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Highly Active Antiretroviral Therapy (HAART) and Metabolic Complications

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1. Introduction

The overwhelming impact the Human Immunodeficiency Virus (HIV) has on the world is undeniable – by the end of 2009 there were 33.3 million people living with HIV in the world, with 1.8 million deaths in that year alone (WHO 2010). In addition, the high rate of deaths can be directly attributed to the lack of available medications – only 36% of the infected population received adequate antiretroviral therapy (WHO 2009). Besides the known political and monetary issues at hand, the multiple number of HIV virus subtypes and sub-subtypes that have been described are overwhelming pharmaceutical availability. In fact, most research completed on HIV therapies has occurred, and continues to occur, in Europe and America, targeting the HIV-1 strain, although much of the world population is also afflicted by HIV-2.

To combat viral strain mutations, Highly Active Antiretroviral Therapy (HAART) has increased in complexity and effectively decreased deaths from opportunistic infections in those that are candidates for this treatment. However, these advances are tainted with metabolic long-term side effects, some of which are directly attributed to HIV Protease Inhibitors (PIs).

HAART has been linked to cardiovascular complications in HIV-1 patients, and recent studies have shown that HIV PIs play critical roles in insulin resistance, dysregulation of lipid metabolism, and inflammation, which are all cornerstones of cardiovascular complications. In addition, HIV PI-induced atherosclerotic cardiovascular disease is becoming the leading cause of mortality in HIV-1 infected persons in developed countries. During the last decade, an extensive effort has been put forth to study HAART-induced side effects. Both *in vitro* and *in vivo* animal studies from our laboratory and others' have linked HIV PIs with the activation of endoplasmic reticulum (ER) stress and oxidative stress as well as an increase in inflammatory cytokine production from several cell types including macrophages, hepatocytes, intestinal epithelial cells and adipocytes. However, the underlying cellular and molecular mechanisms remain to be fully identified and therapeutic strategies are currently unavailable. Understanding the root causes of HAART-associated metabolic syndrome and its potential implications for HIV-infected patients will be critical to the design of effective interventions to combat the metabolic and cardiovascular diseases

in a population chronically exposed to HAART. This chapter will discuss the current findings of HAART-associated metabolic complications and therapeutic challenges in the clinic.

2. FDA approved antiretrovirals in sequence of viral cycle

The first essential step in HIV infection of immune cells is fusion of the viral and cellular membranes. After an initial interaction, viral proteins utilize secondary receptors, especially CXCR4 (T cells) or CCR5 (macrophages), to gain entrance into the host cell. **Maraviroc** is a recently approved antiretroviral agent that inhibits fusion via binding and therefore prevents interactions with CCR5. Another, less commonly prescribed agent is **Fuzeon** (enfuvirtide), which binds to viral gp41. Due to the recent FDA approval of these two therapies, they will not be discussed in this chapter as there is little information on the long term clinical side effects of their usage. However, there is some positive evidence that there are minimal adverse metabolic effects.

After entry into the cellular cytoplasm, the viral genome (two copies of a single-stranded RNA) is reverse-transcribed into DNA by the viral enzyme, reverse transcriptase (RT). The first anti-HIV drugs to come on the market inhibited this enzyme. There are currently three classes of RT inhibitors **(RTI)**, **nucleotide and nucleoside analogues (NRTI)**, as well as **non-analogues (NNRTI)**. NRTIs are analogues to deoxynucleotides which are incorporated into the growing DNA chain, but lack one significant motif, a 3′-hydroxyl group, which is essential in linking each deoxyribose in the chain. Without this hydroxyl group, NRTIs cause a halt in synthesis, terminating chain growth. NNRTIs, on the other hand, bind directly to the RT itself, inhibiting the function of this essential enzyme.

The next step is integration of the newly synthesized DNA strand into the host genome by viral integrase. In 2007, the FDA approved **Raltegravir**, which directly inhibits this enzyme, and therefore does not allow latency of the virus in host cells. There has been some exciting evidence that Raltegravir has minimal side effects and may even reduce the side effects of some HIV PIs, as discussed later.

