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AZT CAUSES TISSUE-SPECIFIC INHIBITION OF MITOCHONDRIAL BIOENERGETIC FUNCTION

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The mitochondrial myopathy associated with long-term AZT therapy is a factor that limits the clinical efficacy of this compound in the treatment of AIDS. The biochemical basis for this tissue-specific pathology was investigated by measuring the effect of AZT on various aspects of bioenergetic function in mitochondria isolated from rat skeletal muscle, brain, and liver. AZT induced a dose-dependent inhibition of both NADH-linked respiration in intact mitochondria and NADH-cytochrome c reductase activity (but not succinate-cytochrome c reductase activity) in freeze-thawed mitochondrial preparations isolated from all three tissue types (½ maximal inhibition was obtained at 2 mg/ml and between 0.3 and 0.8 mg/ml AZT, respectively). These data demonstrate that high concentrations of AZT inhibit electron transfer through respiratory enzyme complex I. Moreover, AZT was shown to induce a tissue-specific inhibition of succinate-linked respiration in intact mitochondria isolated from rat skeletal muscle (½ maximal inhibition at 0.5 mg/ml AZT) and possibly brain, but not liver. The data suggest that this inhibition possibly occurs at the level of succinate transport. These results may help to explain the tissue-specific mitochondrial effects that are induced by long-term zidovudine treatment of AIDS patients and suggest that the anti-retroviral activity exhibited by AZT may be distinct from its mechanism of mitochondrial toxicity.

AZT (3'-azido-3'-deoxythymidine; zidovudine), a potent inhibitor of human immunodeficiency virus (HIV) replication, is the drug of choice in the treatment of acquired immunodeficiency syndrome (AIDS). Long-term treatment with this compound, however, has been shown to induce a muscle pathology characterized clinically by myalgia, muscle weakness and elevated serum creatine kinase levels, and morphologically by various mitochondrial abnormalities including enlarged size, abnormal cristae and proliferation of the organelle (1-4). In addition, long-term AZT treatment has been shown to induce abnormalities in mitochondrial biochemical function both *in vivo*, as determined by ^{31}P magnetic resonance spectroscopy in AZT-treated HIV-positive patients (5), and *in vitro*, in mitochondria isolated from skeletal muscle and brain (but not liver) of rats which had been treated with daily intraperitoneal injection of the compound (6). It has been hypothesized that the mitochondrial toxicity associated with long-term zidovudine therapy is a result of inhibition by AZT of γ-DNA polymerase (7,8,9), the matrix enzyme responsible for mtDNA synthesis. However, AZT may affect mitochondrial function at more than one level.

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The purpose of this study was to investigate possible additional mechanisms of AZT-induced mitochondrial toxicity. This was accomplished by measuring the effect of AZT on various aspects of energy metabolism in mitochondria isolated from rat skeletal muscle, brain, and liver. The results demonstrate that high concentrations of AZT inhibit mitochondrial bioenergetic function differently in different tissues and suggest a basis for explaining the selective mitochondrial toxicity observed after long-term zidovudine treatment. This information helps to provide a more thorough understanding of the cellular basis for AZT-induced myopathy, and other systemic toxic effects.

MATERIALS AND METHODS

Materials - AZT ((3'-azido-3'-deoxythymidine; AZT) was purchased from Sigma Chemical Co. (St. Louis, MO). A stock concentration of 50 mg/ml, prepared in water with moderate heating, was used in each of the assays.

Isolation of Mitochondria - Male CD-1 Sprague Dawley rats were used as the source of fresh tissue from which mitochondria were isolated. Skeletal muscle mitochondria were isolated according to a modification of the procedure of Max *et al.* (10). Six g skeletal muscle from a rat hind limb was dissected free of fat and connective tissue, and placed in 40 ml of ice-cold 250 mM sucrose containing 50,000 units heparin/liter (sucrose-heparin). The muscle was minced on ice and homogenized using a polytron. The resulting homogenate was diluted 1:3 in ice-cold sucrose-heparin, and centrifuged at 800 x g for 10 min at 4°C. The supernatants were decanted and centrifuged at 10,000 x g for 10 min at 4°C. The light fluffy layer surrounding each of the mitochondrial pellets was loosened by gentle swirling with a few drops of sucrose-heparin and aspirated; the pellets were combined and resuspended in 10 ml sucrose-heparin solution, and centrifuged at 10,000 x g for 10 min at 4°C. The resulting pellet was resuspended in the sucrose-heparin medium to a final concentration of 5-10 mg/ml. Liver mitochondria were isolated by differential centrifugation as described previously (11). Rat brain (cerebrum) mitochondria were isolated utilizing a discontinuous Ficoll density gradient essentially as described by Clark and Nicklaus (12).

