



# 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  $\pm$  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  $\pm$  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<sub>2 $\alpha$</sub> ) 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<sub>2 $\alpha$</sub>  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<sub>2 $\alpha$</sub>  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.

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## 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; Lebeck 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 S260 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  $\mu$ m 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  $-80^{\circ}\text{C}$  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,  $\text{CaCl}_2$  1.87 mmol/L,  $\text{MgSO}_4$  1.20 mmol/L, NaHEPES 20.0 mmol/L,  $\text{K}_2\text{HPO}_4$  1.03 mmol/L,  $\text{NaHCO}_3$  25.0 mmol/L, and D(+)-Glucose 11.1 mmol/L, pH 7.4), aerated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$  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  $\mu\text{M}$  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<sub>2 $\alpha$</sub> levels

Plasma samples (0.2 mL) were mixed with an equal volume of potassium hydroxide and were incubated at  $40^{\circ}\text{C}$  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  $2400 \times g$  for 10 min, and was further purified by solid-phase extraction (SPE), as described by Zhao et al. (2001), before 8-iso-PGF<sub>2 $\alpha$</sub>  measurements. Specifically, 100  $\mu\text{L}$  plasma was combined with 200  $\mu\text{L}$  methanol and was centrifuged at  $1500 \times g$  for 10 min at  $4^{\circ}\text{C}$  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<sub>18</sub> 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<sub>2 $\alpha$</sub>  EIA kit obtained from Cayman Chemicals (Ann Arbor, MI).

## 2.6. Measurement of plasma drug levels

Determination of plasma drug levels in the mice treated with antiretrovirals using high performance liquid chromatography (HPLC) proved problematic due to the co-elution of the peak representing pentobarbital, the anesthetic used for these studies, and that for indinavir. Thus, an additional group of mice were obtained and were acclimated to the room for 1 week. The mice were then administered either AZT (10 mg/kg), indinavir sulfate (15 mg/kg), or AZT plus indinavir sulfate (10 + 15 mg/kg) in their drinking water for 2 weeks, to ensure that the steady state plasma levels had been reached. For consistency, the animals were fed the same high fat diet used in the prior experiments. Before sacrifice, the mice were anesthetized using 100 mg/kg ketamine and 10 mg/kg xylazine by i.m. injection. Blood was withdrawn from the inferior vena cava and was placed in EDTA tubes. Plasma was collected by centrifugation and was stored at  $-80^{\circ}\text{C}$  until analysis.

The HPLC determination of plasma antiretroviral levels was by modification of the method described by Notari et al. (2006). Protein was first precipitated by addition of 100  $\mu\text{L}$  ice cold methanol to 100  $\mu\text{L}$  plasma. After centrifuging, the supernatant was collected and centrifuged a second time, before applying to a pre-equilibrated Oasis HLB 1  $\text{cm}^3$  extraction cartridge (Waters, Milford, MA). The column was then washed with 2 mL 5% methanol and then 550  $\mu\text{L}$  10 mM  $\text{KH}_2\text{PO}_4$ . The antiretrovirals were eluted with 2 mL 25% methanol and then 2 mL 100% methanol. The latter two fractions were combined, evaporated to dryness under vacuum, and resuspended in 100  $\mu\text{L}$  methanol. For each determination, 30  $\mu\text{L}$  was injected onto HPLC. The chromatography was accomplished using a method described elsewhere (Jiang et al., 2006). A standard curve using authentic standards was constructed to quantitate the antiretroviral concentrations in the plasma samples.

## 2.7. Assessing early determinants of oxidant injury

As a confirmatory test for changes in vascular redox status, levels of ROS production and glutathione-to-glutathione disulfide (GSH/GSSG) ratios were measured in aortas of mice treated for 2 weeks with antiretrovirals plus a high fat diet, as described above. Following anesthesia using ketamine and xylazine and euthanasia by pneumothorax, the aorta was excised. The area from the aortic origin to the arch was dissected and frozen in OCT medium for assessment of early changes in reactive oxygen species. For the determination of glutathione redox status, the descending aorta was quick-frozen on dry ice and was stored at  $-80^{\circ}\text{C}$ . For quantitating ROS, OCT-embedded frozen tissues of the aortic arch were sectioned at a thickness of 10  $\mu\text{m}$  and were incubated with 2  $\mu\text{M}$  dihydroethidium (DHE) for 30 min at  $37^{\circ}\text{C}$  in the dark. The sections were imaged using an Olympus IX RL fluorescence microscope and a 40 s exposure time. The fluorescence intensity in each image was compared using Image J software (National Institutes of Health). Briefly, a constant threshold was applied to each image to eliminate nonspecific fluorescence, the image was converted to an RGB stack, the area of the artery wall was selected, and mean gray values per unit area were calculated.

