O14484) are also p. MT-ND3:114A. This alanine might facilitate rotenone access to CI Q-site. However, it has been reported that cleaved caspase-3 levels are lower in 100 nM rotenone-treated osteosarcoma 143B cybrids from mtDNA haplogroup B5 than those from haplogroup B4. They are, apparently, more resistant to this high toxic concentration, but B5 cybrids harbor a p. MT-ND3:114A amino acid (Liou et al., 2016). Thus, considering that the same osteosarcoma 143B nDNA background has been used in these three publications, other factors must be also involved in rotenone susceptibility.

We have also observed that m.3460G > A cybrids are more susceptible to capsaicin and rolliniastatin-1. The bis-tetrahydrofuran motif of rolliniastatin-1 bind the third matrix-side loop connecting the fifth and sixth transmembrane helices in the p. MT-ND1 subunit (Kakutani et al., 2010; Murai et al., 2007, 2009; Nakanishi et al., 2011; Sekiguchi et al., 2009). This third matrix loop is at the same side of p. MT-ND1 position 52, in which alanine has changed by threonine in m.3460G > A mutants (Fig. 7). Maybe this new amino acid favors a tighter interaction with acetogenins and other compounds, such as capsaicin. The interface between the 49 kDa and p. MT-ND1 subunits forms the inhibitor/Q-site, although it is considered that different CI inhibitors act on the same Q-site with different but partially overlapping sites (Okun et al., 1999; Sinha et al., 2015).

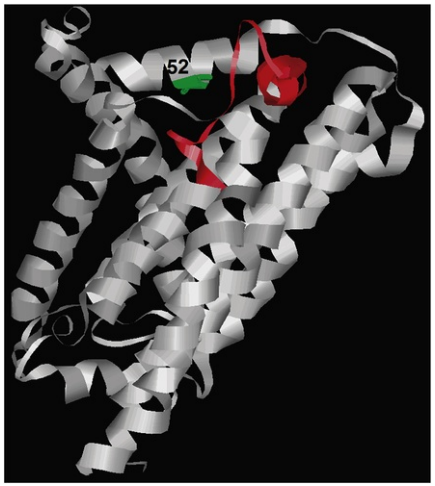

Fig. 7 Molecular model of the bovine p. MT-ND1 subunit, ortholog of human p. MT-ND1 subunit. The third matrix-side loop, which binds rolliniastatin-1 bis-tetrahydrofuran motif, is shown in red color. The alanine at position 52, which is mutated to threonine in m.3460G > A, is represented in green color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

alt-text: Fig. 7

On the other hand, m.11778G > A and m.14484T > C mutations cause p. MT-ND4:R340H and p. MT-ND6:M63V amino acid substitutions, respectively. These polypeptides are not part of the Q-site, and this would explain why toxic effects of these compounds are very similar in Owt, O11778 and O14484, and also in Awt and A11778, cybrids. Some small differences must be due to other population polymorphisms in p. MT-ND1 and 3 (Table 1C). Similar polymorphisms could explain why m.11778G > A osteosarcoma 143B cybrids were more sensitive to rotenone (IC_{50} 4.7 nM) compared to wild-type cybrids (Datta et al., 2016). In this sense, it was also found that a man harboring the m.11778G > A mutation developed LHON after his exposure to n-hexane. He had increased concentrations of urinary 2,5-hexanedione (2,5-HD), an n-hexane metabolite, in the period immediately preceding the visual loss (Carelli et al., 2007). Sensitivity to 2,5-HD induced cell death was greatly increased in osteosarcoma 143B cybrids carrying the m.11778G > A or the m.14484T > C mutations on haplogroup J, whereas the m.11778G > A mutation in association with haplogroups U and H significantly improved cell survival. Thus, mtDNA genetic background can modify the interaction with environmental factors (Ghelli et al., 2009).

Capsaicin is found in food. Foods can be a frequent source of compounds that differentially act as risk factors for LHON depending on the mtDNA genetic background. However, capsaicin is also a pharmacologic drug currently used to treat various diseases (Ying et al., 2013). Other pharmacologic compounds show similar behavior. For example, benzalkonium chloride is the most commonly used eye drop preservative, but it has been associated with toxic effects. Pharmacologically relevant concentrations of this compound inhibit CI activity and reduce oxygen consumption in osteosarcoma143B cybrids (Datta et al., 2017). Moreover, the m.11778G > A mutation conferred significantly increased sensitivity to benzalkonium chloride (Datta et al., 2017).

We have previously suggested that mtDNA genetic background in the cytochrome *b* gene might affect the interaction with pesticides or pharmaceutical drugs (Gomez-Duran et al., 2011). Moreover, we found that mtDNA genetic variation modifies the off-target effects of ribosomal antibiotics used to fight pathogenic bacteria (Pacheu-Grau et al., 2013), and that the widely distributed pollutant tributyltin chloride, an ATP synthase inhibitor, modified the phenotypic effects caused by a pathological mtDNA mutation (Lopez-Gallardo et al., 2016). Thus, considering the interaction between mtDNA and environment factors is going to be important to explain the pathogenicity of mitochondrial disease due to mtDNA mutations.

Conflicts of interest

The authors declare no conflict of interest.

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Multimedia Component 1

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Highlights

- Some mitochondrial DNA (mtDNA) mutations cause Leber hereditary optic neuropathy (LHON).
- Some oxidative phosphorylation system inhibitors cause mitochondrial optic neuropathies.
- Food derived xenobiotics modify the penetrance of LHON mtDNA mutations.
- The m.3460G > A mutation increases rotenone resistance but capsaicin and rolliniastatin-1 susceptibility.
- Gene-environment interactions must be considered to explain mitochondrial disease pathogenicity.

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