Respiration - Mitochondrial respiration was measured polarographically using a Clark oxygen electrode inserted into a 1-ml water-jacketed chamber maintained at 30°C (13). The assay medium consisted of 225 mM sucrose, 10 mM potassium phosphate (KH_2PO_4/K_2HPO_4), 5 mM MgCl, 10 mM KCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4. An initial rate of oxygen consumption (state 2) was recorded following the addition of a substrate, either glutamate plus malate (5 mM each) or 10 mM succinate (+ 2 µg/ml rotenone), and a state 3 rate was recorded following the subsequent addition of 100 nmol of ADP. After a measurable state 4 rate (i.e., the rate after ADP is phosphorylated) was obtained, an 80 µM concentration of the uncoupling agent 2,4-dinitrophenol (DNP) was added to obtain a rate of oxygen consumption in the absence of coupled oxidative phosphorylation. When testing for the effect of AZT on respiratory rates, a specified concentration of the compound was introduced prior to the addition of substrate. AZT was thus present continuously in the assays as the initial, state 3, state 4, and uncoupled rates were successively determined.

Enzyme Assays - Rotenone-sensitive NADH cytochrome c reductase and succinate cytochrome c reductase activities were determined spectrophotometrically, as described previously (14). When AZT was tested for effect on these enzyme activities, it was added before the addition of cytochrome c.

Protein Determination - Mitochondrial protein concentrations were determined by the method of Lowry *et al.* (15) using bovine serum albumin as the standard.

RESULTS

Mitochondrial Respiration - The effect of AZT on respiratory activity was determined polarographically using functionally intact mitochondria isolated from rat skeletal muscle, brain and liver. When glutamate + malate was used as the respiratory substrate, the effect of AZT on mitochondrial respiration was similar in all three tissue types. As shown in Fig. 1A (muscle), 1B (brain), and 1C (liver), AZT inhibited both ADP and DNP-stimulated respiratory rates in a dose-dependent manner, with ½ maximal inhibition obtained at about 2 mg/ml. Since the inhibition of ADP-stimulated respiration was not relieved by addition of uncoupler, this rules out ATP-synthetase as the site of state 3 inhibition by AZT. Over the range of concentrations tested (0-5 mg/ml) AZT did not significantly effect either state 2 (data not shown) or state 4 rates of glutamate + malate oxidation in intact mitochondria isolated from rat skeletal muscle, brain or liver.

When succinate was used as the substrate, there were notable differences in the effect of AZT on respiratory function in mitochondria isolated from the three tissue types. In rat skeletal muscle mitochondria (Fig. 2), AZT induced a dose-dependent inhibition of both ADP and DNP-stimulated respiratory rates, with ½ maximal inhibition obtained at about 1 mg/ml. In mitochondria isolated from rat brain (Fig. 2B), there was no significant effect of AZT on succinate-linked state 3 respiration, however, DNP-stimulated respiration was inhibited by approximately 40% at the highest concentration of AZT tested (5 mg/ml). In rat liver mitochondria (Fig. 2C), AZT had no effect on either ADP or uncoupler-stimulated succinate oxidation, even at a concentration of the drug sufficient to induce maximal inhibition of respiration in mitochondria isolated from skeletal muscle or brain. These results demonstrate a tissue-specific sensitivity of succinate-linked respiration to inhibition by AZT in intact mitochondria. As was the case for glutamate + malate, 0-5 mg/ml AZT had no significant effect on either state 2 (data not shown), or state 4 rates of succinate oxidation in any of the three tissue types.