To determine the GSH/GSSG ratio, the descending aorta that had been fast frozen on dry ice was homogenized in 0.4 N perchloric acid containing 100 nM EDTA. After centrifuging, 50  $\mu\text{L}$  of the acid supernatant was injected onto a Waters Alliance 2695 HPLC system and a Supelco Supercosil LC-8, 3  $\mu\text{m}$ , 25  $\text{cm} \times 4.6$  mm reversed phase column. The mobile phase consisted of 25 mM sodium dihydrogen phosphate, pH 2.65, containing 1.4 mM 1-octanesulfonic acid and 6% acetonitrile. The sample was separated using isocratic elution, with a flow rate of 0.5 mL/min. Under these conditions, GSH and GSSG eluted at approximately 15 and 32 min, respectively. With the HPLC system interfaced to an ESA Coullarray electrochemical detection system (ESA, Inc., Chelmsford, MA) consisting of a single boron-doped diamond electrode, the analytes were detected at a potential of 1500 mV.

Finally, for comparison to earlier experiments, an aliquot of plasma collected in EDTA was utilized for the measurement of 8-isoprostanes, as describe above.

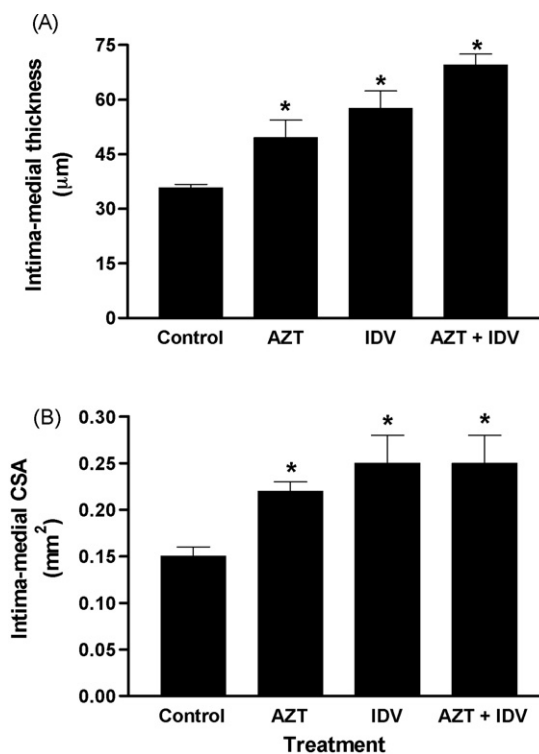
## 2.8. Statistical analysis

All data are expressed as means  $\pm$  standard error. Statistical analyses were performed using SPSS for Windows 10.0 (SPSS, Inc., Chicago, IL). Comparisons between the control and antiretroviral-treated mice were conducted by one-way analysis of the variance (ANOVA), followed by Tukey or Fisher LSD post hoc *t*-tests, and *p* values of  $<0.05$  were accepted as statistical significance.

## 3. RESULTS

### 3.1. Antiretrovirals increase intima-media thickness and cross-sectional area

Intima-media thicknesses and cross-sectional areas were quantified in OCT-embedded sections of the aorta taken near the origin. Fig. 1 demonstrates that in comparison to control mice, AZT treatment increased aortic IMT by  $\sim 38\%$  and CSA by  $\sim 47\%$ . Indinavir, when administered alone, increased aortic IMT by  $\sim 61\%$  and CSA by



**Fig. 1.** Intima-media thickness (A) and intima-media cross-sectional area (CSA; B) in aortas of C57BL/6 mice fed a high fat, high cholesterol diet  $\pm$  antiretrovirals for 20 weeks. Values are means  $\pm$  standard error. (\*) Indicates significant differences in the antiretroviral-treated mice as compared to controls, as revealed by one-way ANOVA with Tukey post hoc *t*-tests.

