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HAART Drugs Induce Mitochondrial Damage and Intercellular Gaps and gp120 Causes Apoptosis

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Abstract

HIV-1 infection is associated with serious cardiovascular complications, but the roles of HIV-1, viral proteins, and highly active antiretroviral therapy (HAART) drugs are not understood. HAART decreases the overall risk of heart disease but leads to metabolic disturbances and possibly coronary artery disease. We investigated toxicities of HIV-1, HIV-1 glycoprotein 120 (gp120), and HAART drugs for human coronary artery endothelial cells (CAECs), brain microvascular endothelial cells, and neonatal rat ventricular myocytes (NRVMs). HIV-1 and gp120, but not azidothymidine (AZT), induced apoptosis of NRVMs and CAECs. Ethylisothiourea, an inhibitor of nitric oxide synthase, inhibited apoptosis induction by gp120. AZT, HIV-1, and gp120 all damaged mitochondria of cardiomyocytes. HAART drugs, AZT, and indinavir, but not HIV-1, produced intercellular gaps between confluent endothelial cells and decreased transendothelial electrical resistance. In conclusion, HIV-1 and gp120 induce toxicity through induction of cardiomyocyte and endothelial cell apoptosis. HAART drugs disrupt endothelial cell junctions and mitochondria and could cause vascular damage.

Key Words: HAART toxicities; azidothymidine; indinavir; gp120; coronary artery; endothelial cells; interendothelial junctions; mitochondria; cardiomyocyte apoptosis; nitric oxide.

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Introduction

HIV patients suffer from increased cardiovascular, pulmonary, and metabolic morbidities. A variety of potential etiologies have been postulated in HIV-1-related

cardiovascular disease, including HIV-1 itself, other viruses, and autoimmune response to viral infections; abuse of cocaine, tobacco, and alcohol; nutritional deficiencies; dyslipidemia; and, paradoxically, highly active antiretroviral therapy (HAART) *(1–3)*. Cardiovascular morbidities include HIV-1 cardiomyopathy (HIVCM), acute myocardial infarction, primary pulmonary hypertension, and the lipodystrophy syndrome. Thus, the cellular targets are immune cells *(4)*, cardiomyocytes *(5)*, endothelial cells *(6)*, hepatocytes, and adipocytes *(7)*. Molecular pathogenesis of AIDS is extremely complex because virions, viral proteins, and HAART drugs have distinct molecular and physiological effects, as shown with virions *(8)*, gp120 *(8,9)*, Tat *(10,11)*, Nef *(12, 13)*, and HAART drugs *(14)*. HIV-1 proteins gp120 *(8,15)*, Nef *(16)*, and Tat *(15,17)* are strongly considered to be pathogenic in the cardiovascular system. gp120 induces apoptosis of rat cardiomyocytes and is significantly associated with HIV cardiomyopathy *(8)*. HIV-1 uses apoptosis to benefit its replication with proapoptotic signaling in cytotoxic T cells and antiapoptotic signaling in target CD4 T cells, but cardiomyocyte apoptosis might be a bystander effect by death receptor ligands and viral proteins produced by infected macrophages.

Clinical studies of HAART drug toxicities in HIV-1 patients are difficult because of their conflicting antiviral and metabolic effects. The nucleoside reverse transcriptase inhibitor (NRTI) azidothymidine (AZT) was the first HAART drug suspected of causing cardiovascular complications because AZT inhibits cardiac mitochondrial DNA polymerase-γ *(18)*. The use of AZT has been related to myopathy, particularly ragged-red fiber myopathy, by typical biopsy findings *(19)*. However, HIV-1-positive or HIV-1-negative infants treated with AZT did not have an increased incidence of dilated cardiomyopathy compared with infected or uninfected infants not treated with AZT *(20)*. The introduction of HAART significantly improved the course and life expectation of HIV patients. Some retrospective studies suggested that HAART might reduce the incidence of HIV-1-related heart disease *(21)*. Since the introduction of HAART, the rate of admission of HIV-positive patients for cardiovascular disease in Veterans Affairs facilities has decreased and the use of HAART therapies has had a beneficial effect on admission and death from any cause *(21)*. However, HAART regimens, especially those including protease inhibitors (PIs), may lead to an increased incidence of metabolic and somatic changes *(22)*, which could offset the antiretroviral benefits of HAART. Recent prospective studies of HAART regimens including PIs suggest that an increased risk of coronary artery and peripheral vascular disease is detected with a longer follow-up *(23)*.

To compare cardiomyocyte and endothelial cell effects of gp120 and HAART drugs, we tested gp120 at concentrations approximating those in the serum of infected patients *(24)* and HAART drugs at serum levels achieved after oral administration. To clarify cell-specific toxicities, we investigated their effects on cellular junctions, mitochondria, and apoptosis. HAART drugs affected mitochondria and cell junctions, but their effects appeared more reversible than apoptotic effects produced by HIV-1 and gp120.

