Laboratory Investigation (2009) 89, 1355–1363

Highly active antiretroviral therapy drugs inhibit *in vitro* cholesterol efflux from human macrophage-derived foam cells

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We previously reported that HIV protease inhibitor, ritonavir, could inhibit cholesterol efflux and induce endothelial dysfunction. In this study, we further determined the effects and molecular mechanisms of a clinically relevant combination of highly active antiretroviral therapy (HAART) drugs on *in vitro* cholesterol efflux from human macrophage-derived foam cells. Foam cells derived from human monocyte cell line (THP-1) and periphery blood mononuclear cells (PBMCs) treated with HAART drugs including stavudine, didanosine and indinavir individually or in combination of three drugs (3-plex), followed by the initiation of cholesterol efflux with apolipoprotein A-I (apoA-I). Clinically relevant concentrations of HAART 3-plex significantly reduced cholesterol efflux in foam cells derived from THP-1 and PBMCs. HAART 3-plex significantly reduced the intracellular cholesterol transport molecule caveolin-1, whereas it increased superoxide anion production in THP-1 foam cells as compared with controls. Furthermore, mitochondrial membrane potential was significantly reduced, whereas the expression of NADPH oxidase subunit $p67^{phox}$ was increased in HAART 3-plex-treated macrophages. Consequently, antioxidants including ginsenosides Rb1 and Rg1, S-allyl cysteine sulphoxide (SACS), simvastatin (SVT) and vitamin E significantly abolished HAART 3-plex-induced inhibition of cholesterol efflux. Therefore, HAART drugs significantly inhibit cholesterol efflux from human macrophage-derived foam cells through downregulation of caveolin-1 and increase of oxidative stress.

Laboratory Investigation (2009) **89,** 1355–1363; doi:10.1038/labinvest.2009.85; published online 21 September 2009

KEYWORDS: HAART; macrophage; cholesterol efflux; superoxide anion; antioxidant; ginsenosides

At the end of 2007, 33.2 million persons were living with HIV infection, with 2.5 million newly infected and 2.1 million patients died from HIV.[1] More than 20 antiretroviral drugs now are available in the United States of America, including protease inhibitor (PI), non-nucleoside analogue reverse transcriptase inhibitor (NNRTI) and nucleoside analogue reverse transcriptase inhibitors (NRTIs). Highly active antiretroviral therapy (HAART), a term that refers to use of combinations of three or more antiretroviral agents, has transformed HIV infection into a manageable chronic disease and has dramatically improved the prognosis of HIV-infected patients.[2] However, there is a growing concern regarding risk factors for cardiovascular disease observed in patients receiving HAART.[3–7] Antiretroviral therapies have been shown to increase proatherogenic cholesterol and triglyceride levels and contribute to the development of insulin resistance and accumulation of abdominal fat.[8] HIV PIs in particular have been associated with risk factors for cardiovascular disease and a possible increased incidence of myocardial infarction.[9] There is evidence that antiretroviral medications may directly affect endothelial function, which could lead to the development of premature cardiovascular disease.[6] Recently, a clinical study documented that HAART exposure was significantly and independently associated with the presence of carotid atherosclerosis in the multivariate logistic regression analysis for all HIV-infected patients.[10]

Although clinical studies indicate that HAART may contribute to cardiovascular disease, the molecular mechanisms of this emerging clinical problem are largely unknown. Our previous studies demonstrated that HIV PIs, such as

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Received 30 November 2008; revised 30 June 2009; accepted 3 July 2009

ritonavir, amprenavir or saquinavir could induce endothelial dysfunction including endothelium-dependent vasorelaxation. The oxidative stress in the vessel walls, activation of mitogen-activated protein kinases (MAPKs) signaling pathways, downregulation of endothelial nitric oxide synthase (eNOS) and alteration of other gene expressions are involved in the progression of HIV PIs-induced endothelial dysfunction. Furthermore, endothelial dysfunction induced by HIV PIs can be reversed by antioxidant ginsenosides Rb1 (Rb1).[11,12] In addition to endothelial dysfunction, accumulation of cholesterol in macrophages resulting from inhibition of cholesterol efflux could be another important factor attributing to the progression of atherosclerosis. We have recently reported that HIV PI ritonavir could induce a decrease in cholesterol efflux in human monocyte-derived foam cells through oxidative stress and activation of ERK1/2. Consequently, reducing oxidative stress by ginsenosides Rb1 is able to abolish the ritonavir-induced inhibition in cholesterol efflux.[4]

HAART drugs stavudine (d4T), didanosine (DDI) and indinavir (IDV), and combination of three drugs (3-plex) are used in treating HIV patients. However, there is no report of this combination on vessel wall biology and macrophage functions. To extend our previous discoveries of single anti-HIV drug on cholesterol efflux,[4] the current study was therefore to test our hypotheses that clinical combination of HAART drugs may also inhibit cholesterol efflux in macrophage-derived foam cells through unique molecular pathways. This study will elucidate the molecular mechanisms of HAART drugs-related cardiovascular complications and provide valuable information for the development of effective strategies for the prevention and treatment of such clinical problems.

MATERIALS AND METHODS

Cell Culture

Human THP-1 monocytes (human acute monocytic leukaemia) were differentiated into macrophages by the addition of phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) for 4 days as previously described.[4] Human PBMCs were obtained from Gulf Coast Regional Blood Center (Houston, TX, USA).

acLDL Loading and Pretreatment

Macrophages were transformed into foam cells by incubation with acLDL (50 μg/ml) and [1α,2α(n)-^{3}H]-cholesterol (1 μCi/ml) in the serum-free medium containing 1.5% BSA for 48 h. The macrophage-derived foam cells were then incubated for 24 h in the presence or absence of d4T (2–8 μM), DDI (5.5–22 μM), IDV (6.25–25 μM), HAART 3-plex (d4T: 4 μM; DDI: 11 μM; and IDV: 12.5 μM) and antioxidants at 10 μM, including ginsenosides Rb1 and Rg1, S-allyl cysteine sulphoxide (SACS), simvastatin (SVT), vitamins C and E.

