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HIV-1 antiretrovirals induce oxidant injury and increase intima–media thickness in an atherogenic mouse model

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ABSTRACT

A growing body of evidence suggests HIV patients are at a greater risk for developing atherosclerosis. However, clinical investigations have generated conflicting results with regard to whether antiretrovirals are independently involved in the development of HIV-associated atherosclerosis. By administering antiretrovirals in an atherogenic mouse model, we determined whether two commonly prescribed antiretrovirals, the protease inhibitor indinavir and the nucleoside reverse transcriptase inhibitor AZT, can induce premature atherosclerosis. C57BL/6 mice were administered an atherogenic diet ± AZT, indinavir, or AZT plus indinavir for 20 weeks. Aortic intima–media thickness (IMT) and cross-sectional area (CSA) were determined. Compared to controls, treatment with AZT, indinavir or AZT plus indinavir, significantly increased aortic IMT and CSA. This suggests that antiretrovirals can directly exacerbate atherogenesis, in the absence of interaction with a retroviral infection. To elucidate the role of oxidant injury in the drug-induced initiation of atherosclerosis, a separate group of mice were treated for 2 weeks with an atherogenic diet ± AZT, indinavir or AZT plus indinavir. Aortic reactive oxygen species (ROS) production and glutathione/glutathione disulfide (GSH/GSSG) ratios, as well as plasma levels of 8-isoprostanes (8-iso-$PGF_{2\alpha}$) and lipids were determined. At 2 weeks, aortic ROS was increased and GSH/GSSG ratios were decreased in all antiretroviral treatment groups. Plasma 8-iso-$PGF_{2\alpha}$ was increased in the AZT and AZT plus indinavir-treated groups. At 20 weeks, increased ROS production was maintained for the AZT and indinavir treatment groups, and increased 8-iso-$PGF_{2\alpha}$ levels remained elevated in the AZT treatment group. Cholesterol levels were moderately elevated in the AZT and AZT plus indinavir-treated groups at 2 but not 20 weeks. Conversely, indinavir treatment increased plasma cholesterol at 20 but not 2 weeks. Thus, though effects on plasma lipid levels occurred, with effects of the individual antiretrovirals variable across the treatment period, there was consistent evidence of oxidant injury across both early and late time points. Together with the known metabolic abnormalities induced by antiretrovirals, drug-induced oxidant production may contribute to the development of antiretroviral-associated atherosclerosis.

1. Introduction

HIV patients taking antiretrovirals, especially protease inhibitor-containing regimens, are widely reported to exhibit metabolic complications such as lipodystrophy, hyperlipidemia, central adiposity, insulin resistance and diabetes mellitus (Martinez et al., 2004). These drug-associated side effects are known risk factors for cardiovascular diseases such as atherosclerosis. It therefore raises the concern that HIV patients, especially those taking protease inhibitors, may be at a greater risk for developing premature atherosclerosis. Although conventionally considered a disease of the elderly, atherosclerosis is now commonly observed in HIV patients, even in young individuals who have no other appreciable risk factors (Currier et al., 2003; Bonnet et al., 2004).

Atherosclerosis is a chronic, progressive disease characterized by plaque formation in medium- or large-sized arteries. Although atherosclerosis can remain "silent" and asymptomatic before a major cardiovascular event occurs, several methods are currently used to quantify the progression of atherosclerosis. Vascular intima–media thickness (IMT) is a well-recognized and validated method to access subclinical atherosclerosis (Chambless et al., 1997). IMT is commonly measured in superficial arteries, such as the common carotid or femoral arteries, by high resolution B mode ultrasound for human studies, and in the common carotid artery or thoracic aorta for studies in laboratory animals. Increased IMT mainly represents medial hypertrophy, resulting from vascular smooth muscle cell (VSMC) proliferation and migration. Importantly, studies have shown that carotid IMT is correlated

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with the risk of stroke, death or coronary events (O'Leary et al., 1999).

