Vitamin C prevents zidovudine-induced NAD(P)H oxidase activation and hypertension in the rat

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Abstract

Objective: Cardiovascular risk is increased among HIV-infected patients receiving antiretroviral therapy due to the development of hypertension and metabolic abnormalities. In this study, we investigated the effects of long-term treatment with zidovudine (AZT) and vitamin C, alone and in combination, on blood pressure and on the chain of events linking oxidative stress to cardiac damage in the rat.

Methods: Six adult Wistar Kyoto rats received AZT (1 mg/ml) in the drinking water for 8 months, six vitamin C (10 g/kg of food) and AZT, six vitamin C alone, and six served as controls.

Results: AZT increased systolic blood pressure, expression of gp91phox and p47phox subunits of NAD(P)H oxidase, and protein kinase C (PKC) δ activation and reduced antioxidant power of plasma and cardiac homogenates. AZT also caused morphological alterations in cardiac myocyte mitochondria, indicative of functional damage. All of these effects were prevented by vitamin C.

Conclusion: Chronic AZT administration increases blood pressure and promotes cardiovascular damage through a NAD(P)H oxidase-dependent mechanism that involves PKC δ. Vitamin C antagonizes these adverse effects of AZT in the cardiovascular system.

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Keywords: AZT; Electron microscopy; ESR; Heart; Mitochondria; NAD(P)H oxidase; Protein kinase C

1. Introduction

Zidovudine (3′-azido-3′-deoxythymidyne, AZT), is an antiviral drug largely used in the treatment of HIV infection [1]. It inhibits viral retrotranscriptase activity by blocking the extension of the viral DNA chain [2]. AZT has several adverse effects which can be attributed to mitochondrial damage and mediated by inhibition of DNA polymerase-γ, the matrix enzyme involved in the mitochondrial synthesis, thereby impairing activity of the respiratory chain proteins [3]. In vivo and in vitro studies have demonstrated that AZT-induced toxicity affects the cardiac and the skeletal muscle [4,5].

Ruga et al. showed that chronic AZT treatment increases systolic blood pressure (SBP) and induces hypertrophy of the interventricular septum in the rat [6]. It has been demonstrated that AZT increases reactive oxygen species (ROS) in the liver and in the cardiac and skeletal muscle [7–9]. Abundant evidence supports a role of superoxide anion (O2•−) in the pathogenesis of high blood pressure and target organ damage in animal and human hypertension [10,11]. The increased ROS generation is mainly derived from NAD(P)H oxidases
and it can be prevented by the antioxidants vitamins C (Vit C) and E [13,14]. NAD(P)H oxidase is a multifunctional enzyme formed by two membrane subunits p22phox and gp91phox and by four cytosolic subunits: p47phox, p40phox, p67phox and Rac-1. It is activated by the translocation of p47phox from the cytosol to the membrane, which is induced by protein kinase C (PKC) [15,16].

Therefore, aim of the present study was to investigate the effects of long-term administration of AZT and Vit C, alone and in combination, on blood pressure and on the chain of events linking oxidative stress to cardiac damage in the rat.

2. Methods

The investigation conforms with the Guide for the care and use of Laboratory animals, published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

2.1. Study protocol

Twenty four male normotensive Wistar Kyoto (WKY) rats (weighing 300–350 g at the beginning of treatment), obtained from Charles-River, were housed in temperature- and humidity controlled conditions, exposed to a 12-h light/dark cycle, and given tap water ad libitum and standard rat chow (Rieper-Molini/Industria Mangimi; Vandoies/Bolzano, Italy). Six rats were treated with AZT (100 mg/kg/day) dissolved in the drinking water [6], 6 with AZT and Vit C (ascorbic acid, 10 g/kg chow), and 6 with Vit C alone [9,17], each treatment lasting 8 months. Six untreated rats served as controls. During treatment, they were kept in the Animal Facility of the Department of Pharmacology and Anesthesiology, University of Padova.

