

Combined Antiretroviral Therapy Causes Cardiomyopathy and Elevates Plasma Lactate in Transgenic AIDS Mice

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SUMMARY: Highly active antiretroviral therapy (HAART) is implicated in cardiomyopathy (CM) and in elevated plasma lactate (LA) in AIDS through mechanisms of mitochondrial dysfunction. To determine mitochondrial events from HAART in vivo, 8-week-old hemizygous transgenic AIDS mice (NL4-3Δ *gag/pol*; TG) and wild-type FVB/n littermates were treated with the HAART combination of zidovudine, lamivudine, and indinavir or vehicle control for 10 days or 35 days. At termination of the experiments, mice underwent echocardiography, quantitation of abundance of molecular markers of CM (ventricular mRNA encoding atrial natriuretic factor [ANF] and sarcoplasmic calcium ATPase [SERCA2]), and determination of plasma LA. Myocardial histologic features were analyzed semiquantitatively and results were confirmed by transmission electron microscopy. After 35 days in the TG + HAART cohort, left ventricular mass increased 160% by echocardiography. Molecularly, ANF mRNA increased 250% and SERCA2 mRNA decreased 57%. Biochemically, LA was elevated (8.5 ± 2.0 mM). Pathologically, granular cytoplasmic changes were found in cardiac myocytes, indicating enlarged, damaged mitochondria. Findings were confirmed ultrastructurally. No changes were found in other cohorts. After 10 days, only ANF was elevated, and only in the TG + HAART cohort. Results show that cumulative HAART caused mitochondrial CM with elevated LA in AIDS transgenic mice. (*Lab Invest* 2001, 81:1527-1536).

Zidovudine (AZT; 3'-azido-2', 3'-deoxythymidine) was the first nucleoside reverse transcriptase inhibitor (NRTI) to treat HIV-1 infection and AIDS (Chaisson et al, 1986; Yarchoan et al, 1986). Monotherapy with AZT is uncommon in the United States today because highly active antiretroviral therapy (HAART) is a formidable clinical combination. HAART typically includes two NRTI plus an HIV-1 protease inhibitor (DHHS, 2001).

AZT caused a mitochondrial skeletal myopathy (Arnaudo et al, 1991; Dalakas et al, 1990). Characteristic microscopic "ragged red fibers" (Shoubridge, 1994) and ultrastructural paracrystalline inclusions occurred. A high lactate (LA)/pyruvate ratio (consistent with abnormal mitochondrial function) was seen in blood of patients with AZT mitochondrial myopathy (Chariot et al, 1994).

Analogous to AZT-induced mitochondrial skeletal myopathy in humans, cardiomyopathy (CM) and mitochondrial skeletal myopathy occur in AZT-treated rats (Corcuera Pindado et al, 1994; Lamperth et al, 1991; Lewis et al, 1991, 1992) and transgenic (TG) AIDS mice (NL4-3Δ *gag/pol*; Lewis et al, 2000). A relation-

ship exists between the development of AIDS CM in adults and prolonged treatment with AZT (Barbaro et al, 1998, 2000; Herskowitz et al, 1992). The mechanism of CM from AZT is not completely understood, but may relate to altered mtDNA replication, its resultant effects on energetics, and related cellular processes (Lewis, 2000).

Recently, lactic acidemia in AIDS gained attention as an important clinical problem found both in adults (Boubaker et al, 2000; Lonergan et al, 2000; Ter Hofstede et al, 2000) and in children (Church et al, 2000). Treatment with certain NRTI (stavudine, lamivudine) resulted in anion gap acidosis (Miller et al, 2000; Moore et al, 2000).

To determine the effects of HAART on myocardial and mitochondrial structure and function in AIDS, a 2 × 2 protocol employed 8-week-old TG AIDS mice (NL4-3Δ *gag/pol*) and wild-type (WT) FVB/n littermates with and without HAART treatment. HAART included zidovudine, lamivudine, and indinavir. Treatment duration was 10 days and 35 days, respectively.

Molecular markers of cardiac remodeling, echocardiographic evidence of left ventricular enlargement, microscopic and ultrastructural features of mitochondrial CM, and elevated plasma LA were found after 35 days, but only in the TG + HAART cohort. After 10 days, only atrial natriuretic factor (ANF) elevation occurred, and only in the TG + HAART cohort. Together, these data indicate that HAART causes cumulative

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mitochondrial CM and elevated LA in AIDS that follows duration of HAART treatment.

Results

General

TG and WT mice each consumed AZT and lamivudine (3TC) in drinking water. For 35-day protocols, cumulative consumption (TG and WT, respectively) was 141 ± 7 and 113 ± 5 mg for AZT; 7.2 ± 0.3 and 5.8 ± 0.3 mg for 3TC; and 32 ± 0.2 and 32 ± 0.2 mg for indinavir. For the 10-day protocols, consumption (TG and WT, respectively) was 50 ± 3 and 39 ± 1 mg for AZT; 2.6 ± 0.1 and 2.0 ± 0.1 mg for 3TC; and 9 ± 0.1 and 9 ± 0.1 mg for indinavir (Table 1).

Echocardiographic Findings

M-mode echocardiography was performed and evaluated on TG and WT mice with and without HAART. Left ventricular (LV) mass was calculated for both 35-day and 10-day treatment protocols.

Figure 1A is a collage of representative M-mode echocardiograms taken at midventricular level of hearts of WT + vehicle (upper left), WT + HAART (lower left), TG + vehicle (upper right), and TG + HAART (lower right) mice after the 35-day treatment. Significant LV thickening and dilation was found in the TG + HAART cohort.