Upon host cell activation, often in times of stress such as infection and inflammation, the integrated virus cuts itself from the host genome and begins the viral replication cycle. Here, the virus utilizes the host RNA polymerase to produce multiple copies of package-able RNA strands from the DNA sequence. At the same time, the host cell machinery is recruited to translate RNA into viral proteins that are necessary for virion production and release. After protein production, the viral protease cleaves long HIV proteins into functional segments. Viral protease is unique in that it recognizes substrates with multi-folded domains and cleaves between a tyrosine/proline or phenylalanine/proline, something which human host cell enzymes cannot accomplish. This allowed for the specific design of HIV **protease inhibitors (PI)**.

2.1 Trails to current recommendations

Zidovudine, an NRTI, was the first drug approved in 1987, followed by approvals of zalcitabine, stavudine, and didanosine. The NRTIs seemed effective, yet the development of resistant strains caused a rebound of opportunistic infections in patients. In 1995, a major turning point occurred with the approval of HIV PIs ritonavir, saquinavir, and indinavir. Not only did these drugs work independently to help reduce viral load, but combination therapy with NRTIs drastically decreased opportunistic infections. This regimen is now

known as Highly Active Antiretroviral Therapy (HAART), although the combinations have increased in variety. The classic HAART regimen includes two NRTIs with one PI, though 2 NRTIs with 1 NNRTI, or 3 NRTIs are also used. With the constant battle against resistant strains in the current HIV-infected population, addition of integrase or fusion inhibitors increases efficacy of these regimens, thus widening the arsenal of HIV drugs available to physicians.

In addition to different classes of drugs, each class has also increased in variety. For instance, within HIV PIs, there are now two generations of drugs, peptidic and non-peptidic, all of which have a hydroxyl group that mimics the transition state structure of hydrolysis (Mastrolorenzo et al. 2007). The peptidomimetic class is large in itself, and inherently diverse in structure. For example, some (i.e. ritonavir) are less peptidic in nature, but enhance stability of PI binding to the enzyme. In contrast, others are more effective but the viral protease may easily mutate with their use. As PIs are discussed extensively in this chapter due to their correlation with metabolic side effects, we will quickly mention them here.

Saquinavir (SQV) was the first PI approved by the FDA in 1995. Indinavir (IDV) reached the market in 1996 and, due to its efficacy with NRTIs, set the bar for HAART. Ritonavir (RITV) was marketed that same year, but currently is only given as a booster with other PIs as it inhibits cytochrome P450 (enzymes that metabolize most PIs and subsequently decrease their bioavailability). Nelfinavir (NFV) was marketed in 1997, and subsequently became the first PI to be recommended for pediatric patients. In 1999, Amprenavir (AMPV – now prescribed as prodrug fosamprenavir) was introduced. Atazanavir (ATZV) was approved in 2003, and was the first PI approved for once-daily dosing, raising the bar for development of more convenient PIs.

Lopinavir (LOPV) entered the prescription option in 2000. It was structurally designed for viral protease variants, but is similar to RITV. Due to low bioavailability, LOPV is prescribed only with a RITV combination. Now, the only form of LOPV available on the market is as a co-formulation pill, Kaletra (the first drug not available in single formulation). Kaletra was so successful in both drug-naïve and drug-experienced patients that it became first-line therapy in 2006, and now has only been surpassed by the newer non-peptidic PI, darunavir (DRV). Tipranavir (TRV) is the most recent PI, coming onto the market in 2006, but is only used in patients with resistant strains due to a high side effect profile.

2.2 Side effects of antiretrovirals

As the list of FDA approved antiretrovirals is long, so is the inventory of side effects. Even though the life expectancy of HIV-infected patients under HAART has been extended, the various HAART-induced side effects significantly affect quality of life. Therefore, treatment of HIV infection requires the balance between the beneficial effects of viral suppression and the drug-induced toxic effects.

The most common side effects of HAART are attributable to the NRTI/NNRTIs. These include general side effects such as rash, anemia, nausea, vomiting, diarrhea, and sleep disturbances. More severely, it is not uncommon to observe liver toxicities, pancreatitis and neuropathy in certain patients, often with underlying risk factors. Furthermore, in the past decade there has been increasing concern over long-term HAART patients experiencing early-onset cardiovascular risk factors such as hypertension and insulin resistance. With parallel observations in the American population, some attributed these to environmental factors not due to the drugs. However, numerous large clinical trials have determined that

HAART can induce such drastic metabolic changes, the most common of which are components of the clinically defined Metabolic Syndrome.