$\sim 67\%$ . When AZT and indinavir were administered in combination, aortic IMT and CSA were increased by  $\sim 94\%$  and  $67\%$ , respectively. Thus, both AZT and indinavir, which represent two different classes of antiretrovirals, increased vessel wall thickness. In addition, when administered in combination, the drugs exhibited an additive effect, inducing further increases in IMT (94% in the combination treatment group as compared to 38% for AZT alone or 61% for indinavir alone), although additive effects were not observed for CSA.

### 3.2. Indinavir elevates total plasma cholesterol levels

It is well appreciated that indinavir induces metabolic side effects, including lipodystrophy, hyperlipidemia and hypertriglyceridemia, in HIV patients. To determine whether indinavir increases circulating lipid levels in C57BL/6 mice fed the atherogenic diet, we measured the levels of plasma total cholesterol and triglycerides. Table 1 shows that total cholesterol levels were increased  $\sim 2$ -fold (to ca. 200 mg/dL) in mice administered the atherogenic diet, in comparison to reported levels of cholesterol in C57BL/6 on normal rodent chow (less than 100 mg/dL) (Breslow, 1996; Stokes et al., 2007) and that measured previously in our laboratory ( $\sim 80$  mg/dL).

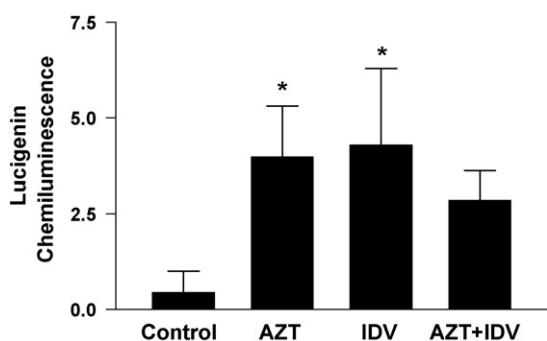
**Table 1**

Plasma total cholesterol and triglyceride levels in C57BL/6 mice treated for 20 weeks with a high fat diet plus antiretrovirals.

	Control	AZT	Indinavir	AZT + indinavir
Total cholesterol (mg/dL)	193.5 $\pm$ 5.7	210.8 $\pm$ 12.8	233.5 $\pm$ 6.8*	222.3 $\pm$ 10.4
Triglyceride (mg/dL)	119.4 $\pm$ 7.0	122.7 $\pm$ 7.7	145.8 $\pm$ 26.4	158.3 $\pm$ 18.6

Values represent means  $\pm$  standard error. One-way ANOVA revealed a significant difference between controls and the drug-treated mice.

\*  $p < 0.05$ .



**Fig. 2.** Vascular reactive oxygen species (ROS) measured by lucigenin-enhanced chemiluminescence in aortic sections from mice treated for 20 weeks with an atherogenic diet  $\pm$  antiretrovirals. Aortas were cut into 3 mm length rings, were added to a microplate containing 5  $\mu$ M lucigenin in 2 mL Krebs's solutions and chemiluminescence was detected for 10 min. AZT and indinavir treatments significantly increased vascular superoxide generation in aortic rings. (\*) Indicates statistical significance versus controls ( $p < 0.05$ ).

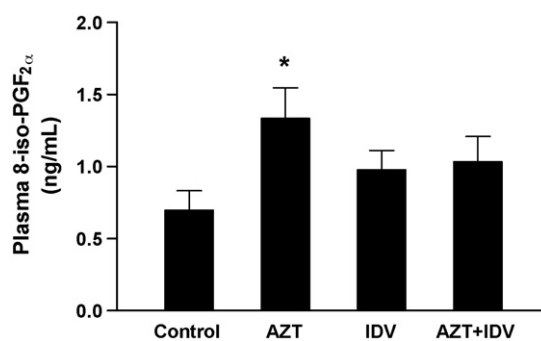
Treatment with indinavir further increased total cholesterol levels by 21% compared to the controls. Indinavir-treated groups also exhibited a trend toward increased plasma levels of triglycerides (a 22% increase compared to controls); however, this effect did not reach statistical significance. On the other hand, AZT treatment had no effect on either plasma cholesterol or triglyceride levels at this 20 week timepoint. Finally, the cholesterol levels in the combination treatment group were similar to that for indinavir treatment alone but were not significantly different from controls.

