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Inhibition of phosphate transport in rat heart mitochondria by 3′-azido-3′-deoxythymidine due to stimulation of superoxide anion mitochondrial production

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Abstract

In order to gain some insight into the mechanism by which 3′-azido-3′-deoxythymidine (AZT) damages mitochondria, we investigated whether externally added AZT can stimulate reactive oxygen species (ROS) production by rat heart mitochondria (RHM). An increase in superoxide anion ($O_2^{\bullet -}$) production was measured in RHM added with AZT, by using a photometrically method which allows an early $O_2^{\bullet -}$ detection by following the absorbance increase at 550 nm due to the ferricytochrome *c* reduction. Such an increase was found to be prevented from externally added superoxide dismutase. The stimulation of $O_2^{\bullet -}$ mitochondrial production induced by AZT was found to occur under conditions in which mitochondrial oxygen consumption was prevented by both inhibitors of electron flow and ATP synthesis. Since ROS can cause mitochondrial carrier impairment, we investigated whether AZT can affect mitochondrial permeability in virtue of its capability to stimulate ROS production. In this regard, we studied the transport of phosphate (P_i), by measuring the mitochondrial shrinkage that takes place as a result of P_i uptake by RHM previously swollen in a calcium acetate medium. As a result of the AZT-dependent $O_2^{\bullet -}$ production, uncompetitive inhibition of the rate of P_i transport in RHM was found (K_i of about 10 μM), consistently, such an inhibition was found to prevent by certain known ROS scavengers, i.e. superoxide dismutase, the antioxidant Vitamin C and reduced gluthatione.

Keywords: 3′-Azido-3′-deoxythymidine; Rat heart mitochondria; AZT toxicity; Superoxide anion; P_i transport inhibition; Antioxidant vitamins

1. Introduction

AZT is one of the mostly used drugs, either alone or in combination with other nucleoside or protease inhibitors, in AIDS therapy [1,2]. Unfortunately, the clinical effectiveness of AZT is limited by its toxic side effects [3].

Mitochondria are cell targets of AZT: long-term as well as short-term treatment with AZT caused many dramatic structural and functional changes of mitochondria [4]. We have shown that adenylate kinase [5], ADP/ATP carrier [6], and nucleoside diphosphate kinase [7] are AZT targets in isolated RLM. On the other hand in rat liver mitochondria, the AZT capability to uncouple mitochondria was ruled out [8]. Finally, the existence of tissue-specific AZT effects have been also reported, with mitochondria isolated by heart and skeletal muscle more impaired than those from other tissues by AZT [9]; moreover, alterations of membrane potential caused by AZT in rat myotubes related to changes on the structural organization of the inner mitochondrial membrane has been recently shown [10].

Specific treatments with certain drugs used in different therapies were found to increase the cellular production of ROS, (see for instance [11]), particularly, ROS have been indicated as important factors in the development of myopathy and cardiomyopathy in AZT-treated rats [12]; consistently, AZT toxicity was prevented by supranutritional doses of antioxidant vitamins [13].

Mitochondria are the main cell sources of ROS [14,15]; as well as they can be targets of ROS generated in several

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Abbreviations: AZT, 3′-azido-3′-deoxythymidine; Fe^{2+}-*cyt c*, ferrocytochrome *c*; Fe^{3+}-*cyt c*, ferricytochrome *c*; GSH, reduced glutathione; $O_2^{\bullet -}$, superoxide anion; P_i, inorganic phosphate; RHM, rat heart mitochondria; ROS, reactive oxygen species; SOD, superoxide dismutase; XO, xanthine oxidase.

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cellular processes as the biochemical events leading to cell death [16,17].

In the light of this, we investigated whether externally added AZT can cause ROS production in isolated RHM. Moreover, since ROS can cause mitochondrial carrier impairment [18], a first investigation was also carried out to determine whether AZT-dependent ROS production can impair the mitochondrial permeability.