Metabolic Syndrome is a diagnosable syndrome that leaves patients at a high risk for heart attacks and strokes. To be diagnosed, according to the National Heart Lung and Blood Institute criteria (Grundy et al. 2006), a patient must have 3 out of the 5 diagnoses:

1. 35" central circumference in women, or 40" in men.
2. A triglyceride level >150mg/dL.
3. An HDL <50mg/dL in women or <40 in men.
4. A blood pressure >130/85.
5. High fasting blood glucose level or insulin resistance.

Importantly, each component is an individual risk factor for atherosclerosis.

In addition, some long term side effects have been given discrete labels, such as HIV-associated lipodystrophy (Caron-Debarle et al. 2010). This has pathophysiologically been defined as selective damage of adipose tissue with subcutaneous fat loss and/or central fat accumulation. A large clinical trial, the Data Collection on Adverse Events of Anti-HIV Drugs (DAD), has provided more insight on this and other phenomena, giving the base for other investigators to continue examining the details. Specifically, lipodystrophy and dyslipidemia are now better explained. Contrary to the belief at the start of HAART, peripheral wasting is no longer attributed to viral wasting, but to NRTIs (stavudine and zidovudine) (Barbaro 2007; Carr et al. 2000; Martinez et al. 2001; Lee, Hanes, and Johnson 2003); and fat accumulation is not a physiological phenomenon, but due to PI treatment (Mallon 2007; Mallon et al. 2003). Additional specific PI-induced side effects include glucose alterations and insulin resistance, which can lead to diabetes (DMII), hypertension, and cardiovascular (CV) dysfunctions (Calza, Manfredi, and Chiodo 2004; Friis-Moller et al. 2003; Group et al. 2007). The bottom line from these investigations is that myocardial infarction is directly correlated with PIs, and not other components of HAART (Friis-Moller et al. 2003; Kaplan et al. 2007).

3. HAART-induced dyslipidemia

Alterations in serum lipids of the HIV infected population have been noted since the beginning of the '90s. Before treatments began, patients often had a decrease in LDL and HDL plasma concentrations. NRTI treatment alone seemed to increase LDL to presumable baseline levels, without any effect on HDL, yet multi-drug treatments tended to increase serum triglycerides. In the late '90s, effort was carried out to tease apart the effect of infection versus particular therapies.

In the past decade, there has been an increase in average serum lipid levels in the general population due to poor diet and increasing age, and the HIV-HAART cohort is no different. In addition, the initial rise in serum lipid levels of HIV patients observed by clinicians was hypothesized to be immune reconstitution phenomena (Floris-Moore et al. 2006), which is a robust inflammatory response when HIV viral load decreases a few months to a year after treatment initiation. However, it has been found in numerous long-term and short-term studies, as well as in healthy versus HIV-infected persons, that HAART regimen, specifically PIs, induces dyslipidemia (Pere et al. 2008; Periard et al. 1999; Tsiodras et al. 2000; Friis-Moller et al. 2003; Calza et al. 2003; Carr et al. 1999; Purnell et al. 2000; Group et al. 2007). Often, clinicians combat this phenomenon with lipid-lowering drugs. At the same

time, research has been attempting to determine which anti-HIV drugs induce the most change in lipid composition, and the mechanism underlying these changes.

Lipid homeostasis is centrally controlled by the liver. When fats are consumed in the diet, lipids are packaged into chylomicrons in the intestines whose final fate is the liver through an apoE endocytosis pathway. In order to effectively transport these to peripheral tissues, the liver packages triglycerides (TGs) and cholesterol into very low density lipoproteins (VLDL). VLDLs circulate and the TGs inside are taken up by muscle and adipose tissue after hydrolysis by lipase. The remnants are called intermediate density lipoproteins (IDL) which can be endocytosed by cells or further converted to low density lipoproteins (LDL) by lipases on the surface of cells. LDLs are cholesterol rich particles endocytosed through apoB-100, mostly in the liver or adipose tissue, and pathologically by macrophages. Another type of lipoprotein is high density (HDL), which is a way peripheral tissues 'send back' lipids, cholesterols, and proteins to the liver in an attempt not to be overloaded with these potentially toxic substances, as well as signal to the liver to stop synthesizing VLDLs.