LV mass was quantified after treatment and striking differences were found among the cohorts (Fig. 1B). As shown in the histogram, after the 35-day treatment, LV mass (normalized to body weight; mg/g) was 2.16 ± 0.22 in TG + HAART, 1.32 ± 0.07 in WT + HAART, 1.47 ± 0.15 in TG + vehicle, and 1.15 ± 0.07 in WT + vehicle cohorts. Normalized LV mass increased 160% above the WT + vehicle controls, and >130% in the TG + HAART cohort compared with either WT + HAART or TG + vehicle ($p < 0.001$ for each comparison; Fig. 1B). In contrast to the 35-day treatment protocols, after HAART treatment for 10 days, echocardiographic changes were absent in the cohorts (Table 2).

Molecular Studies From Hearts of 35-day and 10-day Treatment Protocols

Steady-state abundance of mRNA encoding ANF and sarcoplasmic calcium ATPase (SERCA2) were determined by Northern blot analysis of ventricular tissue.

Representative Northern blots (two samples from each cohort) from the 35-day protocol are presented as a collage in Figure 2A. No difference in ANF mRNA signal was found in cardiac ventricular samples obtained from WT + HAART or TG + vehicle when radioactive signals were compared with those obtained from samples from the WT + vehicle cohort. In contrast, a profound effect on abundance of ventricular ANF mRNA was seen in samples from the TG + HAART cohort (Fig. 2A). Quantitative analysis of cardiac ventricular ANF mRNA abundance revealed a 2.5-fold increase in the TG + HAART cohort compared with untreated controls ($p < 0.05$; Fig. 2B).

Reciprocal changes were observed in the abundance of SERCA2 mRNA. As seen in Figure 2, SERCA2 mRNA levels in TG + vehicle were $56 \pm 16\%$ of those values from WT + vehicle ($p < 0.05$; Fig. 2B). This effect of TG expression per se on ventricular SERCA2 abundance was reported previously (Lewis et al, 2000). HAART treatment further reduced SERCA2 mRNA abundance in the TG animals to $43 \pm 7\%$ of the level observed in untreated, WT animals.

In the HAART 10-day treatment experiment, similar alterations in ANF mRNA abundance (a sensitive index of cardiac molecular changes) were observed in the TG + HAART cohort; however, there were no significant differences in SERCA2 mRNA expression among the different cohorts (data not shown).

Plasma LA

Plasma LA was determined in all cohorts in both the 35-day and 10-day protocols. After 35 days (Fig. 3), samples from the TG + HAART cohort revealed strikingly elevated plasma LA (8.5 ± 2.0 mm) compared with values from WT + HAART (3.0 ± 0.4 mm), TG + vehicle (1.6 ± 0.5 mm), and WT + vehicle (2.7 ± 0.5 mm). The difference was significant for the TG + HAART cohort compared with the others ($p < 0.001$). No differences in mean plasma LA values were found in samples from the 10-day HAART protocol (Table 3). Mean plasma LA values ranged from 1.3 to 4.0 mm after the 10-day treatment.

Histopathologic Changes

Samples taken from the 35-day experiment revealed differences in the microscopic structure of the myocardium. Samples from the TG + HAART cohort at 35 days exhibited numerous cardiac myocytes with gran-

Table 1. Cumulative Consumption of Antiretrovirals

Agent	35 days		10 days	
	TG	WT	TG	WT
AZT	$141 \pm 7^*$	113 ± 5	$50 \pm 3^{**}$	39 ± 1
3TC	7.2 ± 0.3	5.8 ± 0.3	2.6 ± 0.1	2.0 ± 0.1
Indinavir	32 ± 0.2	32 ± 0.2	9 ± 0.1	9 ± 0.1

AZT, zidovudine; 3TC, lamivudine; TG, transgenic mice; WT, wild-type mice.

Data are mean \pm SEM (mg).

* $p < 0.001$ compared with WT at 35 days; ** $p < 0.05$ compared with WT at 10 days.