We show that the exposure of RHM to AZT causes extra-$O_2^{\bullet -}$ mitochondrial formation that can impair the P_i transport in RHM.

2. Materials and methods

2.1. Chemicals

All chemicals were from Sigma Chemicals Co. Mitochondrial substrates were used as Tris salts at pH 7.0–7.3.

2.2. Isolation of mitochondria

RHM were isolated essentially as described in [19] from male Wistar rats, (200–250 g) fed *ad libitum* and suspended in the standard medium containing 70 mM sucrose, 220 mM mannitol, 5 mM Hepes–Tris pH 7.25 and 0.1 mM EDTA in the presence of BSA (0.5 mg/mL). Mitochondrial protein was determined according to [20].

2.3. Detection of $O_2^{\bullet -}$ produced by RHM

In order to measure $O_2^{\bullet -}$ production in RHM, caused by AZT addition, the Fe^{3+}-*cyt c* reduction method was used, essentially as reported in [21], by monitoring photometrically the absorbance increase at 550 nm due to Fe^{3+}-*cyt c* to Fe^{2+}-*cyt c* conversion. RHM (0.6 mg protein) were incubated at 25° in 1 mL of standard medium with Fe^{3+}-*cyt c* (10 μM) either in the absence or presence of AZT (10–100 μM) which *per se* has no effect on the absorbance measurement. After 3 min of incubation, the amount of $O_2^{\bullet -}$ in each sample was calculated on the basis of the absorbance increase at 550 nm, due to stoichiometric formation of Fe^{2+}-*cyt c*, by using a Perkin-Elmer LAMBDA-5 spectrophotometer. Control was made that AZT does not cause $O_2^{\bullet -}$ formation in the absence of mitochondria and that the oxidation of the formed Fe^{2+}-*cyt c* via mitochondrial cytochrome *c* oxidase was negligible. The extinction coefficient at 550 nm, as determined in our experimental conditions, was 21.5 mM^{-1} cm^{-1}, in a fairly good agreement with [21,22].

2.4. Measurement of P_i transport in RHM

P_i uptake into RHM was measured essentially as in [23,24]. RHM (0.8 mg protein) were incubated at 25° in 2 mL of a medium consisting of 250 mM sucrose, 4 mM $MgCl_2$, 5 mM Tris–Acetate pH 6.5. Then, $CaCl_2$ (160 μM) was added to RHM causing mitochondrial swelling monitored as decrease of absorbance at 546 nm. After adding P_i we observed an increase in absorbance due to water efflux from the mitochondrial matrix caused by formation of insoluble calcium phosphate salts into the mitochondria (see Fig. 2A). Under these conditions, the rate of optical change was taken as a measurement of the P_i uptake into mitochondria via its own carrier [23,24]. The rate of P_i transport, determined as tangent to the initial part of the progress curve, was expressed as ΔA_{546}/min mg protein.

2.5. Computing and statistical analysis

The experimental data were plotted by means of Grafit (Erithacus Software). The standard deviations of data were determined from the mean of three experiments. Measurements made in triplicate with the same mitochondrial preparation gave a SD lower than 3%.

3. Results

3.1. Externally added AZT can stimulate $O_2^{\bullet -}$ production by RHM

In order to ascertain whether AZT can affect ROS production when added to isolated RHM, an experimental procedure was used which allows early $O_2^{\bullet -}$ detection by following the Fe^{3+}-*cyt c* reduction [21], (see Section 2). In the absence of AZT, $O_2^{\bullet -}$ amount in RHM was 0.3 ± 0.02 nmol/mg protein, as measured in three different experiments. As a result of AZT addition (100 μM), that *per se* does not produce ROS when added to the medium in the absence of mitochondria, an increase in $O_2^{\bullet -}$ production was found up to 2.2 ± 0.02 nmol/mg protein. The dependence of $O_2^{\bullet -}$ extra-amount production on AZT concentration was investigated either in the absence or presence of SOD (10 E.U.) (Fig. 1). Saturation-like characteristics were found with a half of the maximum amount of $O_2^{\bullet -}$ measured in the presence of about 20 μM AZT; externally added SOD completely prevented $O_2^{\bullet -}$ formation.