Cholesterol Efflux

Cholesterol efflux from THP-1 macrophages was initiated by the addition of 50 μg/ml apoA-I for 24 h. Radioactivity in both supernatants and cell lysates was measured by TopCount-NXT (Packard Instrument Inc., Downers Grove, IL, USA) as previously described.[4]

Real Time RT-PCR and Western Blot Analysis

The mRNA levels of caveolin, scavenger receptor B1 (SR-B1) and several enzymes related to superoxide anion production including NADPH oxidase subunits p22^{phox}, p47^{phox}, and p67^{phox} were determined by real time RT-PCR, as shown in our previous publication.[4] The protein levels of p67^{phox} and caveolin-1 were determined by western blot analyses.

Superoxide Anion Analysis by Flow Cytometry

Macrophage-derived foam cells were treated with or without HAART 3-plex for 48 h. Cells were incubated with 0.5-ml dihydroethidium (DHE, 10 μM) for 20 min at room temperature. Superoxide anion in the cells was analyzed by FACS Calibure flow cytometry.

Assessment of Mitochondrial Membrane Potential ($\Delta\Psi$m)

$\Delta\Psi$m was assessed by using flow cytometry analysis of cells stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolecarbocyanide iodine (JC-1, MitoScreen kit, BD Biosciences, San Jose, CA, USA).

Measurement of ATP Levels

Adenosine triphosphate levels were measured with an ATPLite kit (PerkinElmer, Wellesley, MA, USA) following manufacturer's instructions.

Statistical Analysis

All data are presented as the mean ± s.e.m. Inter-group differences were analyzed using one-way ANOVA for the comparison of three or more groups. Student's t-test was used for comparison between two groups. A P-value <0.05 was regarded as significant.

Online Supplementary Data

Detailed materials and methods as well as Supplementary table I are provided on the Online Supplementary data.

RESULTS

HAART Drugs Inhibit Cholesterol Efflux from Foam Cells Derived from Human THP-1 and PBMCs

As one of the prominent features of atherosclerosis is the presence of lipid-laden macrophages, we first tested the effects of individual HAART drug and combination of 3-plex on cholesterol efflux from foam cells by measuring ^{3}H-cholesterol. THP-1 macrophage-derived foam cells were treated with d4T, DDI, IDV or HAART 3-plex, and cholesterol efflux was initiated by the addition of apoA-I. Significant reduc-

tions of cholesterol efflux to apoA-I macrophage-derived foam cells were observed in a concentration-dependent manner in response to d4T (Figure 1a) and IDV treatment (Figure 1b). At 8 μM of d4T and 12.5 μM of IDV, the cholesterol efflux from THP-1 showed significant decreases by 41 and 31%, respectively, compared with controls (apoA-I only) ($P<0.05$ for both, $n=6$). Although DDI treatment showed a reduction of cholesterol efflux from foam cells, this difference did not reach the significance (Figure 1c). HAART 3-plex contained 4 μM d4T, 11 μM DDI and 12.5 μM IDV at their clinically relevant concentrations (Supplementary table I). At the individual concentration of drugs, they all showed the trend of inhibiting cholesterol efflux to apoA-1 from THP-1-derived macrophages (IDV showed a statistical significance). HAART 3-plex significantly reduced the cholesterol efflux to apoA-I from THP-1-derived macrophages by 43% (Figure 1d, $P<0.05$, $n=6$), and the reduction magnitude was higher than the effect of individual drug used at the same concentration, suggesting HAART 3-plex may have the additive or synergistic effect on the cholesterol efflux. Furthermore, we determined the effect of HAART on cholesterol efflux from foam cells derived from human PBMCs. Consistent with the effect of HAART 3-plex on THP-1-derived foam cells, the treatment with HAART 3-plex significantly decreased cholesterol efflux from human PBMCs-derived foam cells as compared with the controls (Figure 1e, $P<0.05$, $n=6$).

During the experiment, we did not observe any abnormal changes in cell numbers and the morphology of these cell cultures between the treatment of HAART drugs for 24 and 48 h and untreated controls, indicating there was no potential toxicity of HAART drugs used in this study at the experimental conditions.

HAART Drugs Decrease the Expression of Calveolin-1 in Human THP-1 Macrophage-Derived Foam Cells

To determine why HAART 3-plex could affect cholesterol efflux, the expression of several key molecules involved in cholesterol efflux including caveolin-1, caveolin-2 and SR-B1 was analyzed. Treatment with HAART 3-plex decreased the mRNA level of caveolin-1 in foam cells by 27% when compared with controls (Figure 2a, $n=4$, $P<0.05$). Consistent with mRNA data, the protein level of caveolin-1 by western blot was also significantly reduced in HAART 3-plex-treated cells compared with controls (Figure 2b). In a separate experiment, caveolin-1 mRNA levels were determined after the treatment of individual drug or 3-plex by real time RT-PCR. Individual drugs showed the trend of decreasing caveolin-1 mRNA levels. For example, IDV at 12.5 μM significantly reduced the mRNA level of caveolin-1 in foam cells as compared with controls (Figure 2c, $n=4$, $P<0.05$). HAART 3-plex significantly reduced caveolin-1 mRNA levels in THP-1 derived macrophages, and the reduction magnitude was higher than the effect of any individual drug used at the same concentration (Figure 2c). Thus, 3-plex may have additive or synergistic effects on the inhibition of caveolin-1 expression. However, the expression of SR-B1 and caveolin-2 did not