2.2. In vivo studies

Before treatment, the animals were accurately checked to exclude abnormalities of behaviour and deambulation. Body weight, heart rate and systolic blood pressure (SBP) were measured before, during and after 8 month treatment. SBP was measured before (white columns) and after 8 months (black columns) by tail plethysmography. Abbreviations: AZT, zidovudine Vit C, vitamin C Results are mean±SE. *p<0.05 vs basal value by paired T-Test; § p<0.01 by ANOVA analysis.

2.3. In vitro studies

At the end of treatment, the rats were sacrificed by decapitation and the heart quickly removed for ex vivo assessment. The heart was rapidly perfused for 5 min through the aorta at constant flow (8–9 ml g⁻¹ tissue min⁻¹) by a peristaltic pump (Gilson MiniPlus 2 HP²TF, Villers le Bel, France) with a salt solution at pH 7.35 containing (mM) NaCl 125, KCl 5, CaCl2 2.7, MgSO4 2; KH2PO4 0.6, NaHCO3 25, glucose 11, kept at 37 °C, to remove blood from the coronary arteries and optimise heart weighing. The ventricles were then weighted for the determination of cardiac mass, calculated as heart (mg)/body weight (g) ratio (Table 1), and stored at −80 °C for further experiments.

2.4. Antioxidant capacity of plasma and heart homogenates

After decapitation, the trunk blood was collected with EDTA (1 mg/ml) and the plasma, separated by centrifugation, stored at −80 °C.

Fragments of the hearts were homogenized separately in 1 ml lysis buffer (20 mM HEPES, 2 mM EGTA, 10 mM β-glycerophosphate, 2 mM Na3VO4, 10 μM PMSF, 1 μM leupeptin, 5 μM aprotinin, 1 mM DTT), at 4 °C for 10 min with a Branson sonicator. The crude lysate was then sonicated with a Dounce sonicator, 3 times for 20 s at 4 °C and then centrifuged at 2000 rpm for 10 min at 4 °C.

The antioxidant capacity of plasma and heart homogenates was evaluated with Electron Spin Resonance (ESR) spectroscopy, by measuring the ESR intensity decay of the nitroxide probe 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl TEMPO (Aldrich), added to the plasma and heart homogenates (0.25 mM). The samples were mixed with TEMPOL in quartz capillaries and rapidly inserted into the cavity of a Bruker ER200 ESR spectrometer at room temperature. ESR spectra of the nitroxide probe were recorded at 1 min interval for the first 15 min with the following experimental parameters: magnetic

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Body and heart weights</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>Heart weight (mg)</td>
</tr>
<tr>
<td>Control</td>
<td>473±70</td>
</tr>
<tr>
<td>AZT</td>
<td>459±68</td>
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<tr>
<td>AZT + Vit C</td>
<td>437±61</td>
</tr>
<tr>
<td>Vit C</td>
<td>442±75</td>
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Abbreviations: AZT, zidovudine Vit C, vitamin C. Results are mean±SE of six experiments.
field sweep 60 G, microwave power 1 mW, modulation amplitude 1 G, scan time 40 s [18]. The initial ESR intensity decay rate is proportional to the antioxidant level. To convert the decay rate into an ascorbate equivalent concentration, we prepared a calibration curve with ascorbate-TEMPOL samples at known ascorbate concentration. The final ascorbate equivalent concentrations were normalized to the values of control rats, set as 100%, to give relative antioxidant levels [19].

2.5. Preparation of cytosolic and membrane fractions

The heart homogenates were centrifuged at 100,000 × g for 1 h at 4 °C (Beckman, model L5-50B, Fullerton, CA, USA). The supernatant represents the cytosolic fraction, while the pellet is the membrane fraction. The cytosolic fraction and the pellet, resuspended overnight on ice in the same lysis buffer with 1% Triton X-100, were stored at −20 °C. The protein concentration was determined with Lowry’s method, using bovine serum albumin as standard [20].