Since certain electron carriers of the mitochondrial respiratory chain can reduce molecular oxygen to $O_2^{\bullet -}$ [14,15], the AZT stimulation on mitochondrial $O_2^{\bullet -}$ production, was investigated by assaying $O_2^{\bullet -}$ formed by RHM in different respiratory states (Table 1). In the absence of exogenous respiratory substrates, $O_2^{\bullet -}$ production in RHM was stimulated by AZT (100 μM), whereas when RHM were incubated in the presence of either glutamate (5 mM) with malate (1 mM) or succinate (5 mM), no change in the mitochondrial $O_2^{\bullet -}$ production both in states 4 and 3 induced by either ADP with P_i or FCCP was found as a result of AZT addition. In agreement with [14], rotenone, which inhibits electron flow in the

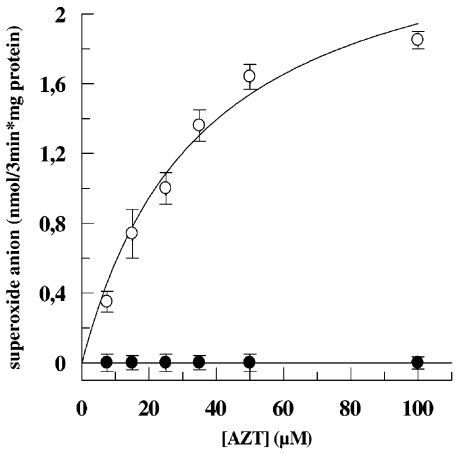

Fig. 1. Dependence of $O_2^{\bullet -}$ production by RHM on AZT concentration. RHM (0.6 mg protein) were incubated at 25° for 3 min, as reported in Section 2. The amount of $O_2^{\bullet -}$ formed was expressed as nmol/mg protein and plotted in function of AZT concentration (10–100 μM) either in the absence (○) or presence of SOD (10 E.U.) (●). Each value was corrected to account for spontaneous mitochondrial $O_2^{\bullet -}$ production. Standard deviations of data were determined from the mean of three experiments.

complex I and, more importantly, antimycin which blocks the complex III, were found to increase $O_2^{\bullet -}$ production. In these cases, as well as in the presence of oligomycin, the ATP synthase inhibitor, a further increase of $O_2^{\bullet -}$ production was caused by AZT.

3.2. Inhibition by AZT of P_i uptake into RHM due to $O_2^{\bullet -}$ production and prevention by ROS scavengers

Since ROS can cause mitochondrial carrier impairment [18], we investigated whether AZT can affect mitochondrial permeability in virtue of its capability to stimulate ROS production. In this regard, we studied the transport of P_i, whose uptake by mitochondria can be followed directly by measuring the mitochondrial shrinkage that takes place as a result of P_i uptake by RHM previously swollen in a calcium acetate medium [23]. It should be noted that under these conditions, i.e. in the absence of respiratory substrates, externally added AZT can increase ROS production by RHM (see Table 1). The addition of P_i (0.075 mM) to RHM caused the mitochondrial shrinkage at a rate equal to 1.3 ΔA_{546}/min mg protein with a complete inhibition due to mersalyl (20 nmol/mg protein), a potent inhibitor of P_i carrier [23]. Interestingly, 30% of inhibition on the rate of P_i uptake was found in the presence of AZT (20 μM) (Fig. 2A). In order to determine the nature of AZT inhibition on P_i transport, the rate of P_i transport was investigated as a function of the P_i concentration either in the absence (Fig. 2B, □) or in presence of 20 μM AZT (Fig. 2B, ■). AZT was found to inhibit P_i transport in RHM in an uncompetitive manner, with K_i value equal to about 10 μM (9 ± 2 μM in three experiments carried out with different mitochondrial preparations). When, in the same