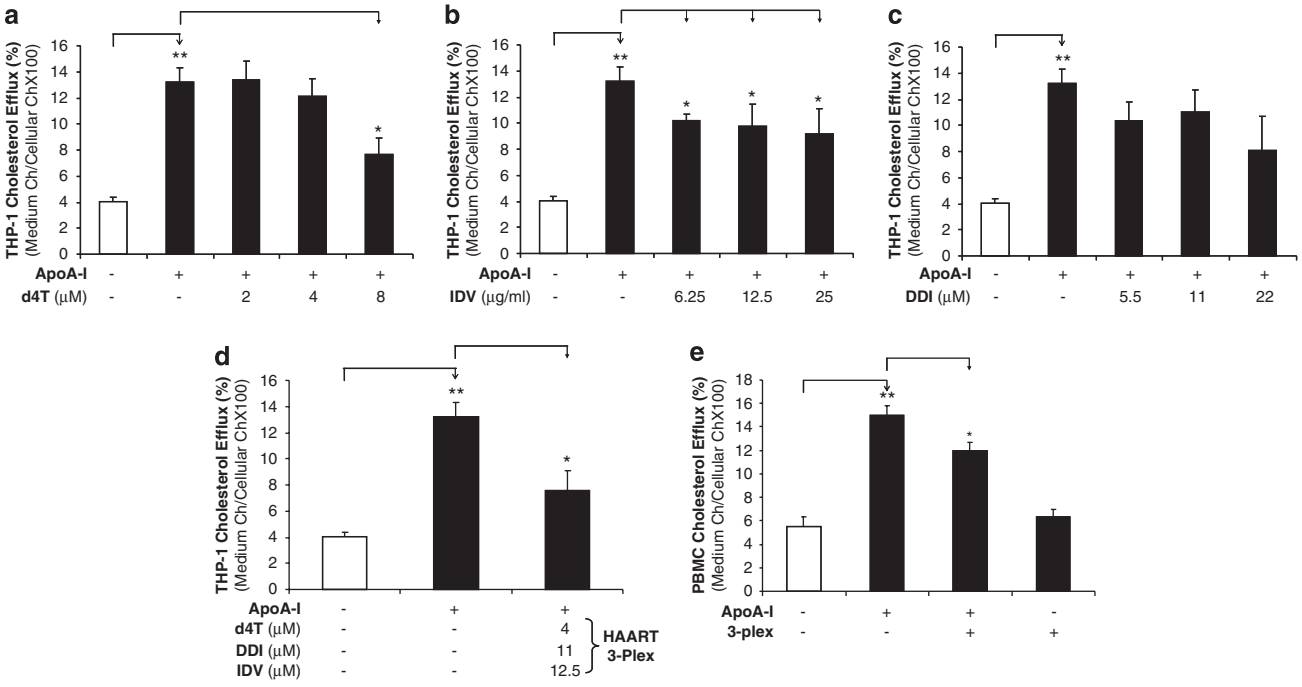

Figure 1 Effects of highly active antiretroviral therapy (HAART) drugs on cholesterol efflux from foam cells derived from THP-1 and human periphery blood mononuclear cells(PBMCs). Effects of d4T (**a**), IDV (**b**) and DDI (**c**) on cholesterol efflux in THP-1 derived foam cells to apolipoprotein A-I (apoA-I) in a concentration-dependent manner as compared with controls. (**d**) Effect of HAART 3-plex on cholesterol efflux from THP-1-derived foam cells. (**e**) Effect of HAART 3-plex on cholesterol efflux from human PBMCs-derived foam cells. Data represent mean ± s.e.m. *$P<0.05$ *vs* controls (apoA-I only), **$P<0.001$ *vs* DMSO controls, $n=6$. d4T: stavudine, DDI: didanosine and IDV: indinavir.