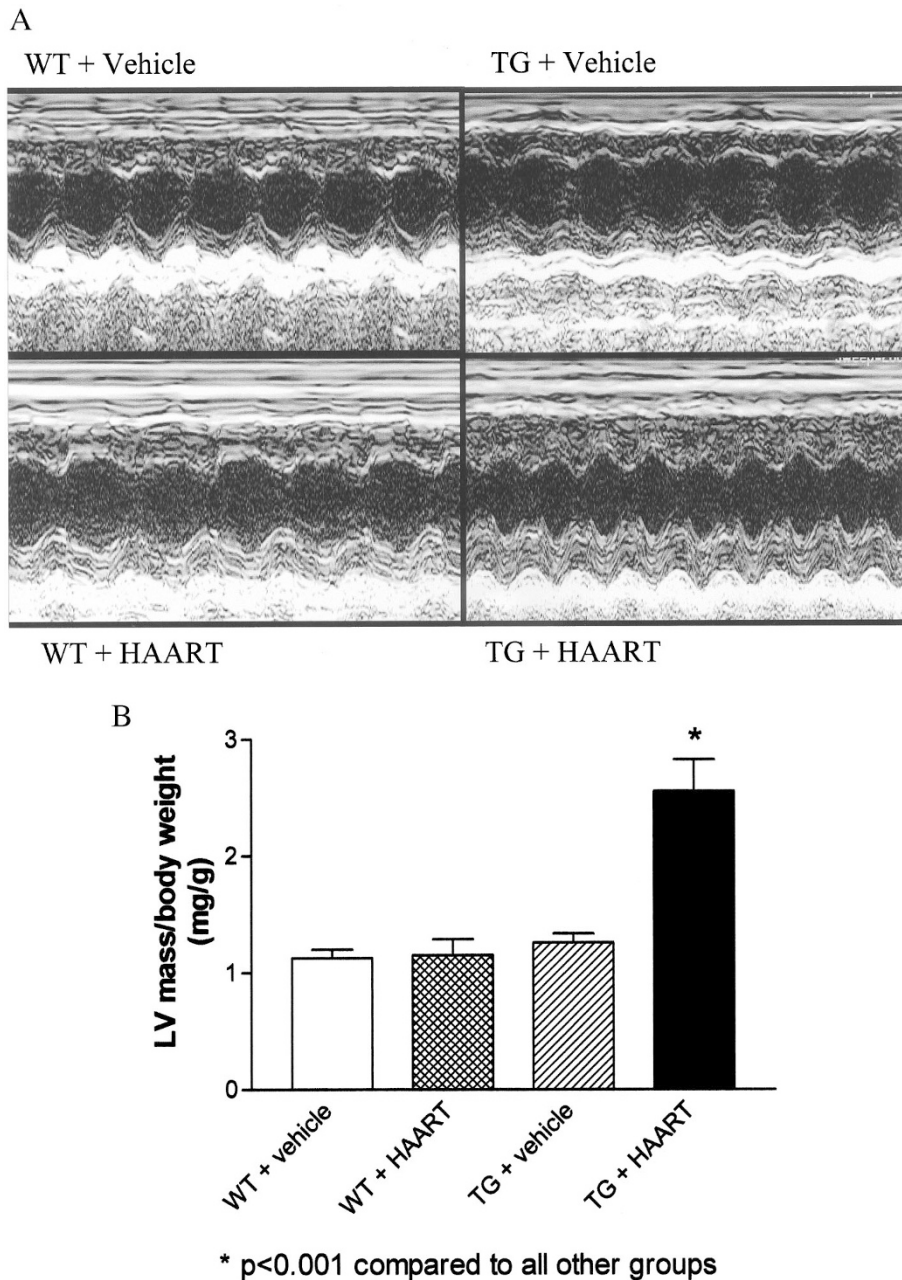


Figure 1.

Echocardiography of transgenic (TG) and wild-type (WT) mice with highly active antiretroviral therapy (HAART) for 35 days. A, Representative M-mode echocardiograms taken at the midventricular level of WT + vehicle (upper left), WT + HAART (lower left), TG + vehicle (upper right), TG + HAART (lower right). TG + HAART demonstrates ventricular thickening and dilation after 35 days compared with all other cohorts. B, Histogram of calculated left ventricular (LV) mass. LV mass was calculated in a blinded fashion, code was broken, and data tabulated. Data are normalized to body weight (mg/g) and plotted as mean \pm SEM. The TG + HAART cohort reveals profoundly increased LV mass after 35 days ($p < 0.001$).

ular cytoplasm (Fig. 4) compared with the samples from the other cohorts at 35 days. Neither interstitial inflammation nor fibrosis was found.

Semiquantitative analysis of cardiac myocyte granularity was performed on scored samples (Fig. 5). Cytoplasmic granularity was a reliable marker of cardiac pathologic change in the TG + HAART cohort ($p < 0.05$). This morphologic feature correlated with biochemical, molecular, and echocardiographic findings in the TG + HAART cohort treated for 35 days (above). Microscopic sections from the

10-day HAART treatment cohorts revealed no differences in granularity of cardiac myocytes (data not shown).

Transmission Electron Microscopy

Ultrastructural changes in cardiac myocytes were determined in the 35-day experiment. Ultrastructural findings paralleled and supported the histopathologic studies. Mitochondria were damaged in cardiac myocytes in the TG + HAART cohort. Pathologic features