### 3.3. Antiretrovirals increase vascular oxidant production at 20 weeks

Vascular ROS production was assessed by lucigenin-enhanced chemiluminescence in aortic rings excised from the mice treated with antiretrovirals plus a high fat diet for 20 weeks. Although a high cholesterol diet is known to increase vascular ROS production *per se* (Erdei et al., 2006), Fig. 2 illustrates that treatment with AZT or indinavir significantly increased vascular ROS production 4-fold compared to controls. ROS production for the AZT plus indinavir treatment group was 3-fold greater than controls; however, this effect did not achieve statistical significance.

### 3.4. AZT induces 8-iso-PGF<sub>2 $\alpha$</sub> production and oxidative stress in C57BL/6 mice

To determine whether antiretroviral-induced oxidant production culminates in oxidative stress in our animal model (Fig. 3), levels of the lipid peroxidation end-product 8-iso-PGF<sub>2 $\alpha$</sub>  were measured in plasma samples of animals treated for 20 wks. AZT treatment significantly increased plasma 8-iso-PGF<sub>2 $\alpha$</sub>  production. However, plasma levels of 8-iso-PGF<sub>2 $\alpha$</sub>  were not increased in indinavir- or AZT plus indinavir-treated animals.



**Fig. 3.** Plasma 8-iso-PGF<sub>2 $\alpha$</sub>  levels in mice fed an atherogenic diet  $\pm$  antiretrovirals for 20 weeks. The 8-isoprostane levels were assessed by ELISA following solid-phase extraction. (\*) Indicates statistical significance versus controls ( $p < 0.05$ ).

### 3.5. Plasma antiretroviral levels

To assess whether our antiretroviral treatment effectively achieved serum concentrations comparable to therapeutic levels in humans and to ensure that drug levels in the combination treatment group were not different compared to individual drug treatments, we measured plasma drug levels by HPLC. The antiretrovirals were administered for 2 weeks to ensure that steady state levels had been reached, and for consistency with our prior experiment, the animals were administered a high fat diet. The plasma levels of indinavir in the indinavir and AZT + indinavir treatment groups were  $2.79 \pm 0.79$  and  $1.51 \pm 0.26$   $\mu$ g/mL, respectively. These concentrations were similar to the therapeutic levels reported for humans—0.32 and 11.1  $\mu$ g/mL for C<sub>min</sub> and C<sub>max</sub> (Kakuda et al., 2001). For the AZT and AZT + indinavir treatment groups, the plasma levels of AZT were  $0.06 \pm 0.02$  and  $0.05 \pm 0.01$   $\mu$ g/mL, respectively. It should be noted that these levels were actually 3-fold lower than the therapeutic concentrations reported for humans (Boyd et al., 2003). Importantly, the plasma levels of each drug were not significantly altered when the two drugs were administered in combination.

### 3.6. Early effects of antiretrovirals on vascular oxidant production and circulating lipid levels

To determine whether changes in circulating lipid levels occurs early after antiretroviral treatment and prior to drug-mediated atherogenesis, total plasma cholesterol and triglyceride levels were determined in mice treated for 2 weeks with antiretrovirals plus the atherogenic diet. As indicated in Table 2, plasma levels of cholesterol were significantly elevated by  $\sim 30\%$  in the AZT and the AZT plus indinavir treatment groups compared to controls. Surprisingly, treatment with indinavir did not alter total cholesterol levels at this early time point, as was observed after 20 weeks treatment (Table 1). On the other hand, antiretroviral treatment did not alter triglyceride levels in any of the treatment groups compared.

To assess whether increases in vascular oxidant production after antiretroviral treatment occurs prior to the onset of atherogenesis, we measured the levels of reactive oxygen species by

**Table 2**

Plasma levels of antiretrovirals as well as lipid levels in C57BL/6 mice administered antiretrovirals for 2 weeks.

	Control	AZT	Indinavir	AZT + indinavir
Total Cholesterol (mg/dL)	132.7 $\pm$ 8.5	174.4 $\pm$ 4.8*	139.9 $\pm$ 0.4	175.2 $\pm$ 7.8*
Triglyceride (mg/dL)	29.4 $\pm$ 1.4	40.2 $\pm$ 5.5	24.1 $\pm$ 3.6	25.4 $\pm$ 0.6
Drug levels ( $\mu$ g/mL)		0.06 $\pm$ 0.02	2.79 $\pm$ 0.79	0.05 $\pm$ 0.01 (AZT) 1.51 $\pm$ 0.26 (IDV)

Values represent means  $\pm$  standard error. One-way ANOVA revealed a significant difference between controls and the drug-treated mice.