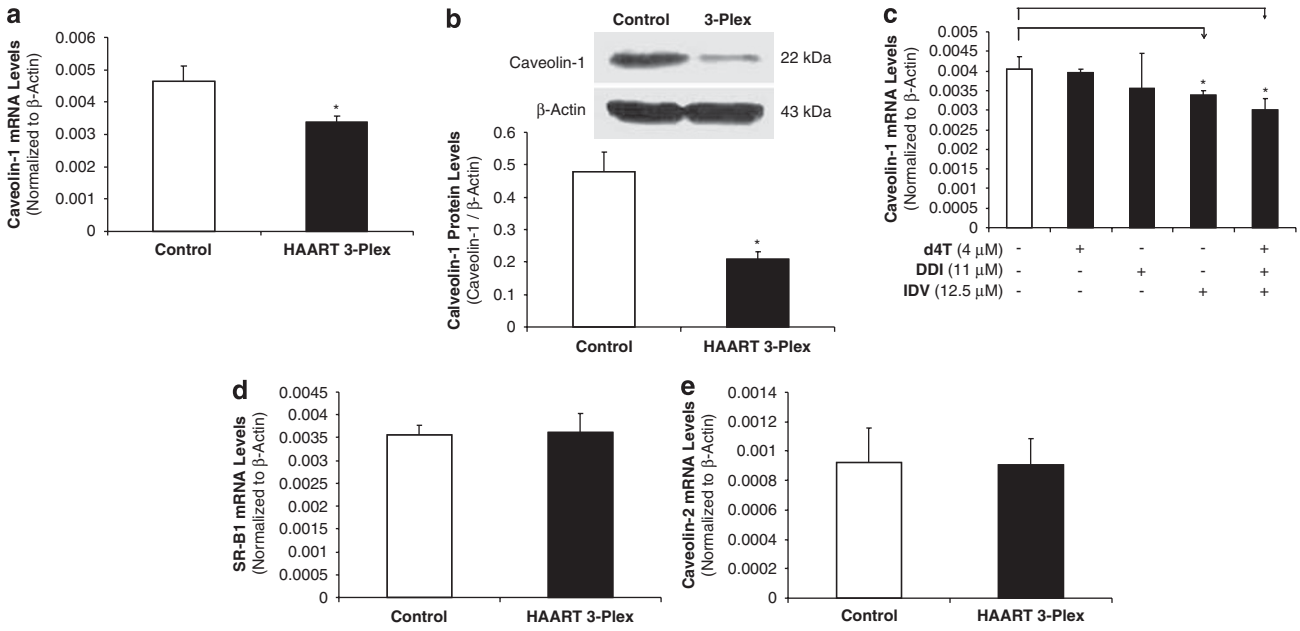

Figure 2 Effects of highly active antiretroviral therapy (HAART) on the expression of calveolin-1 in THP-1-derived foam cells. Macrophage-derived foam cells were treated with HAART 3-plex for 48 h. The expression of calveolin-1 was measured in mRNA level by real time RT-PCR and in the protein level by western blot. HAART 3-plex significantly decreased both the mRNA level (**a**) and the protein level (**b**) of calveolin-1 compared with controls. Effects of individual HAART drug and 3-plex on the mRNA levels of calveolin-1 (**c**) scavenger receptor B1 (SR-B1) (**d**) and calveolin-2 (**e**) by real time RT-PCR analysis. Data represent mean ± s.e.m. $^*P<0.05$, *vs* controls (without HAART 3-plex). $n=4$ for real time RT-PCR. $n=3$ for western blot.

show any significant changes between the HAART 3-plex-treated groups and controls (Figure 2d and e).

HAART Drugs Increase Superoxide Anion Production in Human THP-1 Macrophage-Derived Foam Cells

To determine the possible link between cholesterol efflux and oxidative stress, we evaluated the superoxide anion production from macrophage-derived foam cells in the presence of HAART 3-plex by flow cytometric measurement of DHE staining. Foam cells treated with HAART 3-plex for 48 h showed a significant increase of superoxide anion production by 66% when compared with controls (Figure 3a and b, $P<0.05$). In a separate experiment, the effect of the individual HAART drug on superoxide production was determined. IDV (12.5 μM) significantly increased superoxide anion production in THP-1 derived macrophages, but not d4T (4 mM) and DDI (11 μM) (Figure 3c, $P<0.05$). Compared with HAART 3-plex treatment, IDV had a less effect on the increase of superoxide production. These data suggest that HAART 3-plex may have additive or synergistic effects on the increase of superoxide anion production in THP-1 cells.

HAART Drugs Induce Mitochondrial Dysfunction and Increase the Expression of NADPH Oxidase Subunit P67^{phox} in Human THP-1 Macrophage-Derived Foam Cells

To determine the possible sources of HAART 3-plex-induced superoxide anion production, the mitochondrial function and expression of NADH/NADPH oxidase subunits, a major ROS generating enzyme, were analyzed. Mitochondrial dysfunction could result in the increase of superoxide anion production and the decrease of ATP production. Mitochondrial $\Delta\Psi$m was detected by JC-1 staining and flow cytometry analysis. R2 detects mitochondrial JC-1 aggregates (red fluorescence), whereas R3 detects cytosol monomeric JC-1 (green fluorescence). $\Delta\Psi$m was average 17.07 ± 1.65% positive cells in the control group, whereas $\Delta\Psi$m was reduced to 8.62 ± 0.68% positive cells in the HAART 3-plex treated cells, representing a 51% reduction as compared with the control (Figure 4a and b, $P<0.05$). In addition, treatment of HAART 3-plex significantly reduced ATP levels by 21% when compared with controls (Figure 4c, $P<0.05$). Furthermore, treatment with HAART 3-plex induced a significant increase in NADPH oxidase subunit p67^{phox} in foam cells in both mRNA and protein levels by 83 and 81%, respectively, as compared with controls (Figure 5a and b, $P=0.043$). However, the expression of p22^{phox} and p47^{phox} did not show any significant changes between the HAART 3-plex-treated groups and controls (Figure 5c and d).

Effects of Antioxidants on HAART Drugs-Induced Inhibition in Cholesterol Efflux in Human THP-1 Macrophage-Derived Foam Cells

To further confirm the contribution of increase of superoxide anion on the cholesterol efflux, we evaluated the blocking effects of six antioxidants including Rb1, Rg1, SASC, SVT,