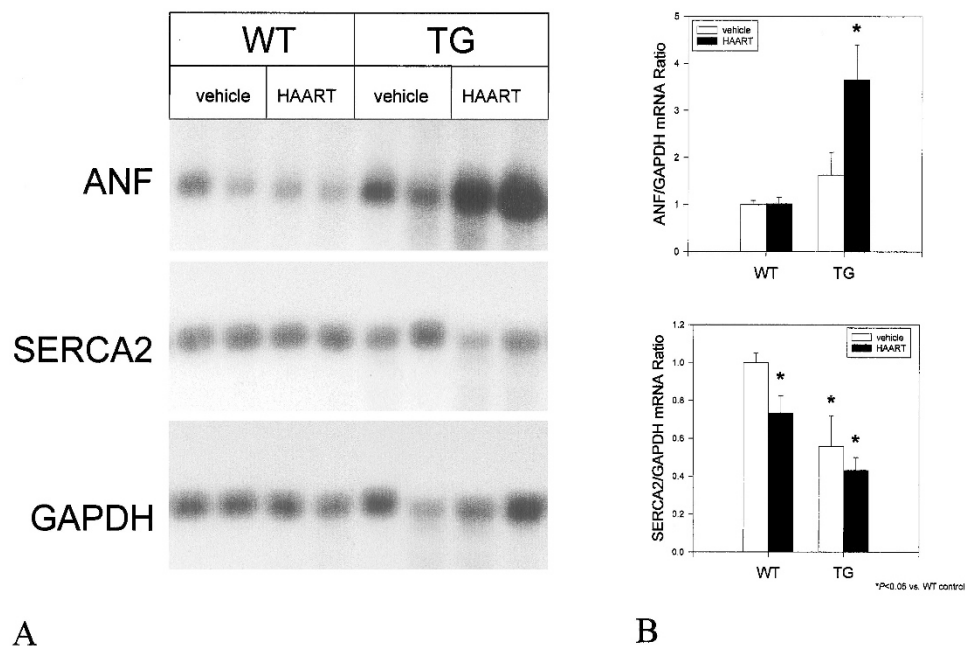
Table 2. Echocardiographic Measurements

Measurement	TG + HAART	WT + HAART	TG + Vehicle	WT + Vehicle
35-day HAART				
LVEDD	3.94 ± 0.14	3.64 ± 0.04	3.65 ± 0.13	3.4 ± 0.06
PWTH	0.35 ± 0.01	0.32 ± 0.01	0.31 ± 0.01	0.3 ± 0.004
R-R	161.8 ± 12.4	150.3 ± 6.1	156.4 ± 8.2	154.4 ± 7.7
LV Mass	41.8 ± 4.4	31.0 ± 1.2	31.0 ± 3.7	26.2 ± 1.1
LV Mass (mg/g)	2.16 ± 0.22*	1.32 ± 0.07	1.47 ± 0.15	1.15 ± 0.07
10-day HAART				
LVEDD	3.32 ± 0.19	3.51 ± 0.13	3.51 ± 0.15	3.32 ± 0.09
PWTH	0.37 ± 0.01	0.35 ± 0.01	0.36 ± 0.03	0.33 ± 0.01
R-R (MS)	156.2 ± 17.0	142.1 ± 7.0	245.7 ± 71.6	153.6 ± 7.1
LV Mass	32.0 ± 3.4	32.8 ± 2.8	35.1 ± 6.1	27.6 ± 1.5
LV Mass (mg/g)	1.44 ± 0.13	1.34 ± 0.12	1.5 ± 0.15	1.19 ± 0.06

HAART, highly active antiretroviral therapy; LVEDD, left ventricular end diastolic dimension; PWTH, posterior wall thickness; R-R, interval between two successive heartbeats (in milliseconds).

Data are mean ± SEM.

* $p < 0.001$ compared with all groups.

**Figure 2.**

Cardiac mRNA in TG and WT mice. Steady-state abundance of mRNA encoding atrial natriuretic factor (ANF), sarcoplasmic calcium ATPase (SERCA2), and glyceraldehyde phosphate dehydrogenase (GAPDH) (top to bottom) were determined in cardiac samples from mice in the 35-day HAART protocol. A, Representative Northern blots for WT + vehicle, WT + HAART, TG + vehicle, and TG + HAART cohorts are presented from two animals in each cohort. B, Histogram of data obtained from all animals in each cohort. Steady-state abundance of ANF RNA increased 250% in the TG + HAART cohort compared with WT or WT + HAART and compared with TG + vehicle ($p < 0.001$ for each) (B). After 35 days, SERCA2 mRNA abundance decreased significantly in the TG + HAART cohort compared with untreated WT mice ($43 \pm 7\%$ decrease) (B).

included mitochondrial swelling and fragmentation of cristae (Fig. 6).

Discussion

Cardiac mitochondrial dysfunction was manifested as molecular, structural, and functional changes of CM and elevated plasma LA in AIDS TG mice treated with HAART for 35 days. The regimen consisted of two NRTI plus indinavir. Echocardiographic, molecular, pathologic, and biochemical evidence support the diagnosis and indicate mitochondrial dysfunction plays a key role in CM development.

Data from these experiments allow two important conclusions to be drawn. First, compared with monotherapy with AZT, treatment with two NRTI plus a protease inhibitor resulted in worse cardiac mitochondrial toxicity that included CM with elevated LA. Second, HAART treatment of TG mice yielded profound LV changes after 35 days, but not after 10 days, when only ANF mRNA abundance was elevated. This suggested a cumulative effect of HAART on the development of mitochondrial dysfunction.

A pharmacologic relationship occurred between CM development, elevated plasma LA, and total HAART

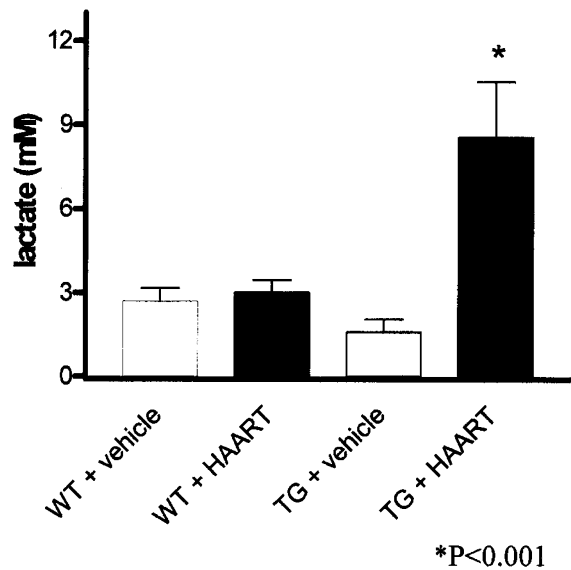


Figure 3.

Histogram of plasma lactate levels in TG and WT mice treated with HAART or vehicle for 35 days. Plasma lactate levels were determined colorimetrically in all cohorts. Samples from the TG + HAART cohort reveal elevated plasma lactate (mean = 8.5 ± 2.0 mM; $n = 8$) compared with values from the WT + HAART cohort (3.0 ± 0.4 mM; $n = 12$), the TG + vehicle cohort (1.6 ± 0.5 mM; $n = 4$), and the WT + vehicle cohort (2.7 ± 0.5 mM; $n = 11$). The difference is significant for each group compared with TG + HAART ($p < 0.001$).

consumption over 35 days. Of the parameters examined, only elevated cardiac ventricular ANF mRNA was found after 10 days, and only in the TG + HAART cohort. Based on current HAART regimens for adult humans (DHHS, 2001), NRTI doses for mice were somewhat higher (mg/kg/d). Indinavir doses resembled doses for humans.