Enzyme Assays - NADH-cytochrome c reductase activity is a measure of electron transfer from NADH to mitochondrial respiratory enzyme complex I, through coenzyme Q, to complex III and finally to cytochrome c. This activity was inhibited by AZT in a dose-dependent manner in preparations of mitochondria isolated from all three tissue types (Figure 3). Fifty percent inhibition of activity was obtained within the range of 0.3 (in brain) to 1.0 mg/ml AZT (in muscle and liver), concentrations 2-6 fold less than that necessary to achieve comparable inhibition of state 3 and uncoupled respiratory rates in intact mitochondria. These data indicate

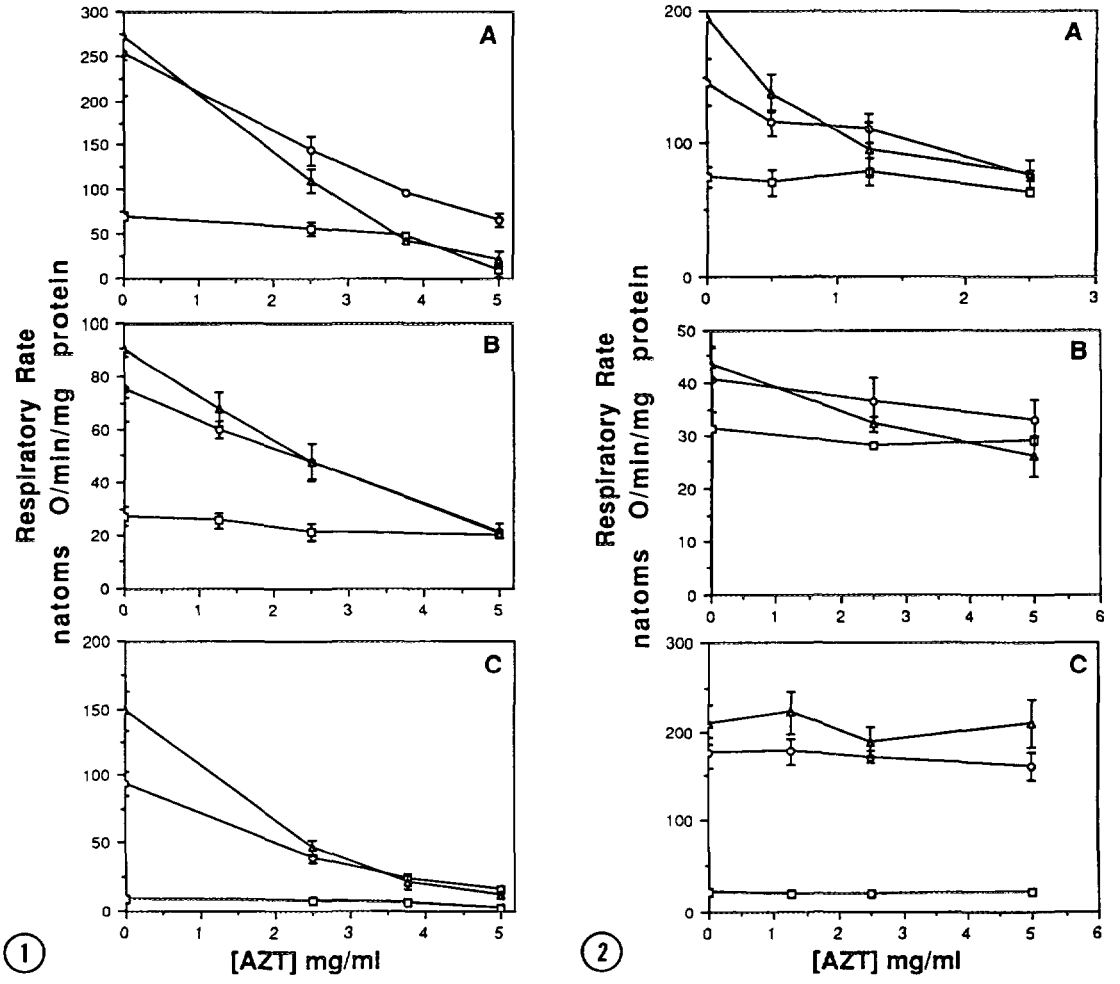

Figure 1. Effect of AZT on mitochondrial respiration using glutamate plus malate as the respiratory substrate. Mitochondria were isolated from the following rat tissues: (A) muscle, (B) brain, and (C) liver. State 3 (O) is the rate obtained upon addition of ADP; the state 4 rate (□) is obtained after conversion of all added ADP to ATP; and the uncoupled rate (Δ) is obtained after addition of 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. Values are the mean of 3 separate experiments ± S.E.

Figure 2. Effect of AZT on mitochondrial respiration using succinate as the respiratory substrate. Mitochondria were isolated from the following rat tissues: (A) muscle, (B) brain, and (C) liver. The rates obtained are indicated as follows: state 3 (O), state 4 (□), and uncoupled (Δ), as described for Figure 1. Values are the mean of 3 experiments ± S.E.

that the inhibition of NADH-linked respiration by AZT occurs at the level of electron transfer through one of the steps between NADH and cyt c.

Succinate-cytochrome c reductase activity is a measure of the rate of electron transfer from succinate to complex II, through coenzyme Q, to complex III and finally to cytochrome c. Table I shows that concentrations of up to 5 mg/ml AZT inhibited this activity by only 20% in