2.6. Expression of p22phox and gp91phox

Each membrane fraction (30 μg) was immunoblotted by Western blot with rabbit polyclonal antibodies for the detection of p22phox and gp91phox. It was solubilized in Laemmli buffer and separated by electrophoresis through a polyacrylamide gel (12% for p22phox and 8% for gp91phox). The proteins, separated on the gel, were electroblotted onto nitrocellulose membrane (Hybond ECL-Amersham Biosciences, Buckinghamshire, UK) in blotting buffer containing Tris 48 mmol/l, glycine 192 mmol/l, SDS 0.1%, methanol 20% (vol/vol) for 3 h at 100 V in the cold, using a Transblot cell (Elettrofor, Padova, Italy). The membranes were blocked overnight at 4 °C in T-PBS containing PBS, 0.05% (vol/vol) Tween, and 5% bovine serum albumin (BSA). The membranes were exposed to the primary antibody against p22phox (1:3000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and gp91phox (1:3000 dilution—Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. The membranes were washed (4 × for 5 min) with the same buffer and then incubated with horseradish peroxidase-conjugate secondary antibody (1:7000, Amersham Biosciences, Buckinghamshire, UK). Detection was made with Enhanced Chemiluminescence System (ECL) from Pierce (CEL BIO, Milan, Italy). Blots were analyzed by the Quantity One program of VersaDOC 1000 (Bio-Rad, Fig. 2. Effect of AZT and Vit C on the plasma antioxidant capacity. The plasma antioxidant capacity was measured with ESR spectroscopy. All values are normalized to the control samples (untreated rats) set to 100%. Abbreviations: AZT, zidovudine Vit C, vitamin C. The results are mean±SE of six experiments. § p<0.01.

Fig. 3. Effect of AZT and Vitamin C on gp91phox and p22phox expression in the rat heart. Membrane proteins were isolated from the rat hearts by lysis and ultracentrifugation. The proteins were loaded on SDS-PAGE acrylamide gels for Western Blot determination and ECL revelation of gp91phox and p22phox. The upper panels report typical immunoblots of gp91phox, gp22phox, and GAPDH expression. The lower panels show the densitometric analysis of gp91phox and p22phox expression, respectively, calculated by the gp91phox/GAPDH and p22phox/GAPDH ratio. Abbreviations: AZT, zidovudine Vit C, vitamin C. The results are mean±SE of six experiments. * p<0.05.
Milan, Italy). The results are adjusted to control(s), on the same blot, set at 100%, and by p22phox/GAPDH, gp91phox/GAPDH densitometric ratio. The quantification of the housekeeping GAPDH guarantees equal protein loading of the immunoblot.

2.7. Expression and activation of p47phox

p47phox expression was performed both in the cytosolic and the membrane fractions through Western Blot. The proteins were loaded in a 10% polyacrylamide gel according to the protocol reported above. The membranes were incubated for 5 h at 4 °C with the primary antibody against p47phox (1:3000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and later with the horseradish conjugated secondary anti-rabbit antibody (1:7000 dilution, Amersham Biosciences, Buckinghamshire, UK). The revelation was performed by ECL. The results are adjusted to control(s), set at 100% and expressed as the membrane/cytosol ratio.

2.8. PKC subunits (α, βII, δ, ε) expression and activation

PKC subunits expression was measured both in the cytosolic and the membrane fractions through Western Blot. The proteins were loaded into 10% polyacrylamide gel following the same protocol previously described. The membranes were exposed to the primary antibodies against PKC α, PKC β II, PKC δ, PKC ε (1:3000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and later to the secondary horseradish conjugated anti-mouse antibody (1:7000 dilution, Amersham Biosciences, Buckinghamshire, UK). The revelation was performed by ECL. The results are adjusted to control(s), set at 100% and expressed as the membrane/cytosol ratio.

2.9. Electron microscopy

Sliced pieces of the left ventricle 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. Thin (60–70 nm) sections were cut in a Supernova-LKB ultramicrotome, counterstained with lead hydroxide and examined by a Hitachi H 300 electron microscope.

2.10. Statistical analysis

The statistical analysis was performed with Student’s T for paired and unpaired data and by ANOVA analysis of variance followed by Duncan’s multiple Tests. A P value <0.05 was accepted as statistically significant. Data are presented as mean±standard error (SE).