Materials and Methods

Chemicals

AZT (Retrovir, Glaxo-Wellcome), the non-NRTI (NNRTI) efavirenz (Sustiva, DuPont), and the HIV PI indinavir (Crixivan, Merck) were obtained as capsules from the hospital pharmacy. AZT and efavirenz were purified using a 1- × 1-cm stack of neutral alumina in a 1-cm diameter fritted funnel and eluting with 4:1 dichloromethane/methanol (50 mL per 500 mg powder). The filtrate was then concentrated to yield approximate recovery of 95% relative to the powder. Indinavir was isolated by pouring the contents of one capsule into saturated sodium bicarbonate (10 mL) and water (25 mL) and extracting with dichloromethane (5 × 10 mL). The dichloromethane layers were washed with brine (10 mL), dried over sodium sulfate, filtered, and concentrated to provide the pure material in approximate recovery of 80% relative to the powder. The structures of all compounds were checked by nuclear magnetic resonance spectroscopy at 500 MHz. The drugs were tested in vitro at concentrations achieved in serum of patients after oral administration. Deoxythymidine (Sigma) was used as a control for AZT. Ethylisothiourea, S-nitroso-*N*-acetylpenicillamine (SNAP), and diethylenetriamine/nitric oxide (DETA/NO) were obtained from Sigma.

HIV-1 and Recombinant gp120

HIV-1$_{MN}$ was obtained from the AIDS Research and Reference Reagent Program, NIAID. Recombinant soluble gp120 from macrophage-tropic strain JR-FL was produced in Chinese hamster ovary (CHO) cells essentially as described for simian immunodeficiency virus envelope *(25)*. HIV-1env was inserted into a mammalian expression vector and transfected into CHO cells. A cell clone expressing a high level of soluble gp120 was selected. Concentrated culture supernatants of this clone were purified on a lectin column, eluted with α-mannopyranoside, and then passed through an anti-α-macroglobulin column. A cell line expressing this protein is available through the AIDS Reference and Reagent Program (http://www.aidsreagent.org).

Cell Culture

Testing of gap formation, mitochondrial damage, and apoptosis was performed in cell cultures of human coronary artery endothelial cells (CAECs), brain microvascular endothelial cells (BMVECs) prepared on Lab-Tek chamber slides (Nalge-Nunc), and neonatal rat ventricular myocytes (NRVMs) on plastic coverslips (Nalge Nunc). Primary BMVECs were prepared and used as described *(26)*. Immortalized BMVECs (referred to as BMVEC-II) were obtained from K.-S. Kim, Johns Hopkins University. Both primary and immortalized BMVECs were propagated in DME/F12 with 10% fetal bovine serum, HEPES, endothelial cell growth supplement (Upstate), and penicillin/streptomycin/Fungizone® (BioWhittaker). Primary CAECs were purchased from Clonetics/BioWhittaker and cultured using EGM®-2-MV Bullet Kit medium as described *(27)*. NRVMs were prepared from neonatal rat heart tissues as described *(8)*.

Mitochondrial Transmembrane Potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ damage was detected by confocal microscopy after incubation of unfixed untreated or treated cells with $\Delta\Psi_m$-sensitive dye 5,5',6,6'-tetrachloro-1,1'3,3'tetraethylbenzimidazolecarbocyanine iodide (2 μ*M*;JC-1 Molecular Probes) for 30 min at 37°C and scanning at 525 nm (green) and 595 nm (red) channel to determine the red/green ratio.

Apoptosis

Apoptosis and $\Delta\Psi_m$ damage were induced by HIV-1 (7.6 ×10^7 virions), gp120 (100 ng/mL), or AZT (1 μg/mL) exposure for 48 h. Apoptosis of NRVMs and CAECs was detected 48-h posttreatment by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique (Apoptosis Detection Kit with dUTP, Promega) using the FITC-conjugated nucleotide mix and simultaneous staining with DAPI or Hoechst 33342 (Molecular Probes) as recommended by the manufacturer.

Cytochrome c Release

Cells were fixed with 4% paraformaldehyde and stained using monoclonal cytochrome *c* antibody (Pharmingen) as described *(8)*.

Phalloidin Fluorescence of Actin Cytoskeleton

To study gap formation, 10,000 endothelial cells were seeded onto 1% gelatin-coated 8- or 16-well chamber slides and grown several days past confluence. Following the treatment with each HAART drug, the cells were fixed with 1% paraformaldehyde in PBS for 20 min, washed in PBS for 5 min, and then made permeable with 0.3% Triton X-100 in PBS for 10 min. The cells were then stained for 1 h at room temperature with Texas Red-phalloidin (Molecular Probes) or polyclonal rabbit anti-β-catenin (Sigma) and were examined using a Bio-Rad MRC1024ES confocal system equipped with a Nikon E800 microscope and a krypton-argon laser or using a BMax Olympus fluorescence microscope.