Table 1
Effect of externally added AZT on $O_2^{\bullet -}$ production by RHM under different respiratory states

Respiratory chain substrates	Checked compounds	Addition	Superoxide anion (nmol/mg)
None	–	–	0.3 ± 0.01
None	–	AZT	**2.2** ± 0.02
Glutamate + malate	–	–	0.4 ± 0.01
Glutamate + malate	–	AZT	0.4 ± 0.01
Glutamate + malate	Rotenone	–	**0.6** ± 0.02
Glutamate + malate	Rotenone	AZT	**0.8** ± 0.02
Glutamate + malate	FCCP	–	0.3 ± 0.01
Glutamate + malate	FCCP	AZT	0.3 ± 0.02
ADP + P_i	–	–	0.3 ± 0.01
ADP + P_i	–	AZT	0.3 ± 0.02
Glutamate + Malate + ADP + P_i	–	–	0.3 ± 0.01
Glutamate + malate + ADP + P_i	–	AZT	0.3 ± 0.01
Glutamate + malate + ADP + P_i	Oligomycin	–	**0.7** ± 0.02
Glutamate + malate + ADP + P_i	Oligomycin	AZT	**1.0** ± 0.02
Succinate	–	–	0.4 ± 0.02
Succinate	–	AZT	0.4 ± 0.02
Succinate	Antimycin	–	**2.0** ± 0.01
Succinate	Antimycin	AZT	**2.3** ± 0.02
Succinate	FCCP	–	0.3 ± 0.02
Succinate	FCCP	AZT	0.3 ± 0.01
Succinate + ADP + P_i	–	–	0.3 ± 0.02
Succinate + ADP + P_i	–	AZT	0.3 ± 0.01
Succinate + ADP + P_i	Oligomycin	–	**1.9** ± 0.03
Succinate + ADP + P_i	Oligomycin	AZT	**2.2** ± 0.02

RHM (0.6 mg protein) were incubated at 25° under conditions described in Section 2 in order to measure Fe^{3+}-*cyt c* reduction due to $O_2^{\bullet -}$ formation. When present, the respiratory substrates were used at the following concentrations: 5 mM glutamate with 1 mM malate, 5 mM succinate in the presence of 2 μM rotenone, 0.5 mM ADP with 1 mM P_i. Where indicated, the oxidative phosphorylation inhibitors or uncoupler, were added to RHM at the following concentrations: 2 μM rotenone, 1.5 μM antimycin, 5 μM oligomycin and 1.25 μM FCCP. AZT concentration used was 100 μM. Reported data are the mean and the standard deviations of three experiments and are expressed in nmol $O_2^{\bullet -}$/mg protein.