HAART-induced dyslipidemia appears to affect many aspects of this pathway. Some studies have found that NNRTIs may even be able to increase HDL (Garcia-Benayas et al. 2001), a clinical advantage for dyslipidemic patients leading some to want to alter regimens to decrease PIs and increase NNRTIs (Barragan, Fisac, and Podzamczer 2006; Walli et al. 2001). In fact, there were successful studies in switching from PI-based treatments to NNRTI or NRTI-only regimes with success in attenuating dyslipidemia (Walli et al. 2001; Ruiz et al. 2001; Martinez et al. 2003). However, the effectiveness of PIs against HIV cannot be disputed. When PIs were added to the regimen in the mid-90s, there was a drastic decrease in patients who succumbed to opportunistic infections. The benefits of PIs far outweigh the side effects, but determining the mechanism behind these side effects may lead to alternative therapies in conjunction with PI use, or better-designed PIs.

4. HAART impact on liver lipid metabolism

The liver is a key organ in multiple processes, as well as the first organ to come into contact with ingested HAART medications. Hepatocytes are critical cells involved in lipid homeostasis, bile acid synthesis, gluconeogenesis, and metabolism of drugs. Therefore, alterations of normal cellular function can lead to global physiological consequences.

In regard to hepatic lipid metabolism, the endoplasmic reticulum (ER) is a central player. The ER is a critical organelle in cellular function as it is responsible for proper protein folding, cellular calcium level, lipid synthesis, and the secretory pathway. Inducing ER stress is thus relatively simple via depletion of ER calcium stores, changes in ER lipid membrane composition, reactive oxygen species (ROS), or accumulation of misfolded proteins. When triggered, the ER signals to the cell through the unfolded protein response (UPR) to cope with the increased accumulation of unfolded or misfolded proteins by downregulating protein synthesis and upregulating the degradation pathway. However, the extended activation of UPR will result in apoptosis.

UPR components identified in mammalian cells include three transducers [ER transmembrane kinase/endoribonuclease (IRE1), doubled-stranded RNA-activated protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF-6)], as well as one master regulator [an ER chaperone protein (BiP/GRP78)]. In response to increased proteins in the ER lumen or calcium depletion, BiP/GRP78 releases the transducers, which induce differential control of gene transcription through transcription factors ATF-4 and spliced

XBP-1. Further activation of the downstream transcription factor GADD 153/CHOP will trigger apoptosis.

In addition, sterol regulatory element-binding proteins (SREBPs) are transcription factors that also reside in the ER membrane. There are three isoforms derived from two genes: SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1c and SREBP2 are the predominant isoforms in the liver and play a major role in regulating the expression of key genes involved in lipid and lipoprotein metabolism such as 3-hydroxy-3-methylgutaryl-CoA (HMG-CoA) synthase, HMG-CoA reductase, squalene synthase, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and nuclear receptors. Therefore, ER stress induction not only alters protein production, but also lipid metabolism.

4.1 HIV PIs activate ER stress in hepatocytes

In HAART-induced metabolic dysfunctions, many features are similar to those observed in nonalcoholic fatty liver disease (NAFLD). NAFLD is a clinical term to describe a phenomenon in which patients have a fatty liver similar in all aspects to an equivalent alcoholic subject. Induction of NAFLD has been described in a range of conditions, such as obesity and diabetes, as well as by a variety of drugs. In addition, NAFLD is strongly associated with hepatic insulin resistance, yet it is hard to distinguish which comes first – fatty liver or the insulin resistance. To complicate the issue further, the exact cause of lipid accumulation in the liver can range from increased non-esterified fatty acids (NEFA) coming mostly from adipose tissue, excess lipids from the diet in the form of chylomicrons, impaired lipid processing in hepatocytes (*de novo* lipogenesis or DNL), or decreased release of VLDLs. However, Donnelly and colleagues were able to demonstrate that the majority of hepatic lipids in patients with NAFLD came from peripheral NEFA and DNL, not the diet (Donnelly et al. 2005).

This result is in agreement with HAART-induced NAFLD, as metabolic complications in these patients do not always seem to be correlated with diet. Importantly, although HAART-related NAFLD is not an entity of its own, many patients taking HIV PIs may have NAFLD with no symptoms, as does the majority of the overweight population. However, other patients progress to nonalcoholic steatohepatitis (NASH), which occurs with excess inflammation and scarring, potentially causing severe damage to the liver. Indeed, many studies have found NASH in greater than 50% of HAART-treated patients undergoing liver histopathological assessments (Ingiliz et al. 2009; Lemoine et al. 2006; Akhtar et al. 2008). HAART has been clearly shown to alter lipid and carbohydrate metabolism pathways, the underlying mechanism of NAFLD (Rector et al. 2008). Above all, NAFLD/NASH is most likely part of HAART-induced metabolic syndrome due to the strong correlations of hyperglycemia, insulin resistance, inflammation, and dyslipidemia.