Transendothelial Electrical Resistance (TEER)

CAECs were seeded onto evaporated gold microelectrodes in the 8W10E array comprising eight separate wells on a glass slide with 10 electrodes per well connected to an electrical cell-substrate impedance sensing system (Applied Biophysics). The cells were grown in a humidified CO$_2$ incubator at 37°C in EGM -2-MV Bullet Kit medium (300 μL per well) in order to develop a confluent monolayer covering the microelectrodes *(28)*; resistance was measured daily until approx 1500-Γ resistance was reached. The array was then placed in the electric cell-substrate impedance sensor (ECIS) apparatus and resistance was recorded for 30–60 min, the array quickly

removed from the apparatus (which caused the transitory drop in resistance seen as a V-shaped notch), the drug (3 µL of 100 × final concentration) added, and the resistance measured continuously for at least 15 h. Time-dependent average TEER values from each well (measured in ohms) were normalized as the ratio of measured resistance to baseline resistance.

Statistical Analysis

To acquire the data for analysis, we scanned three random high-power fields for the color of interest using IMAGE-PRO PLUS 4.1 (Media Cybernetics) and analyzed them by ANOVA using the statistical software SPSS, Version 10.0.

Results

HIV-1 and gp120 are potent inducers of cardiomyocyte apoptosis in vivo and in vitro; gp120 expression in heart tissue is associated with HIVCM and apoptosis *(8)*. To determine the preponderance of cardiovascular toxicities, we compared the proapoptotic potential of AZT to HIV-1 and gp120.

HIV-1 and gp120 Induce Apoptosis of Endothelial Cells and Cardiomyocytes but AZT Does Not

HIV-1 induces cardiomyocyte apoptosis by release of tumor necrosis factor (TNF) family ligands from inflammatory cells and by direct effects of the virus and gp120 through the mitochondrial pathway *(8)*. We treated CAECs or NRVMs for 48 h with cell culture medium with 10% fetal calf serum, gp120 (100 ng/mL), HIV-1 (7.6×10^7 virions), or AZT (1 µg/mL) and after 48-h incubation determined TUNEL-positive apoptotic cells. HIV-1 or rgp120 induced apoptosis of both endothelial cells (Fig. 1A) and

cardiomyocytes (Fig. 1B), but AZT alone did not induce apoptosis and did not have an additive effect in combination with gp120. Apoptosis induction by gp120 was inhibited by ethylisothiourea, an inhibitor of NO synthase, and NO donors SNAP and DETA/NO induced cardiomyocyte apoptosis (Fig. 2).

AZT, Efavirenz, HIV-1, and gp120 All Damage Cardiomyocyte Mitochondria

We examined mitochondrial damage in NRVMs with respect to cytochrome c release and JC-1 staining. AZT, efavirenz, HIV-1, and gp120 damaged NRVM mitochondria, as shown by a decrease of the cytochrome c content in treated cells, demonstrated using immunostaining (Fig.1C), and by a decrease in $\Delta\Psi_m$, demonstrated using JC-1 dye (Fig.1D).

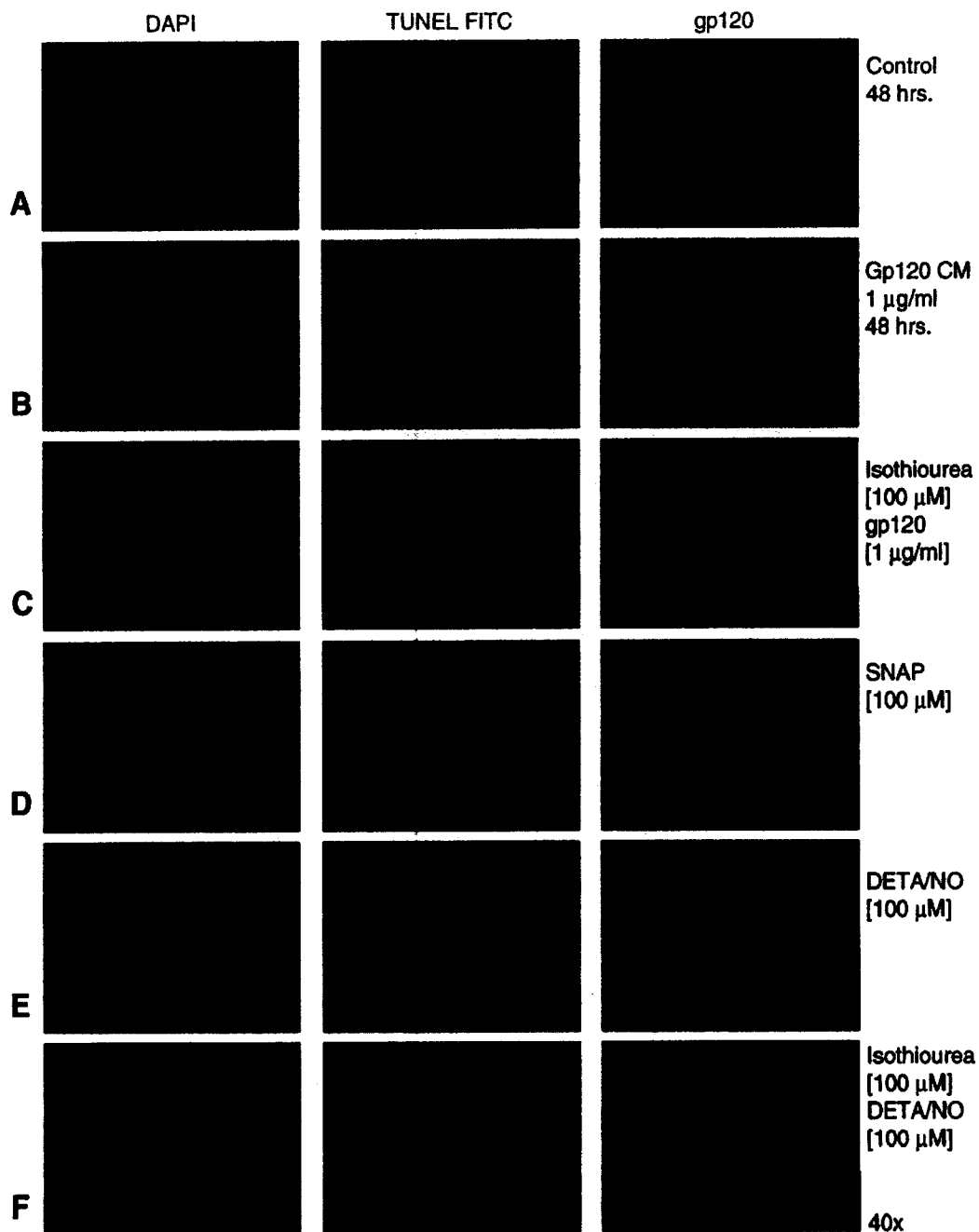### HAART Drugs, but Not HIV-1, Disrupt the Actin Cytoskeleton of Endothelial Cells