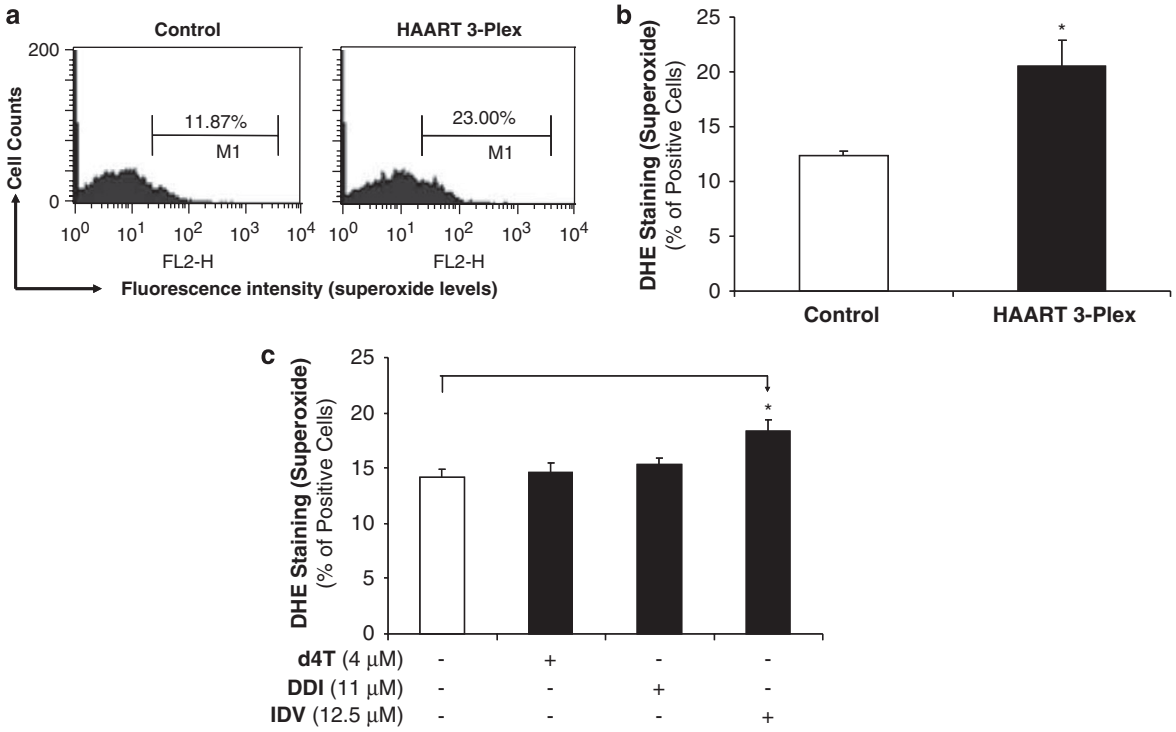

Figure 3 Effects of highly active antiretroviral therapy (HAART) drugs on superoxide anion production in THP-1-derived foam cells. Superoxide anion in the foam cells treated with or without HAART 3-plex was analyzed by flow cytometry with dihydroethidium (DHE) staining. (**a**) Representative histogram of flow cytometry analysis of DHE staining. (**b**) Quantitative data of three samples of DHE staining. (**c**) Effects of IDV (12.5 μM), d4T (4 μM) and DDI (11 μM) on superoxide anion production. Data represent mean ± s.e.m. $^*P<0.05$, *vs* controls. $n=3$.

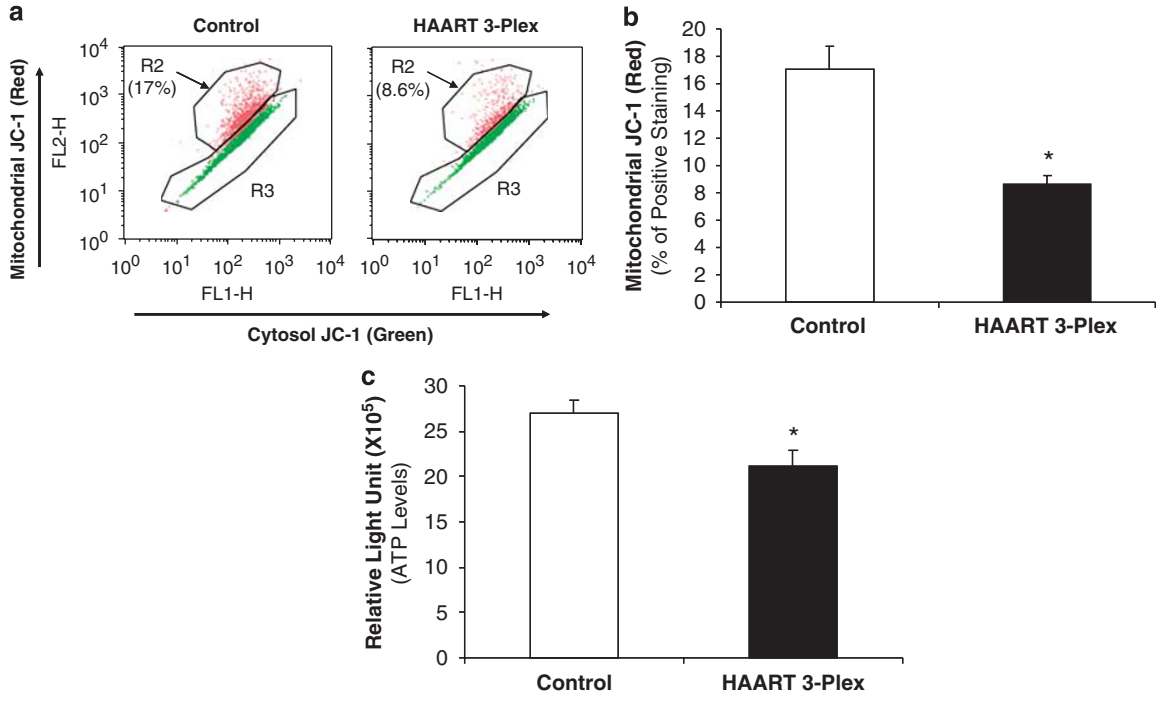

Figure 4 Effects of highly active antiretroviral therapy (HAART) drugs on mitochondrial membrane potential and ATP production in THP-1-derived foam cells. (**a**) Representative histogram of flow cytometry analysis of JC-1 staining. R2 detects mitochondrial JC-1 aggregates (red fluorescence), whereas R3 detects cytosol monomeric JC-1 (green fluorescence). (**b**) Quantitative data of three samples of JC-1 staining. (**c**) Cellular ATP levels were measured with an ATPLite kit. Data represent mean ± s.e.m. $n=6$. $^*P<0.05$.

