Zidovudine-induced alterations in the heart and vascular smooth muscle of the rat

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Abstract

Objective: We investigated the effects of zidovudine (AZT) on cardiac and vascular smooth muscle function and morphology in rats.

Methods: Four adult male Wistar–Kyoto rats received AZT in drinking water for 240 days; four rats served as controls. Echocardiographic examination and systolic blood pressure (SBP) measurement were performed. At the end of treatment the rats were sacrificed, their hearts were weighed and vascular smooth muscle contractile and relaxing properties were evaluated in vitro on endothelium-intact aortic rings. Morphological studies were performed on cardiac and aortic myocytes by light and electron microscopy.

Results: AZT-treated rats (AZT-Rs) showed higher SBP, greater heart weight and, as revealed by echocardiography, greater interventricular septum thickness. Electron microscopy revealed mitochondrial swelling in myocardiocytes in AZT-Rs. Reduced response to contractile stimuli and enhanced relaxation in response to carbachol were observed in the aortic rings of AZT-Rs. The aortic myocytes of AZT-Rs contained apparently unaffected ultrastructural features, but light microscopy suggested their marked enlargement.

Conclusions: AZT treatment for 240 days in rats induces a modest increase in SBP, hypertrophy of the interventricular septum and modified vascular smooth muscle responsiveness. The role of mitochondria in these AZT-induced cardiovascular alterations remains to be established.

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Keywords: Blood pressure; Contractile function; Electron microscopy; Heart; Mitochondria; Smooth muscle; Zidovudine

1. Introduction

Zidovudine (AZT) (3'-azido-3'-deoxythymidine) is an antiretroviral drug largely employed in the treatment of human immunodeficiency virus (HIV) infection. AZT antiretroviral activity is due to the inhibition of the HIV-1 reverse transcriptase, which determines termination of the newly synthesized viral DNA chain [1]. Unfortunately, AZT therapy has been shown to cause several unwanted effects ascribed to mitochondrial damage mediated by an inhibitory effect on DNA polymerase-γ, the matrix enzyme involved in mitochondrial DNA synthesis, with consequent impairment of mitochondrial protein synthesis and alterations of the activity of mitochondrial enzymes, i.e., respiratory chain proteins [2,3]. In vivo and in vitro studies have demonstrated that muscles represent important targets for AZT-induced toxicity [4]. Alterations of either cardiac or skeletal muscle function have been reported in animals chronically treated with the drug [5–8]; these results are in agreement with clinical observations in AZT-treated patients, in whom skeletal muscle weakness and, less frequently, cardiomyopathy have been described [4,9,10]. In both animals and humans, muscle toxicity has paralleled

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ultrastructural mitochondrial damage, suggesting a role of mitochondria in the pathogenesis of skeletal and cardiac alterations [4–7].

Available data concerning AZT-induced toxicity on the cardiovascular system of animals are limited to the heart and refer to treatments lasting from 14 to 90 days [5–7]. Longer animal treatments, more closely representing the duration of AZT therapy in AIDS patients, could offer more reliable information on the features and the extent of AZT-induced cardiac toxicity. Moreover, since it has been shown that AZT slowly accumulates in smooth muscle [7], a longer treatment could allow assessment of possible AZT-related damage in vascular tissue. Accordingly, this study was undertaken to investigate in rats the effects of a 240-day AZT treatment protocol on cardiac and vascular functions, as evaluated in vivo and in vitro, and on the ultrastructural morphology of cardiac and vascular myocytes.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NHI publication No. 85-23, revised 1996).

2.1. General remarks

The experiments were carried out on eight adult Wistar–Kyoto (WKY) male rats (weighing 300–350 g at the beginning of treatment), maintained on 12 h/12 h light/dark cycle at 22 °C and fed with a standard laboratory diet from A. Rieper–Molini/Industria Mangimi (Vandoies/Bolzano). Throughout the 240 days, four animals took AZT in drinking water ad libitum; the other four animals were used as controls. AZT was dissolved in drinking water at a concentration (1 mg/ml) calculated on the basis of the daily water intake of a rat (20–45 ml), so that each rat was treated with approximately 100 mg/kg/day AZT, which closely corresponds to the intake reported in previous studies [6,11]. Prior to drug administration, every 30 days during the treatment period and at its conclusion, the rats were weighed, their systolic blood pressure (SBP) was measured, and echocardiographic evaluation was performed. At the end of AZT administration, the animals were sacrificed by cervical dislocation, and the heart and the thoracic aorta were excised for the ex vivo evaluations.