We investigated the disruption of interendothelial cell junctions by HAART drugs in replicate confluent endothelial CAEC or BMVEC monolayers.

Phalloidin immunofluorescence of untreated CAEC monolayers revealed their confluence during the whole 72-h observation period (Fig. 3A). Treatment of CAEC monolayers with AZT at the concentration range 1 to 20 µg/mL induced gaps between cells, which appeared 24 h after treatment and persisted for 72 h. Efavirenz-treated monolayers developed gaps 24 h posttreatment only at 10 µg/mL and higher concentrations, and the gaps sealed 48 h posttreatment. Indinavir-exposed monolayers showed gaps at the concentration range 10 to 100 µg/mL 24 h and 72 h posttreatment. In endothelial cells surrounding the gaps, the actin cytoskeleton became modified with loss of fine marginal actin filaments and development of dense bands at cell margins.

Fig. 1. HIV-1, gp120, and azidothymidine (AZT) all damage mitochondria but only HIV-1 and gp120 produce apoptosis (original magnification ×400). **(A)** HIV-1 and gp120, but not AZT, induce apoptosis of human coronary artery endothelial cells (CAECs). DAPI (upper row) and TUNEL (lower row) staining was performed on CAECs treated as indicated with control medium, AZT (1 µg/mL), gp120$_{MN}$ (100 ng/mL), or HIV-1 (7.6×10^7 virions) for 48 h. The cells were fixed with 4% paraformaldehyde, stained by DAPI or TUNEL technique, photographed, and O.D. was scanned. The areas of apoptotic cells treated with gp120 or HIV-1 were significantly different from the areas treated with AZT or control medium ($p < 0.001$). **(B)** HIV-1 and gp120, but not AZT, induce apoptosis of neonatal rat ventricular myocytes (NRVMs). DAPI (upper row) and TUNEL (lower row) staining was performed on NRVMs treated with control medium, AZT (1 µg/mL), gp120$_{MN}$ (100 ng/mL), or HIV-1 (7.6×10^7 virions) for 48 h. The areas of apoptotic cells treated with gp120 or HIV-1 were significantly different from the areas treated with AZT or control medium ($p < 0.001$).