Although it is possible to conclude that a relatively high AZT dose was employed experimentally in mice, doses are not always comparable between species. Similar (or even higher) AZT doses were employed in other monotherapy studies in vivo (Lamperth et al, 1991; Lewis et al, 1991, 1992, 2000; Ruprecht et al, 1986). CM was more severe in the present HAART experiments than reported previously (Lewis et al, 1991, 2000; McCurdy and Kennedy, 1998), in which higher AZT doses were used. Data suggest that development of mitochondrial CM depends on cumulative HAART.

HAART cardiac mitochondrial toxicity was documented echocardiographically. A significant increase in LV mass (+160%) was found in the TG + HAART cohort after 35 days. This point was corroborated by pathologic, biochemical, and molecular findings.

Molecular correlates of mitochondrial CM were striking in myocardial samples obtained from the TG + HAART cohort after 35 days. Molecular changes in the cardiac ventricle included increased steady-state abundance of mRNA encoding ANF and decreased mRNA encoding SERCA2, two important markers of CM in vivo (Hunter and Chien, 1999). After 10 days, only ANF mRNA abundance was elevated in the TG + HAART cohort. It is possible to suggest that increased

abundance of ANF mRNA in the TG + HAART cohort at 10 days may have served as a predictor of cardiac molecular changes of CM that were present at 35 days in the analogous cohort (with increased duration of HAART).

As seen in Figure 2, ANF mRNA levels tended to be higher in TG, vehicle-treated animals than in respective WT mice, whereas HAART led to a further, significant increase in the level of its expression. Up-regulation of ANF gene expression in ventricular myocytes was accompanied by a profound decrease in SERCA2 mRNA levels, which was further reduced by HAART treatment. In a previous study from our laboratories (Lewis et al, 2000), we showed that ANF mRNA levels were increased, and SERCA2 mRNA levels were decreased in TG male animals even in the absence of antiretroviral treatment. These reciprocal alterations in gene expression have been found to be sensitive indices of cardiac hypertrophy and failure in both experimental animals and humans with cardiomyopathies of diverse etiology. Indeed, these molecular changes were closely associated with a significant impairment in systolic and diastolic function in TG mice (Lewis et al, 2000). We suspect that these molecular alterations may be important in clinical practice because they may indicate that HIV predisposes affected individuals to further mitochondrial damage induced by retroviral therapy.

Pathologic findings and semiquantitative analyses supported the diagnosis of CM at the cellular level. Granularity of cardiac myocyte cytoplasm was found in the TG + HAART cohort after 35 days. Although not directly addressed in the present study, granularity of cardiac myocyte cytoplasm may correlate with altered mitochondrial biogenesis and CM (Lehman et al, 2000). Classic "ragged red fibers" are found in striated muscle samples from patients with certain mitochondrial encephalomyopathies and in AZT skeletal myopathy (Arnaudo et al, 1991; Dalakas et al, 1990). They correlate with ultrastructural features of mitochondrial damage and proliferation (Shoubbridge, 1994). In the present study, cardiac myocyte cytoplasmic granularity was a correlative feature of ultrastructural evidence of cardiac mitochondrial injury.

Ultrastructural findings bolstered the molecular, histopathologic, and echocardiographic data above. Mitochondrial ultrastructural damage was found in HAART-treated TG mice after 35 days. The damage resembled the damage with AZT monotherapy in the rat and TG mice (Lamperth et al, 1991; Lewis et al, 1991, 2000).