2.2. Measurement of systolic blood pressure

The SBP of conscious, warmed and unrestrained rats was measured by the tail-cuff plethysmographic method (Ugo Basile, Comerio, Italy) as previously described [12]. For each rat, five measurements were made at 10-min intervals, and the mean value of five measurements was calculated.

2.3. Echocardiographic evaluation

To perform echocardiographic studies, rats were anesthetized with an association of ketamine (80 mg/kg) and xylazine (20 mg/kg), and their chests were shaved. The examination was undertaken, with the animals positioned on their backs, using a Hewlett-Packard Sonos 5500 echocardiographer equipped with an S12 (5 to 12 MHz) probe. The following parameters were measured, according to the recommendations of the American Academy of Echography for Humans [13]: left ventricular end diastolic and end systolic dimensions; left ventricular posterior wall and interventricular septum thickness; end diastolic and end systolic dimensions (M-mode); ejection fraction and fractional shortening. Each measurement was performed three times, and the reported values are the average of the three measurements indexed for body weight (Table 2).

2.4. Evaluation of contractile function of vascular smooth muscle

Detection of the developed tension of aortic rings was performed as previously described [14]. Two-mm-long aortic rings were mounted between two metal wires, one of which was connected to an isometric force displacement transducer coupled to a chart recorder (Ugo Basile, Comerio, Italy) for contraction recording. Care was taken to avoid endothelial damage. The rings were dipped into physiological salt solution maintained at 37 °C and at pH 7.35 and bubbled with 95% O₂–5% CO₂. The physiological salt solution was of the following composition (in mM): NaCl 125, KCl 5, CaCl₂ 2.7, MgSO₄ 1, KH₂PO₄ 1.2, NaHCO₃ 25, and glucose 11. The rings were placed under 1.5 g of resting force and left to equilibrate for 45 min before starting the experiment. The aortic contractile function was evaluated by measuring the developed tension in response to either the receptor-coupled vasoconstrictor agent phenylephrine or to the increase in K⁺ concentration to 80 mM in the physiological salt solution. Endothelium-dependent relaxation of vascular rings was evaluated by exposing phenylephrine-contracted vessels to charbacol. In order to correctly compare the results between treated and untreated rats, care was taken to use the same aortic segment from each animal as previously described [15]; each aorta was cut into 2-mm-long rings under a dissection microscope.

2.5. Light and electron microscopy studies and morphometry

Sliced pieces of the left ventricle and the aortic wall were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1 M
phosphate buffer, and embedded in an epoxy resin. Thick (0.5 µm) and thin (60–70 nm) sections were cut by an LKB-III ultramicrotome. Thick sections were stained with toluidine blue, and examined by a camera-connected Leitz Laborlux microscope. Thin sections were counterstained with lead hydroxide and examined by an Hitachi H-300 electron microscope. For each rat, three tissue blocks of left ventricle and aortic wall were examined, and two series of thick and thin sections for each block were selected. Three light micrographs were recorded at a magnification of 300× for each series of thick sections of aortic wall. For each series of thin sections, six electron micrographs at a final magnification of 21,000× were recorded for stereological analysis. The numerical density of aortic myocytes (number/mm² of tissue section) was computed on the light micrographs. The volume of cardiomycyte mitochondria and the surface density of their cristae (µm²/µm² of mitochondria) were measured on the electron micrographs using the conventional stereologic techniques previously detailed [16].

2.6. Statistical analysis

Blood pressure, body weight, heart rate time–courses and echocardiographic parameters were compared by two-way repeated measure analysis of variance (ANOVA). Student’s t-tests was employed to compare heart weight, mitochondrial damage and aortic ring results. Significance was set at P<0.05.

2.7. Drugs and solutions

Commercially available AZT (Retrovir® syrup) was diluted in the drinking water to obtain the final AZT concentration (1 mg/ml). Phenylephrine and charbacol were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were dissolved in bidistilled water. High K⁺ solution used in ring experiments was obtained by adding KCl to the physiological salt solution.

3. Results

3.1. General

No differences were observed in the time–course of the body weight between control rats (CNT-Rs) and AZT-Rs (Fig. 1). The observation of AZT-Rs did not reveal any abnormality in behavior, motor activity and appearance.

3.2. Effects of AZT treatment on SBP

Fig. 2 shows the time–course of SBP in CNT-Rs and AZT-Rs. Starting from the third month, up to the end of AZT treatment, SBP in AZT-Rs was only modestly increased, but significantly higher compared to CNT-Rs.