\*  $p < 0.05$ .

dihydroethidium staining of aortic cross sections. DHE staining of the cross sections demonstrated a pronounced ~12–17-fold increase in red fluorescence across the entirety of the vascular wall in the indinavir, AZT and AZT+indinavir treatment groups compared to controls, with the combination treatment exhibiting a slightly greater effect compared to the individual drug treatments. To determine whether these changes in oxidant production culminated in oxidative stress, levels of the lipid peroxidation end-product 8-isoprostanes and the GSH/GSSG ratios were also measured. Plasma 8-isoprostanes were significantly increased by approximately 30% in all treatment groups, though there appeared to be no additional increase in the combination treatment group. Aortic reduced glutathione-to-glutathione disulfide levels were reduced by ~40% for treatment with either AZT or indinavir alone, and by ~50% for treatment with AZT+indinavir.

#### 4. Discussion

Numerous studies investigating the contribution of antiretrovirals to atherosclerosis have focused on a specific category of antiretrovirals—the protease inhibitors (Maggi et al., 2000, 2004; Depairon et al., 2001; Holmberg et al., 2002; Seminari et al., 2002; Barbaro and Barbarini, 2006). These prior reports suggested that a series of metabolic abnormalities induced by protease inhibitors might be responsible for the increased incidence of cardiovascular diseases observed in HIV patients on combination therapy. Certainly, protease inhibitors have been linked to a variety of metabolic complications, including lipodystrophy, hyperlipidemia, insulin resistance and diabetes mellitus (Carr et al., 1998). These are indeed well known risk factors contributing to atherogenesis. However, antiretroviral-induced alterations in plasma lipid levels may not be the only determinant contributing to HIV-associated atherosclerosis. Stein et al. (2001) showed that while HIV patients treated with protease inhibitors have higher cholesterol and triglyceride levels in the serum, as well as an impairment of flow-mediated vasodilation (FMD), this protease inhibitor-associated endothelial dysfunction was not improved by administration of pravastatin to normalize the plasma lipid levels (Stein et al., 2004). This suggests that the endothelial dysfunction, one of the first vascular changes during atherogenesis, is apparently not directly related to lipid levels in these patients, but that protease inhibitors may be acting through another pathway. Alternatively, other classes of antiretrovirals, perhaps the NRTI, may also contribute to HIV-associated atherogenesis.