A CAEC Apoptosis

B NRVM Apoptosis

C NRVM Cytochrome C

C

D NRVM JC-1 Ratio
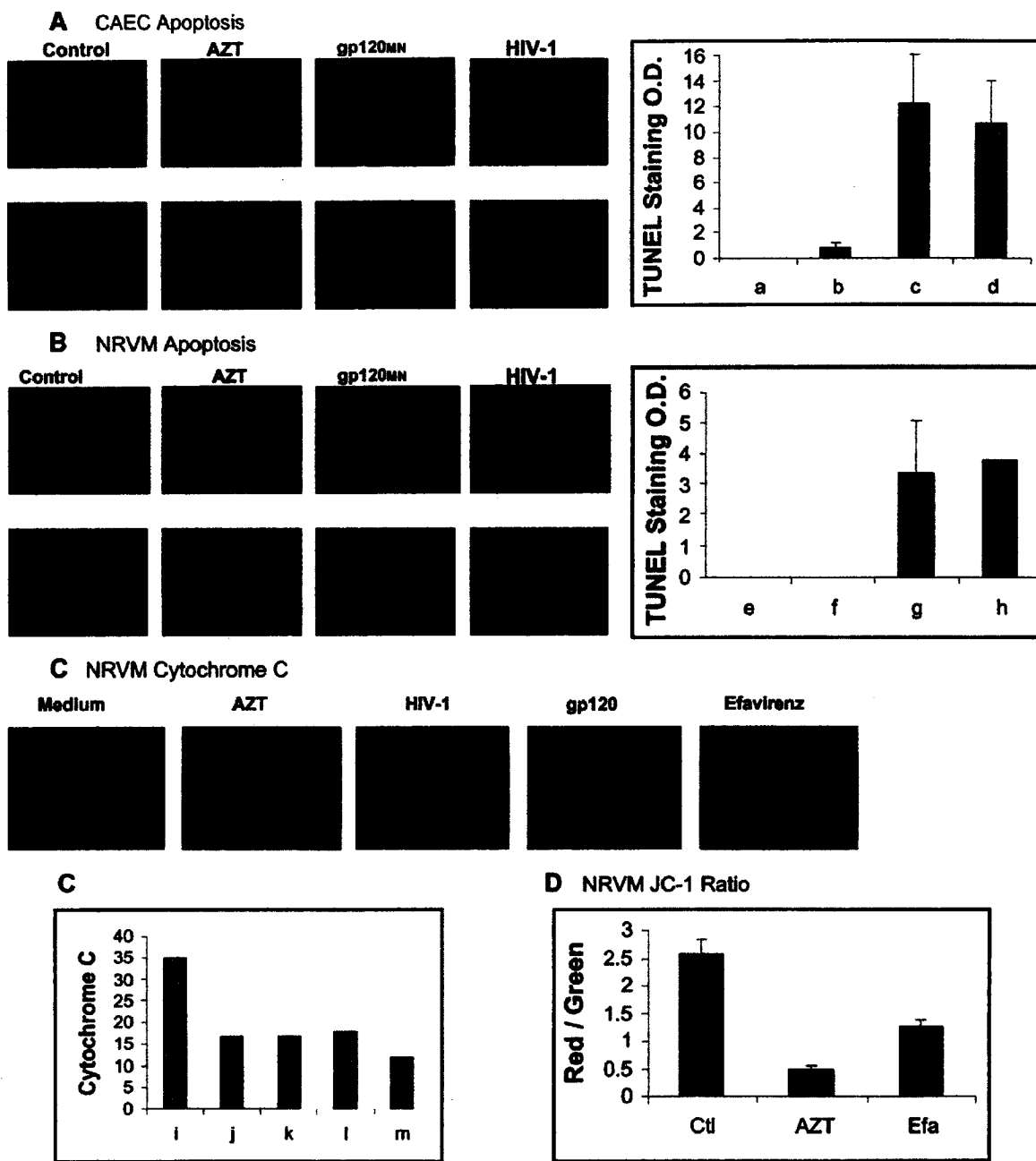
Fig. 1. (*Continued*) (**C**) HIV-1, gp120, AZT, and efavirenz all release cytochrome c from mitochondria. NRVMs were treated for 48 h with control medium, AZT (1 µg/mL), HIV-1 (7.6 × 10⁷ virions), gp120$_{MN}$ (100 ng/mL), or efavirenz (1 µg/mL), fixed, stained using antibody to cytochrome *c*, photographed, and scanned. (**D**) AZT and efavirenz reduce $\Delta\Psi_m$. NRVMs were treated with control medium, AZT (1 µg/mL), or efavirenz (1 µg/mL), fixed, and stained, and the red/green ratio was determined by fluorescence microscopy using fluorescence filters XP100 (Omega) with excitation maximum 475 nm and emission 535/545, and U-N41004 (Chroma) with excitation maximum 560 nm and emission 585 nm. The data were scanned as shown in the graph. The differences between medium control vs AZT ($p < 0.009$) and medium vs efavirenz ($p = 0.012$) were significant.

DAPI   TUNEL FITC   gp120

A   Control
                                                         48 hrs.

B   Gp120 CM
                                                         1 µg/ml
                                                         48 hrs.

C   Isothiourea
                                                         [100 µM]
                                                         gp120
                                                         [1 µg/ml]

D   SNAP
                                                         [100 µM]

E   DETA/NO
                                                         [100 µM]

F   Isothiourea
                                                         [100 µM]
                                                         DETA/NO
                                                         [100 µM]

                                                         40x

Fig. 2. Inhibitor of nitric oxide synthase inhibits gp120-induced neonatal rat ventricular myocyte (NRVM) apoptosis; Nitric oxide (NO) donors also induce NRVM apoptosis. Left column: DAPI staining; middle column: fluorescent TUNEL assay; right column: anti-MF20 staining. (**A**) Control medium. (**B**) gp120 (1 µg/mL). (**C**) Ethylisothiourea pretreatment. (**D**) S-nitroso-*N*-acetylpenicillamine (SNAP). (**E**) Diethylenetriamine/NO (DETA/NO). (**F**) Ethylisothiourea pretreatment followed by DETA/NO.