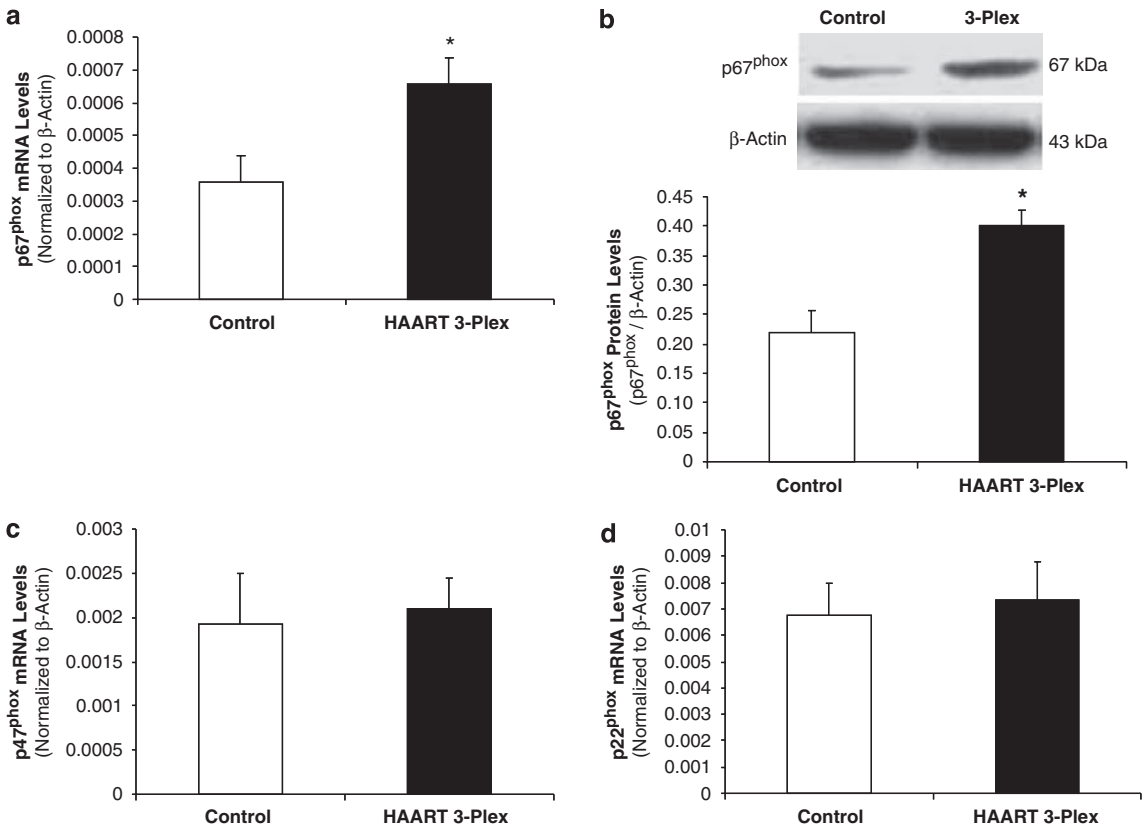

Figure 5 Effects of highly active antiretroviral therapy (HAART) drugs on the expression of NADPH oxidase subunits in THP-1-derived foam cells. Macrophage-derived foam cells were treated with HAART 3-plex for 48 h. The NADPH oxidase subunit p67^{phox} was measured in mRNA level by real time RT-PCR (**a**) and in protein level by western blot (**b**). Effects of HAART 3-plex on the mRNA levels of NADPH oxidase subunits p47^{phox} (**c**) and p22^{phox} (**d**) by real time RT-PCR. Data represent mean ± s.e.m. $^{**}P<0.01$ *vs* controls (without HAART 3-plex). $n=4$ for real time RT-PCR. $n=3$ for western blot.

vitamins C and E on the HAART 3-plex-induced inhibition in cholesterol efflux in THP-1 cells. At 10 μM, five antioxidants (Rb1, Rg1, SASC, SVT and vitamin E) significantly increased cholesterol efflux from THP1 cells to apoA-I by 17, 23, 49, 32 and 42%, respectively, compared with the treatment of HAART 3-plex alone group (Figure 6, $P<0.05$, $n=6$). However, there was no significant effect of vitamin C at this concentration on reversing the HAART 3-plex-induced inhibition of cholesterol efflux in THP-1 cells. Meanwhile, treatments with six antioxidants alone did not show any significant effect on cholesterol efflux in THP-1 cells compared with controls (apoA-I only). These data indicate that oxidative stress has a critical role in HAART 3-plex-induced cholesterol efflux in macrophages. In a separate experiment, we determined whether antioxidant SASC (10 μM) could block HAART 3-plex-induced decrease in caveolin-1 mRNA levels in THP-1 derived foam cells by real time RT-PCR analysis. Caveolin-1 mRNA levels (normalized to β-action) in the control, HAART 3-plex and 3-pex plus SACS (10 μM) groups were 0.00404, 0.003 and 0.0032, respectively. Antioxidant SACS increased caveolin-1 mRNA levels by 6.25% compared with that in HAART 3-plex treated cells, which did not reach statistical difference for three experiments.

DISCUSSION

Clinical reports indicate that the therapeutic benefit of long-term use of HAART drugs is compromised by an increased risk of atherosclerosis. The current data provide further mechanisms for the effect of HAART drugs on the vasculature. This study, for the first time, provides three novel findings: (1) HAART drugs inhibit cholesterol efflux from human macrophage-derived foam cells; (2) HAART drugs decreases expression level of caveolin-1, which is a key molecule mediating cholesterol efflux; and (3) HAART drugs increase superoxide anion production, which may function as signal transduction pathways for HAART drug action in macrophage-derived foam cells. Accordingly, several antioxidants effectively block the effects of HAART drugs on foam cells. The inhibition of cholesterol efflux from human macrophage-derived foam cells by HAART drugs could result in the accumulation of cholesterol in foam cells, thereby attributing to the progression of atherosclerosis. These data provide a better understanding of the molecular mechanism of HAART drug-associated cardiovascular complications and suggest a novel approach in preventing this clinical problem.