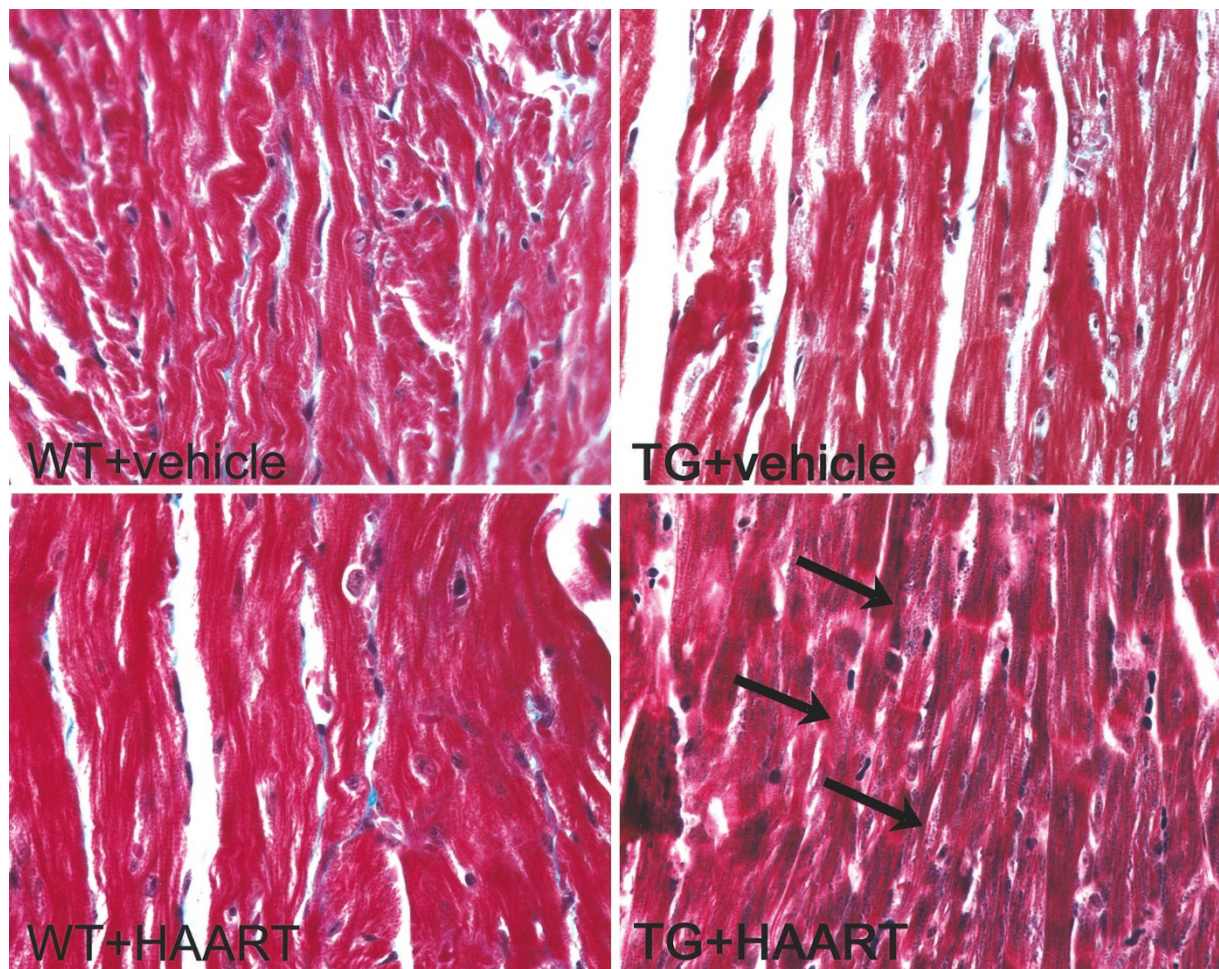
Elevated LA in plasma samples was a noteworthy biochemical derangement that accompanied CM in the TG + HAART cohort at 35 days. It is reasonable to suggest that elevated plasma LA relates to inadequate oxidative metabolism. Myocardium and other tissues may participate in the metabolic derangement. Elevated plasma LA (particularly in the presence of acidemia) is an ominous clinical event. Lactic acidosis per se has detrimental effects on cardiac performance (Adrogué and Madias, 1998). If it is reasonable to consider elevated plasma LA an indicator of worse prognosis, its presence

Table 3. Plasma Lactate with HAART

Protocol Duration	WT + Vehicle (n)	WT + HAART (n)	TG + Vehicle (n)	TG + HAART (n)
35 days	2.7 ± 0.5 (11)	3.0 ± 0.4 (12)	1.6 ± 0.6 (4)	8.5 ± 2.0* (8)
10 days	2.5 ± 0.3 (9)	1.3 ± 0.2 (9)	4.0 ± 0.8 (6)	4.0 ± 0.6 (9)

Data are mean ± SEM (nm).

* $p < 0.001$ compared to 35-day cohorts.

**Figure 4.**

Histopathologic changes and semiquantitative analysis of myocyte granularity in myocardial sections. Cross-sectional slices of myocardium from WT + vehicle (upper left), WT + HAART (lower left), TG + vehicle (upper right), and TG + HAART (lower right) were taken, processed, and examined histopathologically by a reviewer blinded to treatment or TG status. WT + vehicle (upper left) reveals characteristic features of cardiac myocytes. WT + HAART and TG + vehicle are essentially indistinguishable from WT + vehicle. In contrast, cardiac myocyte granularity is increased in TG + HAART (lower right). Original magnification, $\times 400$ each; Masson Trichrome.

in TG + HAART after 35 days supports the concept that the mitochondrial toxicity is cumulative and worsens after long-term HAART treatment (Dalakas et al, 1990; Lewis and Dalakas, 1995). This metabolic change reflects the threshold effect seen with deranged oxidative phosphorylation (OXPHOS) in many genetic mitochondrial diseases (Wallace, 1999) and may help to explain poor cardiac performance in TG + HAART after 35 days.

Support is growing for concepts that link altered mitochondrial DNA replication to mitochondrial toxic-

ity of NRTI (Brinkman et al, 1998, 1999; Morris and Carr, 1999; Swartz, 1995). Accordingly, elevated plasma LA, a general marker of altered OXPHOS, serves as a laboratory test for mitochondrial dysfunction in AIDS patients treated with HAART (Brinkman, 2000).

Recently, mitochondrial changes were found in hearts and skeletal muscles of *Erythrocebus patas* fetuses from dams that received relatively low-dose AZT (Gerschenson et al, 2000). In contrast, echocar-

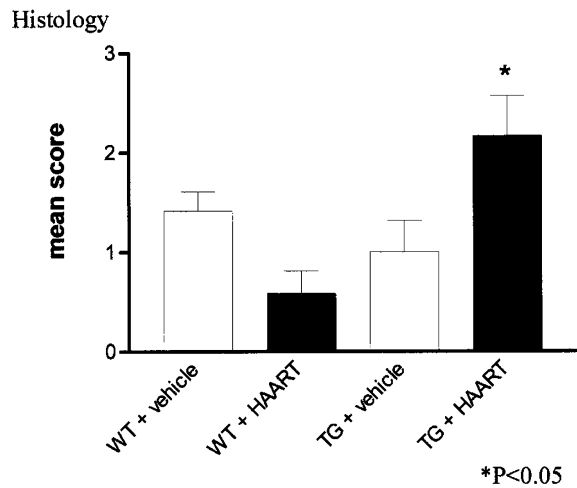


Figure 5.