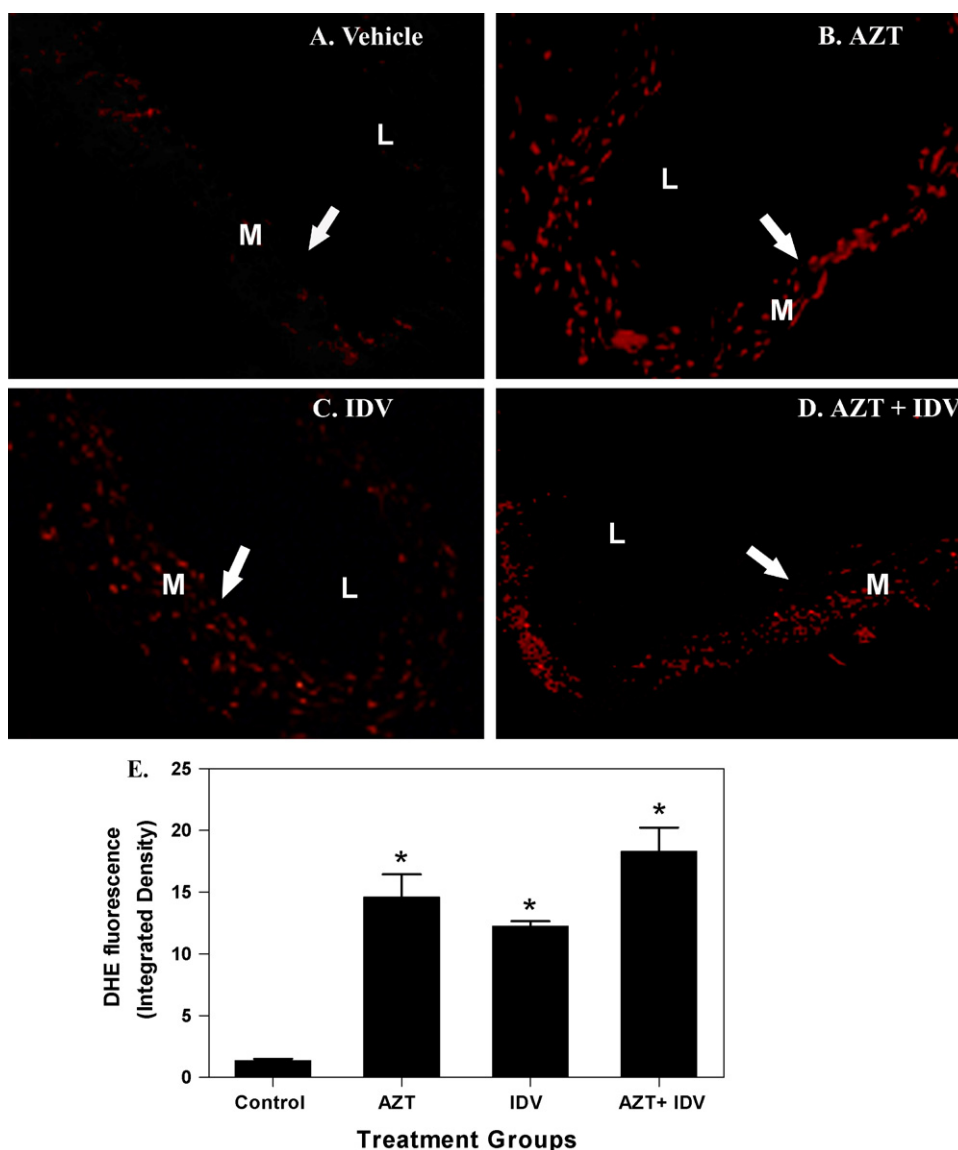
Atherogenesis is a complex disease etiology characterized by vessel wall remodeling and a concomitant accumulation of lipid in the vessel wall. Vascular IMT, or vessel wall thickness, is a valid surrogate for assessing preclinical atherosclerosis. This has been demonstrated by vascular pathology (Pignoli et al., 1986; Persson et al., 1994), as well as epidemiological studies (Chambless et al., 1997). Furthermore, regression of atherosclerotic risk factors was shown to be correlated with decreased IMT (Wiklund et al., 2002). One example is that administration of lipid-lowering therapeutics results in a decrease in IMT in the common carotid artery (Blankenhorn et al., 1993; Furberg et al., 1994).

Rodents are generally considered atherosclerosis-resistant due to their low levels of cholesteryl ester transferase activity (Tall, 1986). As a result, they do not develop hyperlipidemia and hypercholesterolemia, which are key factors for developing atherosclerosis. Induction of atherosclerosis in mice generally requires significant manipulation, either genetically or using a combination of insults. For example, mice deficient in apolipoprotein E can develop atherosclerotic lesions that can be further accelerated by administering a high fat diet. In these studies, we used C57BL/6 administered an atherogenic diet. Certain strains of inbred mice like the C57BL/6 exhibit a 2–3-fold increase in levels of plasma

cholesterol upon feeding a high cholesterol, high fat diet that is supplemented with a low concentration of cholate. The predominant effect on total cholesterol is due to an increase in low density lipoproteins (LDL) or very low density lipoproteins (VLDL). Cholate in the diet presumably facilitates intestinal cholesterol absorption and also interferes with the hepatobiliary excretion of cholesterol, ultimately increasing plasma cholesterol levels in mice (Vergnes et al., 2003). As a consequence of this combination treatment with the high fat diet plus cholate, they develop several layers of foam cells in the subendothelial space (Paigen et al., 1985). As shown here, after feeding this diet for 20 weeks, the plasma cholesterol levels were elevated more than 2-fold (Table 1), as compared to less than 100 mg/dL with a normal rodent diet (Stokes et al., 2007). With this murine model, we demonstrated that treatment with antiretrovirals further increased aortic IMT and CSA (Fig. 1) compared to the atherogenic diet alone.