IMT measurements in the common carotid arteries have been applied to HIV patients. Accumulating evidence suggests that the HIV infection is an independent risk factor contributing to increased IMT and perhaps atherosclerosis (Depairon et al., 2001; Hsue et al., 2004; Mangili et al., 2006). However, when accessing the roles of antiretrovirals on this increased IMT, clinical studies report conflicting results. Some studies suggest that only the traditional risk factors, such as age, male gender, increased plasma cholesterol levels, higher body mass index, and increased systolic blood pressures, are associated with increased IMT in HIV patients, and that antiretrovirals are not a major contributor (Mercie et al., 2002; Chironi et al., 2003; Currier et al., 2005; Lebech et al., 2007). However, other studies indicate that antiretroviral treatment, particularly regimens containing protease inhibitors, significantly increase carotid IMT and thus, may contribute to HIV-associated atherosclerosis (Maggi et al., 2000; Seminari et al., 2002; de Saint Martin et al., 2006; Johnsen et al., 2006; Lorenz et al., 2008). The disparate results of these clinical studies may be due to differing combination drug therapies over the population of patients within a study, changes in drug therapy for individual patients over a given study period, differing sample sizes, as well as the complexity of interactions of drug-induced effects with other atherogenic factors, such as age, sex, serum cholesterol levels, and hypertension, within a given study. Although statistical analysis may be able to reveal the role of a single factor in the final outcome, animal studies in which the drug regimen, treatment time, and other atherogenic factors are well controlled are lacking.

We previously reported that the nucleoside reverse transcriptase inhibitor (NRTI) AZT, administered either alone or in combination with the protease inhibitor indinavir, induces direct endothelial dysfunction in rodents at clinically relevant doses (Jiang et al., 2006). This suggests that in addition to the atherogenic side effects of protease inhibitors, AZT and perhaps other NRTI may also induce an early vascular injury to initiate atherogenesis and may contribute to antiretroviral-associated atherosclerosis. An earlier study conducted in our laboratory demonstrated that both AZT and indinavir could induce VSMC proliferation when co-cultured with endothelial cells. An endothelium-derived mitogen, endothelin-1 (ET-1), was released by antiretrovirals and was shown to mediate VSMC proliferation (Hebert et al., 2004). Though intriguing, it is still unclear whether the antiretroviral-induced VSMC proliferation observed in these studies would translate into increased vessel wall thickness and premature atherosclerosis *in vivo*. In this study, we employed an atherogenic C57BL/6 mouse model to determine whether antiretrovirals can exacerbate atherosclerosis, as indicated by increased IMT in the aorta. We specifically tested the NRTI AZT and the protease inhibitor indinavir. Since few studies have addressed the contribution of NRTI to HIV-associated atherogenesis, and fewer still have compared effects of NRTI versus protease inhibitors, the data generated in these studies will be a significant contribution to our understanding of the factors promoting atherogenesis in the HIV-infected population. Because many studies have suggested an involvement of antiretroviral-induced metabolic disorders in HIV-associated atherosclerosis, and because both drugs utilized here were shown to induce oxidant injury in endothelial cells *in vitro* (Jiang et al., 2007), we furthermore assessed the role of drug-induced effects on circulating lipid levels and vascular oxidant production in exacerbating atherogenesis.

2. Materials and methods

2.1. Treatment of mice with antiretroviral

Six- to eight-week-old male C57BL/6 mice were obtained from Harlan (Indianapolis, IN). The mice were acclimated to the room for 1 week after arrival and were maintained on a normal 12 h light–dark cycle. The mice were then fed an atherogenic diet that contained 15% fat, 1.25% cholesterol and 0.5% sodium cholate (Harlan Teklad, Madison, WI) for 20 weeks, as described previously (Paigen et al., 1985). Since plasma cholesterol concentration is a key factor for developing atherosclerosis, and since mice are resistant to hyperlipidemia and to developing atherogenesis, administration of a high fat, high cholesterol diet is required. This atherogenic diet can be furthermore supplemented with cholate to promote the absorption of cholesterol in the gastrointestinal tract. The diet-induced mouse atherosclerotic model has been well described, with a 2–3-fold increase in plasma cholesterol levels and a significant increase in aortic fatty streak lesions (Liao et al., 1993; Nageh et al., 1997; Paigen et al., 1985). The mice were separated into a control group and three treatment groups administered diet and either AZT (10 mg/kg), indinavir sulfate (15 mg/kg), or AZT plus indinavir sulfate (10 + 15 mg/kg), through their drinking water for 20 weeks. The mice were singly housed and the volume of water consumed was monitored and adjusted to ensure that each animal received the same amount of drug. All experimental procedures and protocols were approved by the Animal Care and Use Committee at the LSU Health Sciences Center at Shreveport.