Not all HAART components are associated with inducing constituents of NAFLD. RTIs can induce liver enzyme dysfunctions, but most studies are inconclusive. The two drugs with the strongest correlation are two NNRTIs, nevirapine and EFV (Waters, John, and Nelson 2007). Nevirapine-based HAART induces a faster liver fibrosis in those co-infected with hepatitis C (Macias et al. 2004), most likely due to a hypersensitivity reaction. In addition, some RTIs can have deleterious effects in the liver due to mitochondrial toxicities, which will not be discussed here due to a lack of correlation with metabolic side effects [reviewed in (Nunez 2010)]. However, HIV PIs are known to induce components of the metabolic syndrome, and hepatic alterations have been subsequently found to be at the core. Indeed, a

connection has been found between western diet-induced NAFLD and HIV PIs – the induction of ER stress (Erickson 2009).

ER stress activation has been linked to various human diseases including NAFLD/NASH. Recent studies have shown that HIV PIs induce ER stress in many cell types including hepatocytes, macrophages and intestinal epithelial cells (Zhou et al. 2006; Zhou, Pandak, and Hylemon 2006; Zhou, Pandak et al. 2005; Zhou, Lhotak et al. 2005; Wu et al. 2010; Djedaini et al. 2009; Cho et al. 2009; Pyrko et al. 2007). We also have identified that HIV PI-induced ER stress is partially due to depletion of ER calcium stores. In addition, activation of ER stress has been linked to the upregulation of SREBPs and dysregulation of lipid metabolism in hepatocytes.

4.2 Autophagy in HIV PI-induced ER stress

Autophagy is similar to the UPR as its main purpose is to protect the cell from deleterious stimuli, but has the ability to increase cellular damage or death when over-stimulated. It can be triggered in a number of ways, but the best described is that of perceived starvation. In a nutrient-rich environment, growth factors stimulate the eukaryotic cell and activate phosphoinositide 3-kinase (PI3K) Class I proteins. In turn, protein Akt-1 becomes activated through phosphorylation, leading to inhibition of autophagosome formation. Consequently, lack of nutrients leads to autophagy, resulting in engulfment of cellular particles or organelles for energy through lysosomal degradation.

In addition to aiding cells during a starvation period, autophagy also helps regulate lipid stores. The exact mechanism behind autophagy-regulated lipid metabolism is not clearly defined, although it is known to be essential. When autophagy is inhibited in hepatocytes, lipids accumulate in droplets (Singh, Kaushik et al. 2009), and this is not due to increased triglyceride synthesis nor decreased VLDL secretions (Singh 2010). In addition, mice lacking autophagy in the liver have enlarged lipid-laden livers with increased triglyceride and cholesterol levels (Singh, Kaushik et al. 2009). Singh and colleagues have coined this process as lipophagy, in which lipid droplets are degraded through autophagy instead of lipolysis. Even further, components of the autophagosome may be necessary for lipid droplet formation, suggesting a flux of lipid metabolism in the cell dependent on this pathway (Shibata et al. 2009).

In recent years, different laboratories focusing on dissimilar topics have independently come upon an autophagy and ER stress link. For one, autophagy offers another pathway other than proteasomes to degrade unfolded proteins (Kawakami et al. 2009; Yorimitsu and Klionsky 2007; Ding and Yin 2008). However, prolonged UPR activation has now been shown to lead to autophagy-induced cell death (Yorimitsu and Klionsky 2007) and inhibition of autophagy increases cell viability with prolonged ER stress (Price et al. 2010; Qin et al. 2010). The exact mechanism of how ER stress induces autophagy is still being investigated. Recently, it was found that ER stress activation can inhibit Akt phosphorylation, which is a dose-dependent response caused by some HIV PIs (Gupta et al. 2007). However, the responsible protein(s) are still not known, and may be cell-type specific [reviewed in (Schleicher et al. 2010)].