Cholesterol efflux is the process that removes excess cholesterol from cells and tissues, including the arterial wall.[13]

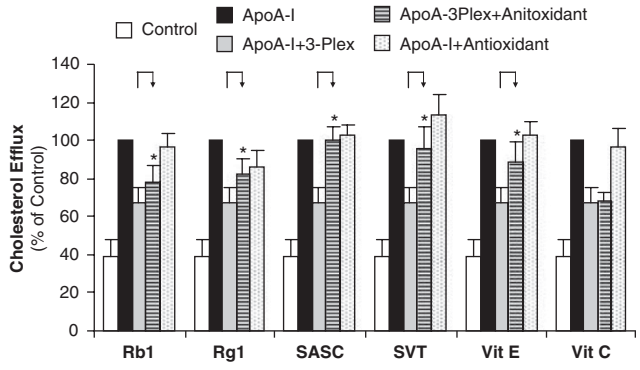

Figure 6 Effects of antioxidants on highly active antiretroviral therapy (HAART) drugs-induced inhibition in cholesterol efflux in THP-1-derived foam cells. The inhibition in cholesterol efflux induced by HAART 3-plex in THP-1 to apolipoprotein A-I (apoA-I) was significantly abolished by antioxidants including Rb1, Rg1, SASC, SVT and vitamin E, but not vitamin C. Data represent mean ± s.e.m. $^*P<0.05$ *vs* controls (HAART 3-plex only), $n=6$. Rb1: Ginsenoside Rb1, Rg1: Ginsenoside Rg1, SACS: S-allyl cysteine sulphoxide, SVT: simvastatin, Vit C: vitamin C and Vit E: vitamin E.

Decreased cholesterol efflux from the arterial wall may potentially promote the progression of atherosclerosis. The formation of foam cells, originated from monocyte-derived macrophages and vascular smooth muscle cells within the arterial wall is thought to have a central role in the development of atherosclerotic lesions.[14] The human monocytic cell line THP-1 can be induced by treatment with phorbol ester to differentiate into macrophage-like cells, which mimic human monocyte-derived macrophages in several aspects, including induction of scavenger receptors, accumulation of cholesterol esters, and secretion of lipoprotein lipase. Thus, THP-1 cells have been extensively used in cholesterol efflux to apoA-I in many studies, as well as in ours.[4,15]

The current data show that HIV protease inhibitor IDV or its combination with d4T and DDI (HAART 3-plex) significantly inhibit cholesterol efflux to apoA-I in foam cells derived from THP-1 and human PBMCs. The HAART combination used in this study is based on clinical trials.[16] Understanding the cellular and molecular mechanisms of HAART associated complications is critical to develop new strategies to prevent or treat these complications. Several relatively new HAART drugs, such as tenofovir, emcitrabine, efavirenz, atazanavir and fosamprenavir, have recently been introduced in clinical use and their clinical complications are still under active investigations. The concentrations of HAART drugs used in this study are clinically relevant maximal plasma concentrations (Cmax) (Physicians' Desk Reference 2006). Consistent with current results, our previous data demonstrated that a single HIV PI ritonavir markedly reduced cholesterol efflux in THP-1-derived foam cells to both apoA-I and HDL.[4] Dressman *et al*[5] also reported that ritonavir could increase the level of cholesterol ester in THP-1 macrophages and human PBMCs. This study demonstrates that the combination of HAART drugs may attribute to the accumulation of cholesterol in macrophages by inhibiting cholesterol efflux, which most likely contributes the progression of atherosclerosis.

Several key molecules including caveolins and SR-B1 have been known to mediate cholesterol efflux.[13] Caveolae are invaginated microdomains at the surface of most peripheral cells, and have been implicated in many cellular activities. Caveolin-1, the main structural protein of caveolae, has been involved in the regulation of cellular cholesterol metabolism and efflux.[17] For example, downregulation of caveolin-1 by treatment with anti-sense oligonucleotides was reported to decrease cholesterol efflux to apoA-I.[18] A recent study suggests that caveolin-1 facilitates cholesterol efflux by inducing caveolae formation in aortic endothelial cells. Overexpression of caveolin-1 by transfection with caveolin-1 cDNA enhanced cholesterol efflux, whereas suppression of caveolin-1 by siRNA reduced cholesterol efflux in aortic endothelial cells.[19] Similarly, cholesterol efflux was increased in L1210-JF cells and human fibroblasts after transfection with caveolin-1.[17,20] This study shows that treatment with HAART 3-plex decreases the expression of caveolin-1, but not SR-B1 and caveolin-2, in foam cells. HAART 3-plex affects more caveolin-1 protein levels than mRNA levels indicating that the post-translational regulation may be more affected. Our previous study shows that ritonavir downregulated both levels of caveolin-1 and SR-B1 in these cells.[4] Thus, ritonavir and HAART 3-plex share some common mechanisms, whereas they also have different molecular pathways in inhibition of cholesterol efflux. Palmer *et al*[15] reported that triglyceride-rich lipoprotein could inhibit cholesterol efflux in these cells to apoA-I, whereas it did not affect levels of SR-B1 and caveolin-1, suggesting that exposure to these lipoproteins inhibits an alternative, anti-atherogenic pathway. A recent study indicates that the expression of HIV Nef can inhibit cholesterol efflux from macrophages.[21] Taken together, we demonstrate that HAART 3-plex inhibits cholesterol efflux through the downregulation of caveolin-1 in macrophages. HIV infection itself may also impair cholesterol efflux in macrophages with unique molecular mechanisms.

Reactive oxygen species are thought to be the key mediators for vascular inflammation and atherogenesis by a variety of mechanisms including regulation of gene expression.[22] ROS include molecules, such as hydrogen peroxide, hypochlorite ion, hydroxyl radical, and superoxide.[23] One of the major sources for ROS is the mitochondrium in cells. Mitochondrial respiration converts carbohydrates into high-energy metabolites such as ATP. This requires the sequential oxidation and reduction of the substrates using respiratory complexes. After oxidation by mitochondrial and plasma membrane oxidases, oxygen is reduced and the superoxide anion is formed. Other important sources of ROS within cells are the enzymatic reactions through dedicated enzymes including NADPH oxidase, which is well characterized as a major superoxide-generating enzyme in phagocytes. NADPH oxidase is a multicomponent enzyme comprised of five

subunits, namely $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$.[23] In this study, we detected a significant increase in superoxide anion production and decreases in the mitochondrial membrane potential and ATP production in HAART 3-plex-treated cells. In addition, treatment with HAART 3-plex increased the expression of $p67^{phox}$ in foam cells. These data indicate that increased superoxide anion production in HAART 3-plex-treated foam cells may result from mitochondrial dysfunction and upregulation of NADPH oxidase. Mondal *et al*[24] provided evidence that exposure of either IDV or nelfinavir combined with zidovudine and efavirenz increased ROS formation in human aortic endothelial cells. HAART drugs zidovudine and IDV disrupt endothelial cell junctions and mitochondria, and could cause vascular damage.[25] The current data provide further understanding of the role of HAART 3-plex in the ROS system of THP-1-derived foam cells, indicating that the oxidative stress may be directly involved in the inhibition of cholesterol efflux induced by HAART drugs. Thus, antioxidant may have the potential to prevent the damage induced by HAART drugs in the vascular system.