2.2. Quantitation of atherosclerotic progression

After treatment for 20 weeks, the mice were anesthetized using intraperitoneal injection of 50 mg/kg pentobarbital. For measurement of plasma analytes, the blood was collected from the inferior vena cava into 4 mL EDTA tubes. For a small number of animals, thoracic aortas were excised in their entirety from the aortic origin to the diaphragm. The adventitia and adipose tissue were carefully removed, and the aortas were cut longitudinally. Atherosclerotic fatty streaks were assessed by Sudan IV staining, as described previously (Ling et al., 2001). The intimal surfaces of the aortas were then visualized using a digital camera interfaced to an Olympus SZ60 stereozoom microscope.

The remaining animals were anesthetized and were euthanized by pneumothorax. Quartile segments from the aortic origin, the ascending aorta, the aortic arch and the descending aorta were excised and embedded immediately in OCT freezing medium (Miles, Elkhart, IN) over dry ice. OCT sections were then cut at a 10 μm thickness for at least 10 consecutive sections, beginning at the proximal portion of the first quartile (aortic origin), and the resulting sections were stained with hematoxylin and eosin. Images were obtained using a Nikon Labophot microscope interfaced to a high resolution digital camera. Aortic intima–media thickness and cross-sectional areas (CSA) were determined in digitized images of cross sections using Metamorph 5 software (Downingtown, PA), and a mean value was computed for each aorta.

2.3. Measurement of plasma total cholesterol and triglyceride levels

At the time of sacrifice, blood was collected into 4 mL EDTA tubes, and the tubes were centrifuged to collect the plasma. Plasma aliquots were then stored at −80 °C until analysis. Plasma total cholesterol and triglyceride levels were determined colorimetrically using total cholesterol and triglyceride reagent kits purchased from Eagle Diagnostics (De Soto, TX).

2.4. Measurement of aortic reactive oxygen species (ROS) production at 20 weeks

Aortic reactive oxygen species (ROS) production was accessed by lucigenin-enhanced chemiluminescence. Aortic segments representing the second quartile from the top were carefully excised, were cut into 5 mm segments, and were incubated in modified Krebs-HEPES buffer (NaCl 99.01 mmol/L, KCl 4.69 mmol/L, $CaCl_2$ 1.87 mmol/L, $MgSO_4$ 1.20 mmol/L, NaHEPES 20.0 mmol/L, K_2HPO_4 1.03 mmol/L, $NaHCO_3$ 25.0 mmol/L, and D(+)Glucose 11.1 mmol/L, pH 7.4), aerated with 95% O_2 and 5% CO_2 at 37 °C for 30 min. The aortic rings were then transferred into wells of 24-well-plates that contained 2 mL of Krebs-HEPES buffer with 5 μM lucigenin. Using a chemiluminescence microplate reader, chemiluminescence was assessed at 1 min intervals over 10 min. The vessel segments were then dried, the dry weight was determined, and the measured luminescence was normalized to the dry weights for each ring.

2.5. Measurement of plasma 8-iso-$PGF_{2\alpha}$ levels

Plasma samples (0.2 mL) were mixed with an equal volume of potassium hydroxide and were incubated at 40 °C for 60 min. To this mixture, 2 volumes of ethanol containing 0.01% butylated hydroxytoluene were added, and the sample was vortexed. After evaporating the ethanol by vacuum centrifugation, the pH was adjusted to 3.0 with 1N HCl and to this, 2 mL of 100 mM formate buffer, pH 3.0, was added. The sample was then centrifuged at 2400 × *g* for 10 min, and was further purified by solid-phase extraction (SPE), as described by Zhao et al. (2001), before 8-iso-$PGF_{2\alpha}$ measurements. Specifically, 100 μL plasma was combined with 200 μL methanol and was centrifuged at 1500 × *g* for 10 min at 4 °C to remove precipitated proteins. The pH of the samples was adjusted to 4.0 using HCl, and the acidified samples were then passed through a Sep-Pak C_{18} cartridge. The samples were eluted using 5 mL ethyl acetate containing 1% methanol and were evaporated under vacuum. The samples were finally dissolved in acetone and were assayed by Enzyme-Linked ImmunoSorbent Assay (ELISA), using an 8-iso-$PGF_{2\alpha}$ EIA kit obtained from Cayman Chemicals (Ann Arbor, MI).