3. Results

3.1. Effects of AZT on systolic blood pressure

As shown in Fig. 1, the chronic administration of AZT for 8 months significantly increased SBP in comparison to basal values, while there were no differences for the other three groups. Vit C inhibited the AZT induced SBP increase but, alone, it did not influence SBP.

3.2. Effect of AZT on antioxidant level in plasma

The antioxidant level in plasma was assessed by the measurement of the ascorbate-equivalent concentration with ESR spectroscopy. As shown in Fig. 2, a large decrease of the antioxidant level was found in AZT-treated rats compared to the control rats (to 47%±12%, p<0.01). The administration of Vit C almost completely restored the antioxidant level in AZT treated rats (to 88%±14%, p<0.01), while it was ineffective in untreated rats (100% ±18%). Similarly, AZT reduced the antioxidant capacity in heart homogenates (to 71%±19%, p<0.05), which was restored by Vit C. No effect was seen with Vit C alone.

3.3. Effects of AZT on NAD(P)H oxidase subunits expression in the heart

The main subunits of NAD(P)H oxidase expressed in the cardiac tissue are p22phox, gp91phox and p47phox. gp91phox is
responsible of the catalytic activity of the oxidase, while p47phox plays a key regulatory role in the oxidase activation following its translocation to the cell membrane, where it associates with gp91phox and p22phox [21, 22].

Fig. 3 shows representative immunoblots of the effects of AZT on gp91phox and p22phox expression in the rat heart. AZT markedly increased immunodetectable gp91phox expression (0.72 ± 0.05 vs 0.43 ± 0.04 arbitrary units, a.u.; \( p < 0.05 \)), which was attenuated by chronic Vit C administration (0.44 ± 0.1 a.u.; \( p < 0.05 \)). Vit C alone had no effect on gp91phox expression. No significant difference was observed in the expression of intracellular p22phox subunit of NAD(P)H oxidase.

Fig. 4 shows a typical immunoblot of p47phox expression in the cytosol and in the membrane fractions of the heart from the four different treatment groups and its densitometric analysis. p47phox expression was significantly increased in the membrane fraction in AZT-treated rats in comparison to the control group (0.71 ± 0.08 vs 0.32 ± 0.06 a.u. \( p < 0.01 \)) and this effect markedly reduced by Vit C (0.37 ± 0.07 a.u. \( p < 0.01 \)). Vit C alone had no effect on p47phox translocation.

3.4. Effect of AZT on PKC expression in the heart

Previous studies have reported that PKC isoforms play an important role in the regulation of the NAD(P)H subunit expression and, in particular, in the translocation of p47phox from the cytosol to the membrane [16, 23]. Therefore, we evaluated the expression of PKC α, PKC βII, PKC δ, PKC ε in the cytosol and in the membrane fraction of the heart. All were immunodetected with specific antibodies (data not shown). AZT increased the translocation of PKC δ from the

Fig. 6. Electron micrographs of longitudinally sectioned cardiomyocytes from the left ventricle of control (panel a), AZT treated (panel b) and AZT plus Vit C-treated rats (panel c). Cardiomyocytes from control rats displayed the classic myofilament organization and mitochondria were provided with abundant parallel laminar cristae embedded in an electron-dense matrix. AZT treatment caused marked mitochondria alterations, involving loss and disorganization of cristae and striking decrease in the matrix electron-density. The classic arrangement of the contractile materials was also far less evident. Vit C administration prevented all the morphological effects of AZT (×12,000).
cytosol to the membrane and this effect was blunted by Vitamin C (Fig. 5a), while no effect was seen for the other PKC isoforms. The densitometric analysis confirmed that the membrane/cytosol ratio of PKC δ was significantly greater and that Vit C prevented AZT induced PKC δ translocation (Fig. 5b).

3.5. Effect of AZT on cardiomyocytes

Ultrastructural analysis of cardiomyocytes of AZT-treated rats revealed marked mitochondrial alterations, indicative of impaired functions: cristae were less abundant and in some organelles their architecture was disrupted; mitochondrial matrix displayed an evident loss of electron-density. The classic arrangement of myofilaments was also far less evident. Vit C administration prevented all these AZT-induced morphological alterations (Fig. 6).