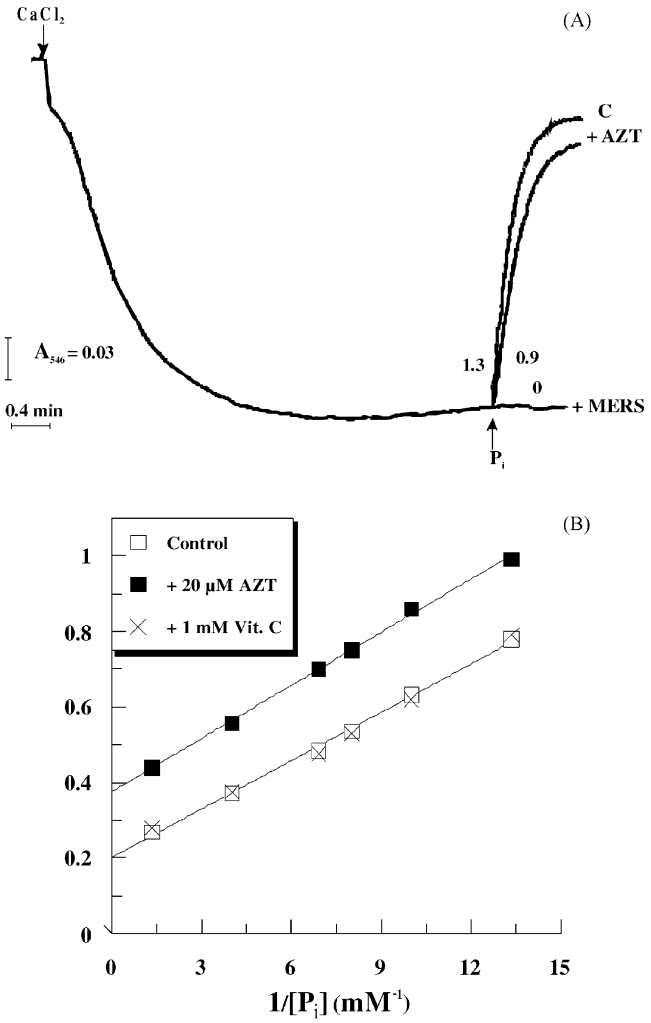

Fig. 2. (A) Inhibition by AZT on P_i uptake into RHM. RHM (0.7 mg protein) were incubated at 25° under conditions described in Section 2. At the arrow, $CaCl_2$ (160 μM) was added. Where indicated, AZT (20 μM) or mersalyl (MERS) (20 nmol/mg protein) were added to RHM before adding P_i (0.075 mM). Numbers along traces indicate the initial rate of P_i uptake (V_o) expressed as $\Delta A_{546\ nm}$/min mg mitochondrial protein. (B) Double reciprocal plot of the AZT inhibition on P_i transport in RHM and prevention of inhibition by Vitamin C. RHM (0.7 mg protein), were incubated under the conditions described in (A). Experimental data are reported as $1/V_o$ *against* $1/[P_i]$, determined either in the absence (□) or in presence of 20 μM AZT, alone (■) or with Vitamin C (1 mM), added 1 min before of AZT (×).

experiment, Vitamin C (1 mM), that *per se* has no effect on P_i uptake, was supplemented to RHM before adding AZT, a complete prevention of the AZT inhibition was observed (Fig 2B, ×).

In order to confirm that AZT inhibition was due to ROS production, the capability of certain ROS scavengers (including SOD (100 E.U.), 1 mM Vitamin C and 1 mM GSH), that *per se* do not affect P_i transport, to prevent AZT inhibition of the rate of P_i transport, was investigated (Table 2). The AZT inhibition proved to be essentially prevented from all the checked compounds. In the same experiment, xanthine (10 μM) with XO (10 E.U.), which

Table 2
Effect of certain ROS scavengers on the AZT inhibition of P_i transport in RHM

Addition	V_o (% of control)
None	100 ± 3
AZT	68 ± 3
SOD + AZT	97 ± 3
Vitamin C + AZT	96 ± 3
GSH + AZT	95 ± 4
Xanthine/XO	65 ± 3
SOD + xanthine/XO	98 ± 3
Vitamin C + xanthine/XO	95 ± 4
GSH + xanthine/XO	96 ± 2

RHM (0.7 mg protein) were incubated at 25° under conditions described in Section 2 to measure P_i (0.075 mM) transport. When present, the added compounds were used at the following concentrations: 20 μM AZT, 10 μM xanthine/XO (10 E.U.), 1 mM Vitamin C, 1 mM GSH, SOD (100 E.U.). When present, SOD, Vitamin C or GSH were added to RHM 1 min before adding AZT or xanthine/XO. The rate of P_i transport (V_o), measured as reported in Section 2, was expressed as percentage of the control. The standard deviations of data were determined from the mean of three different experiments.

are known to produce $O_2^{\bullet -}$ [23], were found to inhibit P_i transport in a manner prevented from the same compounds.