Antioxidants are a group of substances, which are present at low concentrations in relation to oxidizable substrates and significantly inhibit or delay oxidative processes while often being oxidized themselves. In recent years, there has been an increasing interest in the application of antioxidants to many diseases. In the current study, we have demonstrated that HAART 3-plex inhibits the cholesterol efflux, and induces oxidative stress in human macrophage-derived foam cells. This raises the possibility that suppression of oxidative stress may block the inhibition in cholesterol efflux induced by HAART 3-plex. To address this issue, we further determined the functional role of oxidative stress induced by HAART 3-plex in cholesterol efflux process by blocking ROS using several antioxidants including Rb1, Rg1, SACS, SVT and vitamins C and E. Ginsenoside Rb1 and Rg1 are those compounds that are responsible for the activity of ginseng, which is a widely recognized herbal drug from Chinese medicine and is known to have pharmacological effects against certain chronic diseases. Ginsenosides have protective roles in atherosclerotic plaque formation and various vascular injuries due in part to preventing free radical injury and inducing NO release.[26] Garlic is very effective on the basis of its antidiabetic, antibiotic, antiatherogenic and anticancer effects. SACS, a sulfur-containing amino acid of garlic, has been found to have both antidiabetic and antioxidant effects.[27] In addition to efficiently reducing plasma LDL cholesterol, statins have been shown to attribute to various effects on the vascular wall, which include improved endothelial function as well as antioxidant and anti-inflammatory activities.[28] Vitamins are one of the main defense mechanisms of the body's non-enzymatic antioxidant systems, in which vitamins C and E are the most studied natural antioxidants. Vitamin E may act by normalizing aberrant signal transduction and gene expression in anti-oxidant and non-antioxidant manners; in particular, over-expression of scavenger receptors and consequent foam cell formation can be prevented by vitamin E.[29]

Several studies indicate that oxidative stress directly regulates caveolin-1 expression in endothelial cells and macrophages. For example, endothelin-1 increased the ROS production and decreased caveolin-1 expression in human umbilical vein endothelial cells (HUVECs), whereas NADPH oxidase inhibitor apocynin could effectively block endothelin-1-induced caveolin-1 downregulation.[30] Hayashi *et al*[31] reported that high glucose concentrations increased oxidative stress and suppressed caveolin-1 expression in THP-1-derived macrophages. In this study, it is not clear whether antioxidant SACS (10 μM) could block the HAART 3-plex-induced caveolin-1 downregulation at mRNA and protein levels. Further investigations in this regard are needed.

Our current data demonstrate that the inhibition in cholesterol efflux induced by HAART drugs was effectively abolished by Rb1, Rg1, SACS, SVT and vitamin E, but not vitamin C. The reason for selecting these antioxidants in this study is the fact that all of them are commonly used dietary supplements. We have chosen the concentration of 10 μM for all antioxidants because the affectivity of these compounds is readily comparable.[4,32–35] Comparing the therapeutic magnitude of each agent may enhance our knowledge of these agents for the applications of reducing the complications of HAART drugs. In accordance with this study, our previous studies also showed protective effects of Rb1 on ritonavir-induced inhibition in cholesterol efflux.[4] Despite the antioxidant properties of vitamins E and C, their efficacy remains controversial in some recent clinical trials and meta-analyses. Factors responsible for the disappointing and discrepant results from observational and clinical studies on the relationship between antioxidant vitamins and cardiovascular diseases may include differences in the methodology of these studies, criteria for selection of patients such as gender, age, body weight, duration of treatment, vitamin supplement dosages and dietary habits.[36] In addition, different antioxidants may work on different pathways of ROS generation. Different risk factors including HAART drugs may produce oxidative stress with different mechanisms. Therefore, specific study should be conducted to investigate as to which particular pathway is induced by certain HAART drugs, and to test which antioxidant may be best for attenuating the effects of HAART drugs on cholesterol transport.

In summary, the current study demonstrates the functional relationship and molecular mechanisms of the effects of HAART drugs-induced inhibition in cholesterol efflux from human macrophage-derived foam cells. Consequently, antioxidants successfully improved HAART drugs-induced side effects on foam cells. Thus, this study implies an important future therapeutic strategy of using antioxidant-based pharmacotherapy to counteract the detrimental effects of HAART drugs. Despite HAART has substantial benefits to improve HIV patients, it must be borne in mind that with the

advent of HAART, HIV infection has become a chronic disease that requires long-term use of HAART, the need will arise to use drugs with less cardiovascular toxicity or to apply agents such as antioxidants to block the side effects of antiretroviral agents.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

ACKNOWLEDGEMENTS

HAART drugs including d4T, DDI, and IDV were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. This work is partially supported by research grants from NIH (Changyi Chen: HL65916, HL72716 and HL083471) and by the Michael E. DeBakey Department of Surgery, Baylor College of Medicine and Michael E. DeBakey VA Medical Center, Houston, TX, USA. We thank Dr Hong Chai and Dr Shaoyu Yan for their technical assistance.

DISCLOSURE/CONFLICT OF INTEREST

The authors state no conflict of interest.

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