3.3. Effects of AZT treatment on the heart

AZT treatment did not significantly affect heart rate (Fig. 3). Echocardiographic evaluation over the course of the 240 day protocol showed a decrease in the investigated indexed dimensional parameters in both AZT-Rs and CNT-Rs, with the exception of the interventricular septum thickness which was increased only in AZT-Rs (Fig. 4A–C and Table 1). Heart weight, normalized to body weight (BW), was also significantly greater in AZT-Rs compared to CNT-Rs (3.2±0.05 vs. 2.68±0.07 mg/g/BW, n=4, P<0.01) (Fig. 4D). Ultrastructural analysis of the hearts of AZT-Rs revealed mitochondrial enlargement and loss of matrix electron-density, without disruption of cristae architecture (Fig. 5). Stereology showed a 60% increase in mitochondrial volume, coupled with a 35% decrease in the surface density of their cristae. The cristae surface per
Table 1

<table>
<thead>
<tr>
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<th>CNT-Rs (n=4)</th>
<th>AZT-Rs (n=4)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>240 days</td>
</tr>
<tr>
<td>IVSTd</td>
<td>0.32±0.005</td>
<td>0.26±0.002</td>
</tr>
<tr>
<td>LVDD</td>
<td>1.87±0.09</td>
<td>1.36±0.1</td>
</tr>
<tr>
<td>PWTd</td>
<td>0.43±0.05</td>
<td>0.36±0.01</td>
</tr>
<tr>
<td>IVSts</td>
<td>0.55±0.07</td>
<td>0.55±0.01</td>
</tr>
<tr>
<td>LVDS</td>
<td>1.10±0.06</td>
<td>0.63±0.03</td>
</tr>
<tr>
<td>PWTs</td>
<td>0.67±0.05</td>
<td>0.58±0.04</td>
</tr>
<tr>
<td>FS (%)</td>
<td>41±2.0</td>
<td>42±3.1</td>
</tr>
<tr>
<td>EF (%)</td>
<td>77±3.0</td>
<td>81±3.4</td>
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In AZT group, the 240-day value of interventricular septum systolic thickness was significantly greater than the basal value. Values are indexed to the body weight. Data are means±S.E.M., n=4. IVSTd=Interventricular septum diastolic thickness; LVDD=left ventricular diastolic dimension; PWTd=posterior wall diastolic thickness; IVSts=Interventricular septum systolic thickness; LVDS=left ventricular systolic dimension; PWTs=posterior wall systolic thickness; FS=fractional shortening; EF=ejection fraction. * P<0.05 compared to basal AZT-Rs value.

Fig. 3. Time-course of heart rate in CNT-Rs and AZT-Rs. Basal heart rate values were 336±39 beats/min in CNT-Rs and 370±38 beats/min in AZT-Rs. At the end of the treatment, heart rate was 312±8 beats/min in CNT-Rs and 316±24 beats/min in AZT-Rs. Data were analysed by two-way repeated measures ANOVA. Each point represents mean±S.E.M., n=4.

Fig. 4. Short axis view of the left ventricle of a rat at baseline (panel A) and after 240 days of AZT treatment (panel B). The septal thickness increased in treated rats as visualised by the black arrows in the two-dimensional images and by the ratio between left ventricular cavity (LV) and walls (IVS and PW) in the M-mode tracing. The effect of AZT treatment on interventricular septum thickness of each rat is shown in panel C. Panel D shows the effect of AZT treatment on the heart weight. Data are means±S.E.M., n=4. * P<0.01.
Fig. 5. Electron micrographs of cardiomyocytes from the left ventricle of CNT-Rs (panel A) and AZT-Rs (panel B). M, Mitochondria; ER, endoplasmic reticulum; the arrows indicate an intercalated disk. ×15,000.
mitochondrion was unchanged (Table 2), thereby indicating the occurrence of mitochondrial swelling, which accords well with its light matrix.

### 3.4. Effects of AZT treatment on the aorta

KCl-induced contraction (80 mM) was blunted in AZT-Rs compared to CNT-Rs (1120±85 vs. 1435±165 mg, n=12, P<0.01) (Fig. 6A); similarly, the concentration–response curve to phenylephrine (0.01–10 μM) was down-shifted and the maximum contractile response was reduced by 73% (338±85 vs. 1236±170 mg, n=15, P<0.01) in the aortas of AZT-Rs compared to CNT-Rs (Fig. 6B). The EC50 values were 0.85 and 0.35 μM in CNT-Rs and AZT-Rs aortas, respectively. Carbachol-induced relaxation of aortic rings precontracted by 10 μM phenylephrine was significantly enhanced in AZT-Rs as compared to CNT-Rs (73 vs. 54%, n=12, P<0.01) (Fig. 6C).