Double immunostaining with phalloidin and anti-β catenin visualized HAART drug-induced gaps in BMVECs 24 h posttreatment (Fig. 3B). The disruption of phalloidin and anti-β actin was superimposable. AZT and indinavir were the most disruptive drugs, whereas efavirenz was much less disruptive.

HAART Drugs Cause Reversible Decrease in Electrical Resistance

To follow these effects over time, we measured TEER in an ECIS apparatus for at least 15 h. AZT (0.1 μg/mL to 50 μg/mL) caused immediate decrease in electrical resistance that persisted during the observation period in CAECs (Fig. 4A,C) and BMVECs (Fig. 4B,D). BMVEC II cells replicated normally and regained TEER 24 h after treatment when the cell entered a second cell cycle. Indinavir produced an immediate decrease of TEER, which appeared to be proportionate to the dose (Fig. 4F–H). Control treatment with deoxythymidine did not produce any noticeable effect on TEER (Fig. 4E).

Discussion

Cardiovascular complications of HIV-1, such as myocardial infarction and heart failure, continue to develop during HAART treatment despite overall beneficial effects on mortality. It is clear that HIV-1 and gp120 are strongly pathogenic in the cardiovascular system, as shown in a previous study (8) and by pro-apoptotic effect on CAECs, BMVECs, and NRVMs in this study. HAART drugs do not cause apoptosis of these cardiovascular cells and, moreover, suppress viral replication; however, certain of their mechanisms might contribute to cardiovascular complications.

HAART drugs could produce cardiovascular complications through lipodystrophy, dyslipidemia, and insulin resistance (29), as well as mitochondrial and cell junctional effects revealed in this study. Interendothelial gaps induced by AZT and indinavir could increase contact of platelets with tissue factor, thus promoting coagulation and vascular alterations leading to coronary atherosclerosis. Furthermore, these gaps could increase monocyte transmigration, which might also foster atherosclerosis.

This study provides evidence that HAART drug toxicities are characteristically different from HIV-

1. HIV-1 does not disrupt endothelial cell junctions in BMVECs or CAECs but increases transcellular permeability for its own invasion (26,27). HAART drugs, primarily AZT and indinavir, on the other hand, induced gaps between endothelial cells, causing a severe drop in electrical resistance. These acute effects seemed to be reversible both with respect to gaps and electrical resistance. The mechanisms of interendothelial gap formation by HAART drugs might be related to those used by inflammatory mediators (histamine and thrombin), which increase centripetal tension and decrease intercellular and matrix adhesion in the absence of a rise in cyclic adenine monophosphate (30). Thrombin triggers multiple signaling pathways through activation of heterotrimeric G proteins, which inactivate adenylate cyclase by G_i-linked receptor or activate phospholipase C by G_q-dependent action (31). Phospholipase C accelerates cleavage of phosphatidylinositol-4,5-diphosphate to inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 elevates intracellular Ca^{++}, and DAG activates protein kinase C. G proteins also activate small GTP binding proteins, Rho, Rac, and Cdc42 (32). These signals lead to activation of multiple protein kinases, Rho kinase, protein kinase C (33), and mitogen activated protein kinase, which lead to phosphorylation of their target proteins, caldesmon, heat shock protein 27, filamin, and myosin light chain kinase. The target proteins induce interendothelial gaps by contraction of myosin and formation of actin stress fibers (34). In BMVECs, thrombin induced intercellular gaps similar to those made by AZT, although with a faster onset of gap formation, as described by others (35).