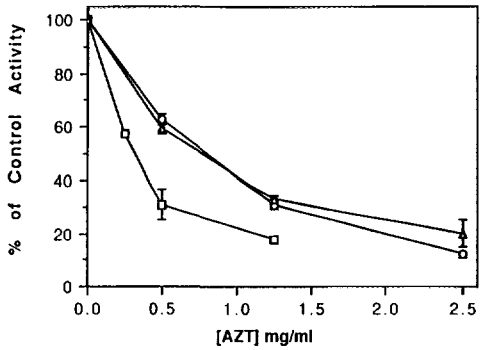

Figure 3. The effect of AZT on NADH-cytochrome c reductase activity in freeze-thawed mitochondria isolated from muscle (O), brain (□), and liver (Δ). Values printed as mean ± S.E. (n = 3).

all three of the tissue types tested. These results demonstrate that: 1) the inhibition by AZT of succinate-linked respiration in intact mitochondria isolated from rat skeletal muscle does not occur at the level of electron transfer; 2) succinate dehydrogenase (a component of respiratory enzyme complex II) is not significantly inhibited by AZT; and 3) the site of inhibition by AZT of NADH-linked electron transfer in rat skeletal muscle, brain, and liver mitochondria can be localized more precisely to respiratory enzyme complex I.

DISCUSSION

The data demonstrate that high concentrations of AZT have a direct and immediate effect on mitochondrial bioenergetic function. AZT was shown to induce a dose-dependent inhibition of NADH-linked respiration in mitochondria isolated from rat skeletal muscle, brain, and liver. The compound also inhibited NADH-linked but not succinate-linked electron transport enzyme activity in freeze-thawed preparations of mitochondria isolated from all 3 tissue types. These data indicate that AZT inhibits electron transport at a site unique to the path of electrons in NADH oxidation, i.e. respiratory enzyme complex I.

Table I: Effect of AZT on succinate-cytochrome c reductase activity in freeze-thawed preparations of mitochondria isolated from rat skeletal muscle, brain, and liver

	Succinate-Cytochrome c Reductase Activity (nmol/min/mg mitochondrial protein)*		
[AZT] mg/ml	**skeletal muscle**	**brain**	**liver**
0.0	356 ± 18	55 ± 5	67 ± 2
2.5	376 ± 44	50 ± 6	60 ± 4
5.0	294 ± 20	44 ± 4	53 ± 8

*Values presented as mean ± S.E. (n = 3).