One of the most important findings of these studies was that, in addition to the protease inhibitor indinavir, treatment with the NRTI AZT also induced a significant increase in aortic intima-media thicknesses and cross-sectional areas in C57BL/6 mice fed an atherogenic diet. The NRTIs are an important component of an initial therapy for the HIV infection and have been widely used as the backbone for antiretroviral therapy since the 1980s. NRTI toxicities have been well documented in hepatocytes (Velsor et al., 2004) and cardiomyocytes (Lund and Wallace, 2004), and an NRTI-associated mitochondrionopathy has been described (Lewis, 2004). Our prior studies investigated the effects of AZT, either alone or in combination with indinavir, on vascular cells in both *in vitro* and *in vivo* experiments. These studies suggested that AZT can significantly impair endothelium-dependent vessel relaxation (Jiang et al., 2006), and can also promote VSMC proliferation subsequent to endothelial dysfunction (Hebert et al., 2004). In this study, we showed that both AZT and indinavir significantly increased aortic IMT and CSA in C57BL/6 mice after 20 weeks of treatment (Fig. 1). Indinavir significantly elevated plasma cholesterol levels after 5 months of treatment (Table 1), and this effect may contribute to the observed increase in aortic IMT and CSA. However, the increases in lipid levels occurred only with chronic treatment, since cholesterol levels were not elevated higher than controls at 2 weeks after the initiation of therapy. AZT, on the other hand, increased aortic IMT and CSA, with only a transient and modest increase in plasma total cholesterol levels measured at 2 weeks after the initiation of treatment (Table 2).

To examine more closely the role of oxidant injury in the observed antiretroviral-induced exacerbation of atherogenesis, we conducted an additional experiment in which animals were administered an atherogenic diet ± antiretrovirals for 2 weeks and levels of ROS in aortic cross sections, as well as plasma levels of 8-isoprostanes and aortic GSH/GSSG ratios were assessed. Though the antiretroviral-induced effects on circulating lipid levels were variable, pronounced increases in ROS production were observed across all antiretroviral treatment groups (Fig. 4). This increased ROS production apparently resulted in an oxidative stress condition, since the vascular GSH/GSSG ratios were significantly reduced and the plasma 8-isoprostane levels were increased (Fig. 5). Note that 8-isoprostane (8-iso-PGF<sub>2α</sub>) is a non-enzymatic lipid peroxidation product that serves as a sensitive marker for lipid oxidation and oxidative stress in biological fluids (Morrow et al., 1990). Furthermore, quantification of 8-iso-PGF<sub>2α</sub> has been used widely as an index of atherosclerosis (Morrow, 2005). That the oxidant injury appeared early after the onset of drug therapy suggests that it may have an important role in the mechanism for antiretroviral-mediated exacerbation of atherogenesis. Though increases in ROS production and 8-isoprostanes were less significant at 20 weeks (Figs. 2 and 3) compared to 2 weeks (Figs. 4 and 5), we rationalize that this could be because the antiretroviral drugs accelerate



**Fig. 4.** Aortic reactive oxygen species production measured at 2 weeks after treatment with a high fat diet (A) or a high fat diet plus, (B) AZT, (C) indinavir, or (D) AZT plus indinavir. Aortic cross sections taken near the origin were incubated with  $2\ \mu\text{M}$  dihydroethidium. Total magnification for all images is approximately  $200\times$ . The arrows indicate vascular endothelium, “M” denotes the medial layer, and “L” indicates the location of the vessel lumen. (E) Quantitation of reactive oxygen species in aortic cross sections of mice treated for 2 weeks with a high fat diet  $\pm$  antiretrovirals. DHE-stained images were analyzed for color density using Image J software. Data represent mean densities  $\pm$  standard error. ANOVA revealed a significant effect of treatment ( $p < 0.05$  versus controls).

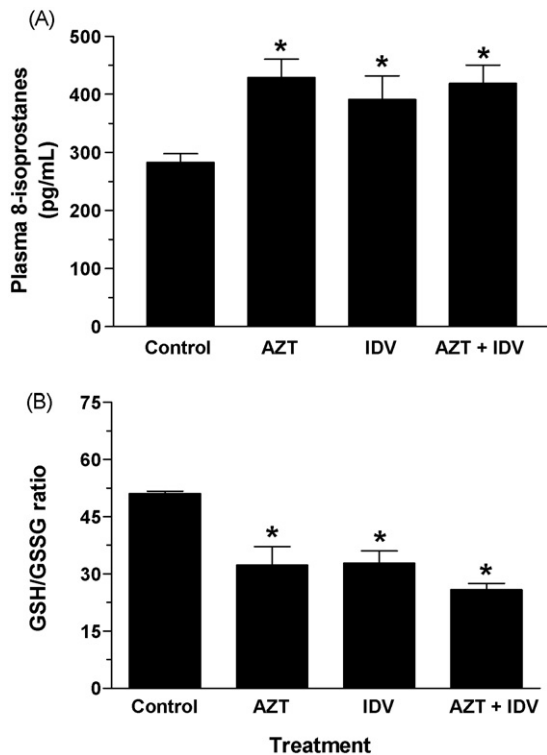
the time course for production of oxidation products and this may peak at an earlier time point than is observed in the diet-only controls. An alternate explanation is that at 20 weeks, the drug effect may be overwhelmed by the effect of the atherogenic diet.