Histogram of semiquantitative analysis of myocyte cytoplasmic granularity after 35-day treatment. Using a blinded review of slides from each of the cohorts, myocyte cytoplasmic granularity was analyzed. Myocyte cytoplasmic granularity is significantly higher in the TG + HAART cohort than in any other ($p < 0.05$).

diographic changes were essentially absent in another recent study of neonatal patients treated in utero with AZT (Lipshultz et al, 2000). It now appears axiomatic to consider AZT treatment of HIV-1-infected mothers as a potent therapeutic tool to prevent vertical HIV-1 transmission (Lewis, 2001; Mofenson, 2000).

In summary, HAART contributed to structural and functional changes of mitochondrial CM in AIDS TG mice after 35 days. The CM changes were worse than those seen with AZT monotherapy. CM was manifested by increased LV mass (echocardiographically), reversion of ventricular myocyte mRNA expression to a more embryonic phenotype (molecularly), mitochondrial damage (ultrastructurally), and elevated plasma LA (biochemically). After 10 days of HAART, only ANF mRNA was elevated in the TG + HAART cohort.

Taken together, these findings are concordant with the concept that mitochondrial toxicity occurs with HAART and is cumulative (Lewis and Dalakas, 1995). TG mouse models of AIDS coupled with antiretroviral treatment allow us to explore features of AIDS CM and help us to understand pathophysiologic mechanisms of mitochondrial cardiotoxicity from AIDS therapeutics.

Materials and Methods

Animals and Treatment

Hemizygous AIDS TG mice were generated from offspring of the colony established by Klotman and colleagues (Kopp et al, 1992). Tg26 is a well-described murine line of NL4-3Δ *gag/pol* bred on FVB/n background. The TG mouse develops nephropathy (Bruggeman et al, 1997; Kopp et al, 1992) and skin lesions in hemizygotes and severe wasting in the homozygotes (Kopp et al, 1993). For this study, a 2 × 2 protocol included hemizygous TG and WT mice

treated for 35 days and 10 days. Cohorts for 35 days included TG + HAART ($n = 10$), WT + HAART ($n = 12$), TG + vehicle ($n = 6$), and WT + vehicle ($n = 12$). Cohorts for the 10-day protocol included TG + HAART ($n = 9$), WT + HAART ($n = 10$), TG + vehicle ($n = 6$), and WT + vehicle ($n = 10$). All procedures complied with guidelines of and were approved by the Institutional Animal Care and Use Committee.

Each mouse received either drinking water alone or water containing AZT (Glaxo Wellcome, Research Triangle, North Carolina; 0.7 mg/ml) plus 3TC (from our Center for AIDS Research, Clinical Pharmacology Unit, Atlanta VAMC, Decatur, Georgia; 0.036 mg/ml) for consumption ad libitum. Volume consumed was determined for individually housed mice. Total and mg/kg/day consumption were calculated for each compound. Fresh water was replenished on alternate days. Indinavir (0.9 mg/day; courtesy of Merck Labs, West Point, Pennsylvania) was administered with vehicle (carboxymethyl cellulose 0.1% in water) by gavage using a curved 20-gauge stainless steel tube and syringe. 3TC and indinavir consumption were determined similarly. Control animals received vehicle without drug.

Echocardiography of TG and WT Mice with HAART

Echocardiographic studies were performed in age-matched (littermate) WT and TG mice that were treated with or without HAART (35-day or 10-day protocols) in the cohorts described above. Echocardiographic methods were detailed previously (Hoit et al, 1995, 1997; Kadambi et al, 1996; Lewis et al, 2000). Echocardiography was performed by an individual observer who was blinded to treatment or TG status.

Briefly, mice were anesthetized with Avertin (0.25 mg/g of body weight) and two-dimensionally targeted M-mode studies (Acuson Sequoia, Mountain View, California) were performed with a 13 MHz imaging and a 7 MHz Doppler transducer. M-mode measurements of end-diastolic dimension (EDD), end-systolic dimension (ESD), end-diastolic posterior wall thickness (EDPWT), and end-diastolic septal wall thickness (EDSWT) were made from original tracings. LV mass was calculated as follows: $1.06 \times [(EDD + EDPWT + EDSWT)^3 - EDD^3]$.

Cardiac mRNA Analysis

Total RNA was isolated from LV tissue samples from the 35-day and 10-day cohorts, respectively, using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987), as previously described (Lewis et al, 2000). RNA integrity was determined by electrophoresis and quantified spectrophotometrically as 260/280-nm absorbance ratio. Total RNA (10 μg) was size-fractionated electrophoretically and transferred to nylon membranes for Northern blotting.