AZT was also shown to induce a tissue-specific inhibition of succinate-linked respiration in mitochondria isolated from rat skeletal muscle but not liver. Since AZT had only a minimal effect on succinate-linked oxidation in freeze-thawed preparations of mitochondria isolated from either of these tissue types, this compound does not significantly inhibit succinate driven electron transport. Rather, the observed tissue-specific inhibition by AZT of succinate-linked respiration in intact mitochondria most likely occurs as a result of inhibition by AZT at some other site, perhaps at the level of succinate transport.

The effect of AZT on succinate-linked respiration in mitochondria isolated from rat brain was less clear cut. While there was no significant inhibition by AZT on the ADP-stimulated respiratory rate, some inhibition was observed for the uncoupler stimulated rate at high concentrations of AZT. However, brain mitochondria have historically exhibited low respiratory control when succinate is used as the respiratory substrate (6,12). The apparent inhibition of uncoupler stimulated (but not ADP stimulated) respiratory rates by AZT may be the consequence of an additive insult (arising from the combination of AZT plus DNP) to an already compromised electron transport system, rather than to a direct effect by AZT alone.

The clinical and morphological changes associated with zidovudine therapy have been well documented, however, much less has been reported regarding AZT-induced biochemical effects. In one recent study (5), ^{31}P magnetic resonance spectra from gastrocnemius muscles of AZT-treated, HIV-infected patients showed a delay in phosphocreatine recovery following exercise, reflecting impaired mitochondrial oxidative metabolism, when compared to untreated, uninfected controls. In another study (6), an assessment of biochemical functions in mitochondria isolated from tissues of AZT treated rats indicated a decrease in both NADH and succinate-linked respiratory activity in skeletal muscle, and an uncoupling of oxidative phosphorylation in brain. These latter results are in agreement with the data presented here, and suggest that the long-term *in vivo* administration of AZT may have additional effects, possibly on mitochondrial biogenesis and enzyme synthesis.

It has been hypothesized that the biochemical changes resulting from long-term zidovudine treatment are consistent with *in vitro* studies which show AZT inhibits γ-DNA polymerase, the matrix enzyme responsible for mitochondrial DNA synthesis (7,8,9). This study is the first to investigate the direct effect of AZT on mitochondrial biochemical function. The data show that AZT can have a significant and immediate effect on certain aspects of mitochondrial energy metabolism. The concentration of AZT necessary to obtain acute maximal inhibition of bioenergetic function is much greater than that necessary to obtain comparable inhibition of γ-DNA polymerase (7,9). However, covalent binding of the compound's chemically reactive azido group to biochemical target sites could produce a cumulative effect which, subsequent to long-term treatment with low doses, results in an observable inhibition of

bioenergetic function. Therefore, the impaired mitochondrial enzyme function resulting from long-term AZT treatment may have a primary cause, in addition to a secondary cause related to inhibition of mitochondrial DNA synthesis.

While the effect of AZT on NADH-linked respiration and γ-DNA polymerase is presumably identical in all mitochondria, the tissue-specific inhibition of succinate-linked respiration may prove to be the basis for the tissue-specific pathology resulting from long-term zidovudine therapy. AZT is not the first therapeutic agent shown to display tissue-specific mitochondrial toxicity. High concentrations of the synthetic glucocorticoid methylprednisolone have also been shown to induce a tissue-specific inhibition of succinate oxidation in isolated mitochondria in a manner quite similar to that of AZT (16). Interestingly, steroid-induced myopathy is a potential toxic side-effect in patients receiving high daily doses of these drugs (17).

The myopathy induced by long-term AZT therapy is a factor that significantly limits the clinical efficacy of AZT in the treatment of AIDS. The results obtained in this study provide a more thorough understanding of the cellular basis for AZT-induced myopathy, and other systemic toxic effects. The data presented here suggest that the antiretroviral activity (i.e. inhibition of DNA synthesis) exhibited by AZT may be distinct from its mechanism of mitochondrial toxicity. Studies are underway to more precisely define the structure/function relationship involved in the inhibition by AZT of both respiratory enzyme complex I and mitochondrial succinate-linked respiration. The information derived from this and future studies is fundamental to the rational search and/or design of more potent, less toxic antiretroviral agents and to the development of compounds that might counteract the toxic side effects of AZT.

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