Light microscopy evidenced a net decrease in the number of myocytes in the unit area of the aortic wall of AZT-Rs. Morphometry showed a 24% decrease in the numerical density of myocytes (control 2105±185 vs. AZT 1608±151; P<0.05), thereby suggesting an increase in their average volume (Fig. 7). Conversely, electron microscopy showed a reduction in the number of cristae mitochondriales per mitochondrion (73 vs. 54%, n=12, P<0.01) (Table 2).
evaluation. Indeed, as far as energy supply and hence contractile function are concerned, the increase in the number of mitochondria may compensate the functional alteration likely associated with their swelling.

The increased thickness of the interventricular septum in AZT-Rs indicates a hypertrophic response and may be an early manifestation of the generalized hypertrophy which characterizes many metabolic disorders involving alterations of myocardial energy metabolism [17]. This hypertrophic response, paralleled by an increase in heart weight, is in accordance with the previous report that describes the development of cardiac hypertrophy evaluated echocardiographically, accompanied by an increase in heart weight, in mice treated for 21 days with AZT [5]. However, the observed increase in SBP of this study might also be related to the development of cardiac hypertrophy in AZT-Rs. In fact, the AZT-induced increase in SBP represents a completely new finding since it has not been previously described. Our data in vitro also show for the first time that AZT treatment affects vascular smooth muscle function, inducing a marked reduction in the contractile responses to either high K+ or phenylephrine. The high K+-induced contraction is due to membrane depolarization and is completely dependent on Ca2+ entrance through voltage-operated calcium channels [18]. At variance, the agonist-induced contraction is due to the activation of a receptor, and the increase in cytoplasmic Ca2+ concentration triggering the response is consequent both to Ca2+ release from intracellular stores and Ca2+ entrance through a variety of Ca2+ channels [18]. The finding that AZT treatment blunts contractile responses of the aorta to either phenylephrine or KCl suggests an alteration in the ability of vascular myocytes to contract, rather than a defect in a specific receptor mechanism, since tracheal smooth muscle contractile responses were also blunted in AZT-Rs in the course of the present study (Bova et al., unpublished data). It remains to be ascertained whether the reduced aortic contractile responsiveness in AZT-Rs is related to the reduced number of myocytes. An additional, endothelium-dependent mechanism could be implicated in the decreased contractile responses to phenylephrine. It is well known that α-adrenergic receptors are also located on the endothelial cells, where they induce the release of the relaxing factor nitric oxide (NO) [19,20]. Therefore, the α-adrenergic-induced contractile responses of endothelium-intact vascular preparations are the net result of actions mediating two opposing effects in the vascular smooth muscle, i.e., contraction and relaxation. The finding that the phenylephrine-precontracted aortas of AZT-Rs more strongly respond to the muscarinic agonist carbachol with greater relaxation as compared to CNT-Rs, suggests an increased capability of the endothelium of AZT-Rs to produce NO. This could occur not only in response to muscarinic stimulation, but also to α-adrenergic activation, thus contributing to the decreased contractile response to phenylephrine. This hypothesis can be
Fig. 8. Electron micrographs of myocytes of the aortic wall of the CNT-Rs (panel A) and AZT-Rs (panel B). N, Nuclei; M, mitochondria; the arrows indicate dilated profiles of endoplasmic reticulum. ×16,000.
supported by the finding that increased expression of NO synthase has been demonstrated both in cardiac myocytes and in the endothelium of small vessels of endomyocardial biopsy specimens from AZT-treated HIV patients with cardiomyopathy [21]. However, it cannot be ruled out that the great reduction in the phenylephrine contractile response may independently influence, at least in part, the extent of the response to carbachol in AZT-Rs aortic rings.

The decreased contractile responsiveness of the AZT-Rs aortas seems in contrast with the increased blood pressure observed in these animals. However, a reduced responsiveness to vasoconstrictor agents has been reported in hypertensive rats [22–24], suggesting that a direct relation cannot be observed between blood pressure values and vascular responsiveness to vasoconstrictors in vitro. Furthermore, as far as endothelial function is concerned, considering that the increased SBP observed in the AZT-Rs of this study is modest, an analogy may be proposed with borderline hypertensive rats in which the relation between blood pressure and nitric oxide (NO) activity is inhibited competitively and non competitively by reactivity to endothelin: a comparison between young and old rats [9].

In conclusion, this study shows for the first time that a 240-day treatment with AZT raises SBP, reduces in vitro vascular responsiveness and induces a cardiac hypertrophic response confined to the interventricular septum. Moreover, our results confirm that AZT treatment causes ultrastructural alterations in rat cardiac mitochondria, although they are minor compared to those reported in both rats and mice following much shorter treatments. Finally, vascular smooth muscle dysfunction cannot be related to evident mitochondrial damage in vascular myocytes, but it may be, at least in part, a consequence of a decreased number of myocytes and of an altered endothelial function.

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References