On the other hand, HIV-1 and gp120 cause irreversible damage in the cardiovascular system by induction of apoptosis of cardiomyocytes and endothelial cells. HIV-1 and gp120 induce cardiomyocyte apoptosis through the mitochondrial pathway (8). Although HIV-1, gp120, and AZT all damaged mitochondria, only HIV-1 and gp120 caused apoptosis, suggesting that acute AZT effects are reversible. Whether chronic AZT administration during HAART therapy might lead to other effects on vessel wall, predisposing to atherosclerosis, is not clear from cell culture experiments. AZT effects could be enhanced in vivo by coexistent HIV-1 infection. Indeed, HAART toxicity was noted only in an HIV transgenic model but not in nontransgenic animals (36). Moreover,
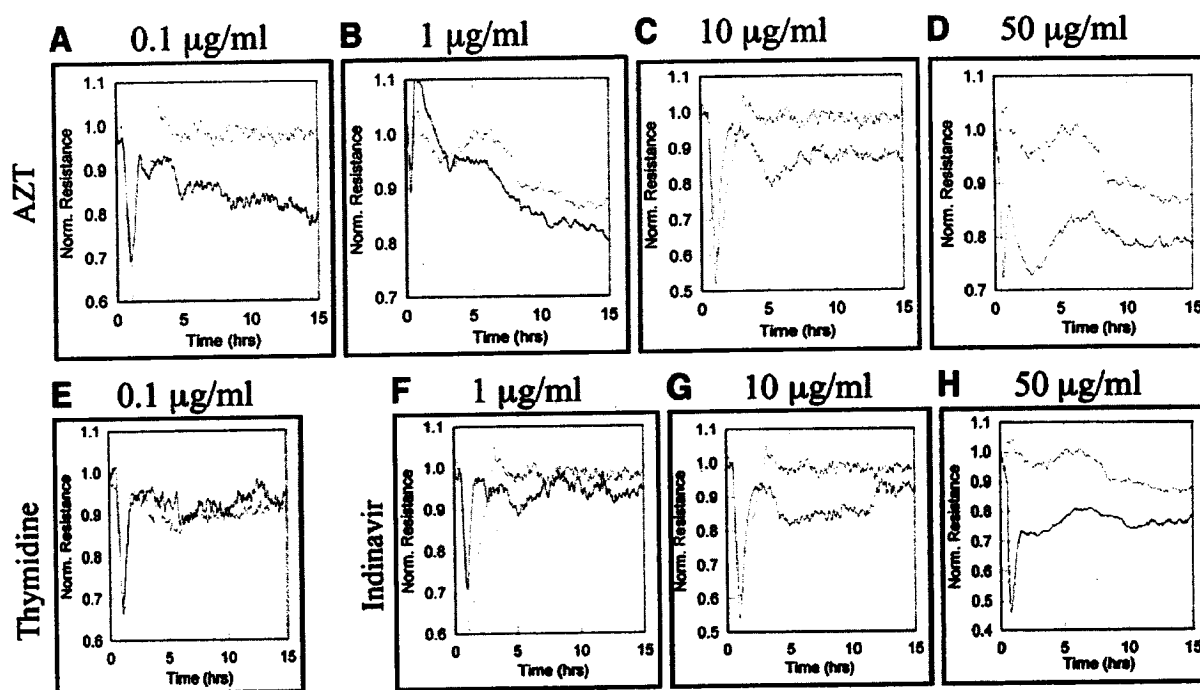
A   **Phalloidin**