4. Discussion

The present study demonstrates in rats that long term AZT administration increases SBP and promotes oxidative stress and NAD(P)H oxidase activation, leading to mitochondrial damage in the heart. All these effects were prevented by Vit C, which could exert a protective role against the cardiovascular complications associated with AZT therapy.

Hypertension and its associated cardiovascular complications, such as cardiac hypertrophy and metabolic syndrome, are characterized by increased oxidative stress due to upregulation of ROS production [24,25]. ROS are generated in the vasculature mainly by NAD(P)H oxidase, through an Angiotensin II-dependent mechanism, and their effects are mediated by redox-sensitive targets, which play a central role in vascular function, vascular reactivity, hypertension, and cardiovascular remodeling [10,26,27].

ROS increase mainly when there is an imbalance between their occurrence and the antioxidant defense mechanisms [28]. The present study, showing a significantly reduced antioxidant capacity of plasma and cardiac homogenates, indicates that at least part of the cardiovascular effects of AZT are caused by increased oxidative stress.

The mitochondrial chain system regulates the balance of ROS production in the cell. It has been shown that AZT causes cardiac mitochondrial damage, which increases ROS generation, the primary cause of AZT-induced skeletal myopathy [9,29]. The present study extends those observations to the cardiac cells and suggests that AZT increases ROS generation also by NAD(P)H oxidase activation, which is the main system involved in oxidative stress production in the heart and in the vasculature [10,30]. In fact, we have shown increased expression of gp91phox and activation of p47phox, which translocates from the cytosol to the membrane, where it associates with gp91phox [31].

A variety of pathological stimuli, such as Angiotensin II, stretch, endothelin-1, thrombin, catecholamines, hypercholesterolemia, growth factors, insulin and serum acutely activate NAD(P)H oxidase in the vasculature [16,32,33]. We have not measured angiotensin II levels, but we did not find any change in p22phox expression after AZT chronic treatment, which rules out a major role of Angiotensin II in AZT induced hypertension and NAD(P)H activation. Interestingly, hydrogen peroxide and lipid peroxide stimulate NAD(P)H oxidases, leading to a feed-forward increase of ROS production [34,35]. The results of the present study suggest that ROS production promote p47phox and gp91phox activation through PKC δ translocation. In fact, AZT promoted PKC δ translocation, which is a novel PKC isoform, involved in accelerated neointima formation, resulting in complete occlusion of the vessel lumen in vein grafts [36]. Furthermore, PKC δ interferes with glucose metabolism by affecting energy reserves and promoting ROS production [37]. We, therefore, suggest that PKC δ mediates NAD(P)H oxidase activation and is involved in AZT-induced alteration of metabolic pathways and mitochondrial structure [16,38].

Vit C in AZT treated rats increased the antioxidant capacity of plasma and cardiac homogenates, prevented the increase of gp91phox expression, p47phox, and PKC δ activation, as well as mitochondrial damage and SBP rise. The main mechanism through which Vit C acts relies on its antioxidant properties. In SHR rats long term Vit C administration significantly reduced SBP and simultaneously reduced oxidative stress mediated by NAD(P)H oxidase activation [39,40]. Vit C has beneficial effects not only on blood pressure but also on endothelial function in hypertensive and diabetic patients [41,42]. Vit C is a soluble compound and it prevents protein and lipid oxidation in the extracellular environment [43]. In vivo studies confirmed that Vit C administration improves arterial vasodilatation by increasing NO production [44]. All these findings suggest, therefore, a feed-forward ROS production by NAD(P)H oxidase, as a key determinant of hypertension and cardiovascular damage in AZT treated rats.

5. Conclusions

Chronic AZT therapy induces cardiovascular system dysfunction by enhancing ROS production through PKC δ and NAD(P)H oxidase-dependent activation. These adverse effects may be reversed by chronic administration of the antioxidant Vit C. Its clinical efficacy against AZT induced cardiovascular alterations deserves further investigations.

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References