4. Discussion

We show that $O_2^{\bullet -}$ production in RHM increases as a result of AZT addition. Since AZT cannot produce ROS, both *per se* and when added to respiring mitochondria (Table 1), we conclude that ROS are generated by RHM and that AZT-dependent increase of ROS generation is due to AZT interaction with certain mitochondria component/s, perhaps the highly reactive electron carriers of the respiratory chain, including flavins, non-heme iron proteins, quinols and semiquinones, that can produce ROS [14,15]. Interestingly, we found that AZT effect depends on the mitochondria conditions: we show that AZT can stimulate $O_2^{\bullet -}$ mitochondrial production only in conditions of low respiratory rate, i.e. in the absence of exogenous substrates and in the presence of both substrates and the specific inhibitors of their oxidation via the respiratory chain. In these cases, both oxygen concentration and the levels of electron chain radical intermediates remain high, leading to reduction of the molecular oxygen to superoxide anion [14,15]. On the other hand, owing to respiratory substrate oxidation by RHM, both oxygen concentration and electron chain radical intermediates proved to decrease; this results in ROS production decrease with no AZT effect. Thus, we propose that AZT causes an additional damage to mitochondria in which the physiological electron flow could be somehow impaired. Consistently, short-term AZT damage on electron flow in rat skeletal muscle, but not in RLM was reported with impairment of respiratory chain capability in mitochondria isolated from AZT-treated rats as well as in mitochondria with externally

added AZT [25]. It is possible that our *in vitro* conditions could reproduce the AZT-dependent inhibition of the respiratory chain observed in muscle mitochondria [25]. In this regard, we have used AZT concentrations similar to those measured in human and animal fluids under AZT therapy (20 μM) [4].

Since the ubisemiquinone autoxidation at the outer side of the complex-III ubiquinone pool can give $O_2^{\bullet -}$ in the intermembrane space [26], the transport proteins are expected to be special ROS targets [18]. Thus, we used the P_i carrier as transport protein ''model'' to gain a first insight into the capability of AZT-dependent ROS production to impair the mitochondrial permeability. In this case, differently from [5,6], we studied P_i transport under conditions in which ROS production occurs, i.e. in the absence of succinate. Thus, AZT inhibition of P_i transport into RHM depends on $O_2^{\bullet -}$ production caused by AZT–RHM interaction, as shown by the prevention of AZT inhibition by different scavenger compounds (see Table 2 and Fig. 2, ×). The complete prevention of AZT inhibition by SOD on P_i transport excludes the possibility that H_2O_2, (formed in SOD reaction) and, consequently, hydroxyl radical, can impair P_i translocator, even though H_2O_2 removal by mitochondrial catalase cannot be ruled out [26].

The mechanism of the process leading to the uncompetitive inhibition must be a matter of further speculation: since Vitamin C does not affect the P_i uptake, we can ruled out its involvement in prevention of AZT binding to the carrier molecule, thus, we can assume that AZT inhibition is due only to $O_2^{\bullet -}$ formation that can affect the P_i carrier. Considering the observed uncompetitive inhibition, we conclude that ROS generating AZT–P_i carrier interaction takes place only when the substrate is bound to its own carrier. Such a conclusion is consistent with the fact that no significant change in the inhibition takes place when AZT incubation with RHM is allowed in a range of 1–3 min (data not shown). The AZT failure to inhibit the P_i translocator in rat liver mitochondria [6] shows mitochondrial-tissue specificity in AZT sensitivity.

It should be noted that we have already found AZT competitive inhibition of enzymes/carrier in RLM [4–7] and, more recently, in RHM [27]; in these cases, the structural analogy between substrate and AZT is consistent with the nature of the inhibition.

Since the P_i carrier plays an important role by filling up the intramitochondrial P_i pool allowing movement of several metabolites across the mitochondrial membrane [28] and affects the distribution of flux control among the enzymes of oxidative phosphorylation in rat skeletal muscle mitochondria [29], we suggest that the impairment of P_i carrier might play a major role in the process leading to the high sensitivity of heart mitochondria to AZT.

According to [13] and to above-mentioned results, use of antioxidants in AIDS therapy with both AZT and AZT containing drug cocktails could be strongly recommended.

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