A recent study has found a strong link between activation of ER stress, increased autophagy induction, and increased SREBP activation leading to lipid overload in hepatocytes (Nishina et al. 2010), although the exact pathway linking these three was not experimentally addressed. Our laboratory has recently found that HIV PIs induce ER stress in hepatocytes,

leading to an increase in SREBP translocation to the nucleus, as well as induction of autophagy. However, the contribution of autophagy to HIV PI-induced metabolic syndrome remains to be determined and is the focus of our current studies [Figure 1].
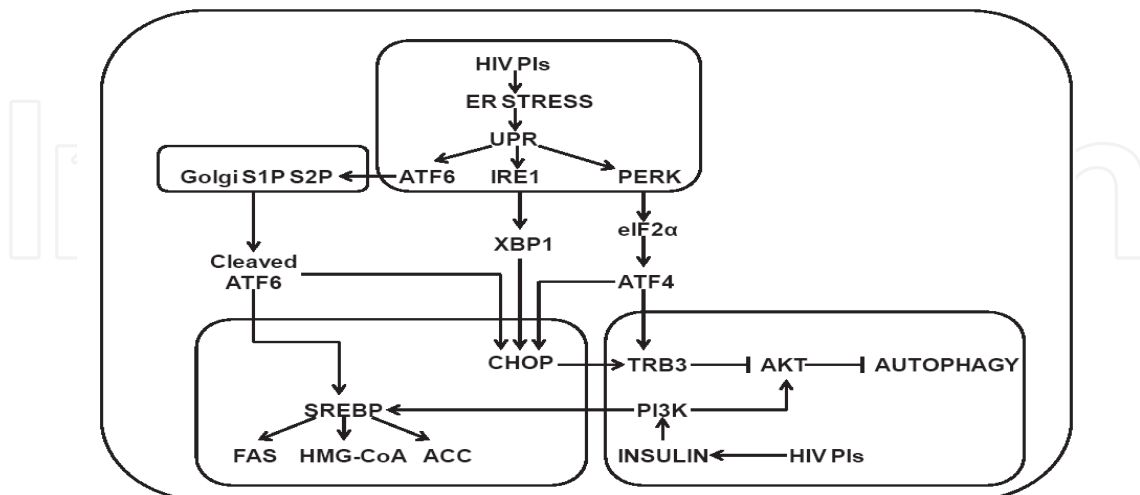

Fig. 1. Potential mechanisms of HIV PI-induced dysregulation of hepatic lipid metabolism.

4.3 HIV PI-induced hepatic insulin resistance

As discussed in the second section, HIV PIs have been directly linked to induction of HAART-metabolic syndromes, including lipodystrophy, glucose intolerance, and insulin resistance. These metabolic changes significantly increase the risk of DMII. There does not appear to be any one instigator in the spiral towards disease, but rather a mixture of increased lipolysis in the periphery, increased fat deposition in the liver and muscle, and peripheral and hepatic insulin resistance. In an attempt to tease these apart, a few clinical trials have found that HIV PIs first target a decrease in peripheral glucose uptake, although chronic treatment alters hepatic glucose production (Lee, Rao, and Grunfeld 2005; Woerle et al. 2003). Of specific note was an increase of fasting glucose – explained by endogenous liver production and decreased uptake in the periphery.

DNL occurs in the absence of insulin, when the body is in a presumably fasting state. This pathway may be directly activated without absence of insulin as the instigator, such as through growth hormones activating Akt (potentially linking the findings of ER stress above) or simply through cellular insulin insensitivity. In particular, diabetic mice demonstrated an increase of Tribbles 3 (TRB3). Du and colleagues have found that TRB3 complexes with Akt, causing an inhibition of its activity (Du et al. 2003). As Akt mediates insulin signaling, the inhibition of its action results in inducing cellular insulin resistance. Importantly, ER stress can activate TRB3 directly through transcription factors ATF-4 and CHOP. Again, TRB3 activation would also lead to autophagy through inhibition of Akt activity.

In addition to activating TRB3, ER stress also activates c-Jun N-terminal Kinase (JNK). JNK phosphorylates insulin receptor substrate (IRS-1), inhibiting its action of insulin-stimulated cell signaling (Ozcan et al. 2004). However, no current studies have been able to definitively show that HIV PI-induced ER stress leads to JNK activation, giving more support to the activation of TRB3 leading to both insulin insensitivity as well as autophagy.