B
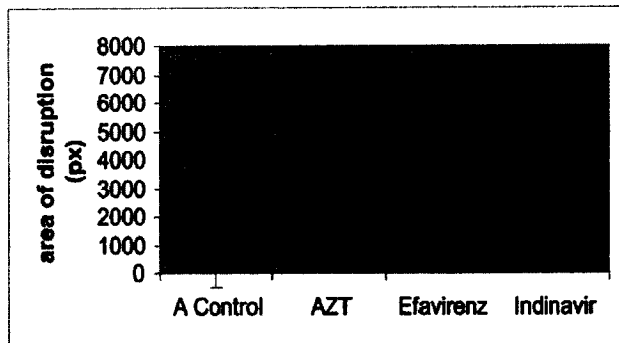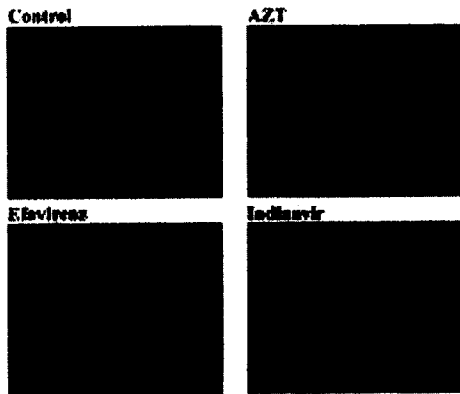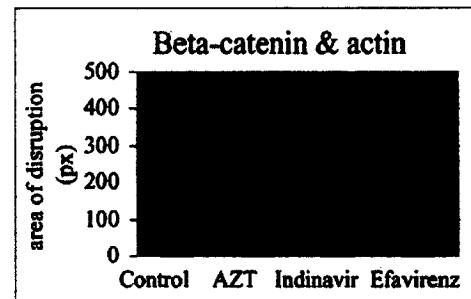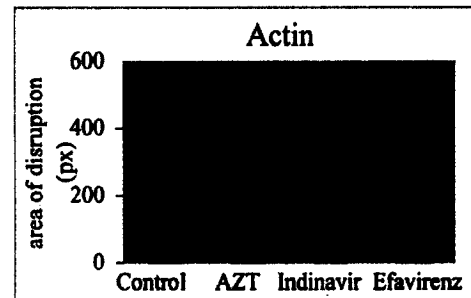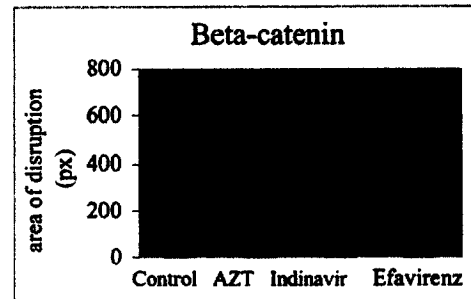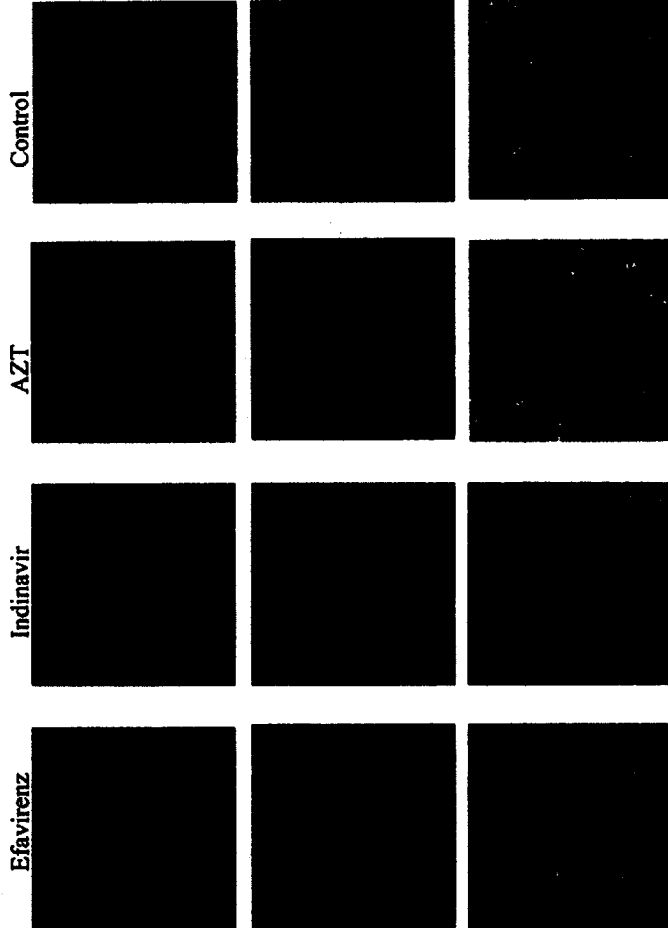
Fig. 4. Highly active antiretroviral therapy drugs induce drop in transendothelial electrical resistance (TEER). **(A–C)** Treatment of human coronary artery endothelial cells (CAECs) with AZT (0.1 µg/mL and 10 µg/mL) causes immediate drop in TEER. **(A and D)** Treatment of immortalized brain microvascular endothelial cells (BMVEC-II; (1 µg/mL and 50 µg/mL) causes immediate drop in TEER; the cells continue to replicate (see increase in TEER in [D]). **(E)** Deoxythymidine does not affect TEER. **(F–H)** Treatment of CAEC with indinavir (1 to 50 µg/mL) causes dose-dependent drop in TEER.

mitochondrial loss of cytochrome *c* could contribute to heart failure even without noticeable apoptosis of cardiomyocytes *(37)*.

The role of NO in HIVCM was first suggested by the observations that patients with HIVCM have increased levels of inducible NO synthase (iNOS) *(38)* and HIV-1 myocarditis is associated with infiltration by macrophages, some positive for iNOS and cyclooxygenase-2 *(4)*. NO is a potent inducer of cell death by shifting the cellular redox potential to a more oxidized state *(39)*. Assays of NO and iNOS mRNA expression in cardiomyocytes showed that

gp120 modulates interleukin-1β-induced NO production, leading to p38-mediated activation of nuclear factor (NF)-κB pathway *(40)*. NF-κB factors are involved in modulation of apoptosis.

It is likely that each class of HAART drugs, NRTI, NNRTI, and PI, has specific cardiovascular effects that need to be individually evaluated and managed. Although the overall balance of beneficial antiviral effects and adverse cardiovascular effects of HAART drugs is not clear from these in vitro experiments, our results point to a dominant effect of uncontrolled virus replication on the heart.

Fig. 3. *(Opposite page)* Highly active antiretroviral therapy drugs produce interendothelial gaps and restructure the actin cytoskeleton. **(A)** Human coronary artery endothelial cells were treated for 24 h with medium, AZT (10 µg/mL), or efavirenz (10 µg/mL), or for 72 h with indinavir (100 µg/mL). The actin cytoskeleton was visualized by immunostaining with phalloidin-FITC (×40) and the areas of intercellular gaps were scanned. Significance of all differences between medium control and AZT, efavirenz, or indinavir $p < 0.0001$. **(B)** Brain microvascular endothelial cells were treated for 24 h with medium, AZT (10 µg/mL), efavirenz (10 µg/mL), or indinavir (100 µg/mL). The cells were fixed, stained with antibodies to β-catenin or actin, and photographed, and areas of disruption were scanned. Original magnification ×400.

Acknowledgments

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