It is noteworthy that when the animals were co-administered AZT and indinavir, the drug treatment exhibited an additive effect on IMT. This may suggest that combination treatment with these two antiretrovirals may predispose HIV-infected patients to additive adverse cardiovascular effects. Although many antiretroviral drugs are now available, and it is likely that there are both inter- and intra-class differences, our findings strongly suggest that the pro-atherogenic effects of antiretroviral drugs extends beyond the protease inhibitors. It should furthermore be emphasized that the plasma drug levels measured in these experiments were somewhat low compared to therapeutic levels in humans, which may indicate that effects in HIV patients may be even greater than what was observed here.

It was also notable that the combination treatment did not potentiate oxidant injury in the vessels compared to single drug

administrations (Figs. 2–5). It is possible that these two antiretrovirals may interact *in vivo* so as to attenuate the extent of vascular oxidant production, thus contributing to the lack of an additive effect on ROS production. An example might be that one drug may increase the metabolism of the other, such that the serum level of a given drug is lower for a combination treatment, and its toxic effect is diminished. However, in these experiments, this evidently did not occur (Table 2), as serum drug levels were not significantly different between the single drug, compared to the combination treatments. On the other hand, we did observe an additive effect on IMT. This additive effect on IMT may suggest that the drug combination triggers an alternate mechanism, for example, elevations in circulating lipid levels (Table 2) or an inflammatory response, in addition to oxidant generation, such that the collective effect was additive.

Prior studies by our laboratory and by other groups showed that both AZT and indinavir significantly impair vascular endothelium-dependent relaxation, indicating drug-induced endothelial dysfunction (Shankar et al., 2005; Jiang et al., 2006). We also showed that the endothelial dysfunction was not correlated with the plasma



**Fig. 5.** Plasma 8-isoprostane levels and glutathione-to-glutathione disulfide (GSH/GSSG) ratios for aortic tissues of mice treated for 2 weeks with a high fat diet  $\pm$  antiretrovirals. Data represents means  $\pm$  standard error. ANOVA revealed a significant effect of treatment compared to controls ( $p < 0.05$ ).

lipid levels, and *in vitro* studies suggested a mitochondrionopathy and a concomitant production of ROS may be a mechanism for this drug-induced endothelial dysfunction (Jiang et al., 2007). It is well accepted that endothelial dysfunction is an initiating event in atherogenesis that is correlated with mature atherosclerosis (Busse and Fleming, 1996). Studies presented here demonstrate that the next stage of atherogenesis, vessel wall thickening, e.g., hyperplasia, was also induced by these two antiretrovirals. However, because the model utilized here exhibited mainly an increase in medial area and not truly a growth of neointima, it is not well understood whether the antiretrovirals can modulate vascular neointimal hyperplasia by increasing VSMC migration and proliferation after an initiating endothelial dysfunction. Current studies in our laboratory, using a carotid artery endothelial denudation model on C57BL/6 background mice, are thus aimed at determining effects on neointimal formation following endothelial dysfunction.

In summary, taken together, results presented here demonstrate that in the absence of retrovirus, antiretrovirals such as AZT and indinavir can induce premature atherosclerosis. Antiretroviral-induced changes in lipid profiles may be important in the development of atherosclerosis, but ROS production and oxidative stress may also be an important mechanism contributing to antiretroviral-associated cardiovascular diseases. These and our prior studies (Hebert et al., 2004; Jiang et al., 2007) suggest that this direct mechanism may involve antiretroviral-induced ROS produced, perhaps *via* a mitochondrionopathy.

Though the clinical use of indinavir has diminished significantly in recent years, use of AZT is increasing, owing to its use in a dual NRTI, single capsule formulation marketed as Combivir (Soriano et al., 2008). Our results demonstrating the cardiovascular toxicity of AZT are thus of high clinical significance. On the other hand, our findings with respect to indinavir and the AZT/indinavir combination may further highlight the problems with the use of indinavir

and may underscore some contributing factors for its diminished use.

#### Conflict of interest statement

None.

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