Northern blots were sequentially probed with ³²P-labeled cDNA probes specific for the SR Ca ATPase (SERCA2, a 2.3-kb cDNA fragment of the rat cardiac

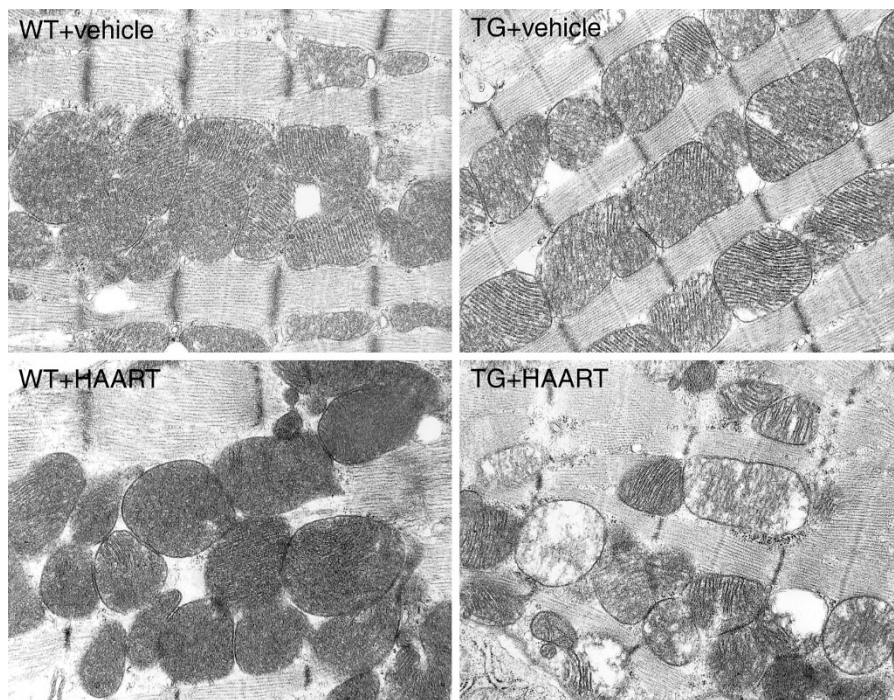


Figure 6.

Ultrastructural changes in cardiac myocytes. LV myocardial samples were processed from WT + vehicle (upper left), WT + HAART (lower left), TG + vehicle (upper right), and TG + HAART (lower right) in the 35-day protocol. In TG + HAART, significant mitochondrial structural damage is found. Changes include cristae dissolution and disruption. Magnification, $\times 14,000$ for each.

SERCA2, provided by Dr. W. Dillmann, University of California San Diego, and ANF (a 0.8-kb cDNA fragment of rat ANF courtesy of T. Inagama, Vanderbilt University, Nashville, Tennessee). Equal loading and uniformity of transfer were insured by normalizing hybridization signal intensity to that of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA (human GAPDH cDNA, obtained from the American Type Culture Collection, Manassas, Virginia). cDNA probes were radiolabeled and hybridized as previously described (Eleftheriades et al, 1993). The amount of each respective mRNA relative to the amount of GAPDH mRNA was quantified by solid-phase scintillation spectroscopy (Instant Imager; Packard Instruments, Meriden, Connecticut)

Plasma LA

At termination of the experiments, blood samples were obtained by retro-orbital bleeding of anesthetized TG and WT mice. Plasma was separated, stored briefly at 4° C, and aliquots (1–4 32 μ l) were analyzed for lactate colorimetrically using a kit (Sigma Scientific, St. Louis, Missouri). All assays were performed on a Versamax microtiter plate reader (Molecular Devices, Sunnyvale, California) and run in triplicate with external standards with each run.

Histologic Examination

At termination of the experiments, samples of the hearts were immediately immersion-fixed in neutral buffered formalin. For histopathologic evaluation, each heart slice

was dehydrated through a graded series of alcohols, and paraffin embedded (uniformly oriented apex down). Serial sections from the paraffin embedded tissue slice (6 μ m) were stained with Masson's trichrome. Each slide was examined microscopically by an observer who was blinded to treatment or TG status.

A semiquantitative scale was used for histopathologic comparisons within the experiments. Myocyte cytoplasmic granularity was an index of mitochondrial enlargement and damage in cardiac myocytes in methods analogous to those used in skeletal muscle samples from some mitochondrial diseases (Shoubridge, 1994). Granular and total myocytes were counted in each field examined. Granularity was measured using the following schema: zero granular myocytes per high-power field in ventricular sections was scored as grade zero; one to three granular myocytes per high-power field was scored as grade 1; four to six granular myocytes per high power field was scored as grade 2; seven to nine granular myocytes per high power field was scored as grade 3; more than 10 granular myocytes per high-power field was scored as grade 4. Averages from 10 high-power fields were taken from each slide. Numerical data obtained from this scoring system were statistically analyzed and plotted on a histogram. Photomicrography of slides was performed with a Nikon photomicroscope (Nikon, Garden City, New York).

Transmission Electron Microscopy (TEM)

A single 2 mm-thick slice was made at the ventricular apex. The LV was identified, immersed immediately in

a drop of chilled 2% glutaraldehyde-cacodylate, and sectioned longitudinally and transversely to generate 1 mm cubes that were further fixed for TEM in 2% glutaraldehyde-cacodylate for 16 to 24 hours at 4° C. Each heart sample provided approximately 20 blocks for embedding. Myocardium was rinsed in cold Ringer's solution and post-fixed in 1% OsO₄ (Sigma) in PBS, pH 7.4, for 2 to 3 hours. After osmication and rinses, tissue was dehydrated with graded ethanols, and embedded in Spurr (Spurr, 1969). Myocardial samples were sectioned (100 nm), stained with uranyl acetate, and examined on a JEOL-JEM-100CX electron microscope (JEOL, Tokyo, Japan). For each embedded sample of myocardium, 10 random sections were selected and photographed at a uniform magnification (14,000×). Photomicrographs were enlarged to 5×7-inch prints and reviewed by a single investigator for structurally abnormal mitochondria. Structurally abnormal mitochondria were operationally defined as those with loss or dissolution of more than 25% of cristae area (Lewis et al, 2000).

Statistics

ANOVA was employed to compare data from the different groups.

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