The link between insulin insensitivity in the hepatocyte and that of metabolic consequences seen in HAART patients is now coming together. It is understood that insulin suppresses

VLDL secretion, most likely through inhibition of ApoB100 from packaging TGs. Therefore, loss of insulin stimulation will invariably lead to increased VLDL secretions, contributing to dyslipidemia. In addition, insulin actively stimulates SREBP-1c, but it cannot be assumed that loss of the insulin activation will lead to a decrease in SREBP-1c and thus a decrease in lipogenesis. In contrast, HIV PIs may be continually stimulating the UPR, leading to a continuation of SREBP-1c activation and lipogenesis. Therefore, HIV PI effects in the liver may, in fact, contribute directly to dyslipidemia and insulin insensitivity.

5. Impact of HAART on lipid metabolism in adipocytes

Although the average individual despises adipose tissue due to the displeasing aesthetics, there is no denying its importance in overall health. Adipose tissue is a much more complex and important endocrine organ than originally thought, composed of adipocytes, macrophages, and endothelial cells communicating in a matrix of complex extracellular proteins and proteoglycans. To make issues more complex, there is a constant turnover of cells in tissue (10% of adipocytes renew each year (Spalding et al. 2008)) and an influx of macrophages from the periphery at times of adipocyte hypertrophy or stress (Sell and Eckel 2010).

Different depots in the body have differing physiology (Hocking et al. 2010; Ray et al. 2009), most likely due to inconsistent derivations of tissue (Laharrague and Casteilla 2010; Billon et al. 2007). The differences between depots are seen both chemically and physiologically. It was found in the late '90s that preadipocytes isolated from different depots have different adipogenic induction responses (Adams et al. 1997) and gene expressions (Lefebvre et al. 1998), but these differences are still not well understood. Physiologically, it has been long known that those with increased visceral adipose mass have a higher risk of cardiovascular disease, although those with the same BMI, but more subcutaneous mass do not have the same risk. In HIV treatment, different drugs have differential effects on fat tissue - the RTIs seem to decrease subcutaneous depots while the HIV PIs increase visceral adipose mass (Mallewa et al. 2008). The result is a substantial increased risk of CV disease in HAART-treated patients, patients, which can be directly attributed to PIs can be attributed directly to PIs (Group et al. 2007).

However, the differences that lead to detrimental risks are still not understood. Basic researchers have probed the physiology of visceral versus subcutaneous depots for a decade now with no concrete results. Nevertheless, these studies have provided more information on the metabolism of adipocytes and adipose tissue than ever before, allowing more understanding not only of HAART side effects, but also those of diabetes.

5.1 Reverse transcriptase inhibitors

Most persons that are not treated for HIV infection experience wasting syndrome, or the involuntary loss of a significant amount of weight with associated weakness. With the advent of RTIs, physicians were able to combat this uncomfortable aspect of the disease, but soon realized another phenomenon – that of a patient gaining weight only centrally or even directly loosing fat mass from the legs, arms, and face. In the mid '90s when clinicians first began observing this occurrence, they attributed it to the PI component of HAART. This explanation was in good logic as the observance came soon after PIs were added to the regimen. After further clinical studies, they were able to tease apart this phenomenon termed HAART-induced lipodystrophy, finding that RTIs induce peripheral fat loss while

PIs increased central obesity. The next phase of studies determined which drugs were most likely to induce these aesthetic mishaps. It is now known that thymidine analogue-NRTIs in particular are most strongly associated with lipo-atrophy of the extremities (Mallon 2007; Stankov and Behrens 2007; Mallal et al. 2000). Even more so, the mechanism behind this attribution seems to stem from mitochondrial toxicity leading to adipose cell death.

Thymidine analogues are not initially active when they enter host cells as they must be phosphorylated before they can inhibit RT. This occurs in three steps, and generates two pharmacokinetic NRTIs in patients – active phosphate in cells and inactive drug in the plasma. Within the cells, intermediates occur that can have added effects, but the triphosphorylated NRTI is of most concern here, as it has been shown to directly inhibit mitochondrial DNA polymerase in many organs, including adipose (Kakuda 2000; Collins et al. 2004; Cote 2005). In fact, mitochondrial DNA was found to be depleted in HAART patient adipose biopsies (Nolan et al. 2003; Shikuma et al. 2001). However, this hypothesis seems insufficient to explain the total sum of physiological peripheral lipo-atrophy in patients. Therefore others have proposed supplementary mechanisms that may play a part in inhibition of the DNA polymerase for an additive effect on cells (Anderson, Kakuda, and Lichtenstein 2004; Lund and Wallace 2004). Most of these mechanisms are related to mitochondrial dysfunction, and may be partially due to the intermediate NRTIs. In addition, the balance of endogenous substrate with triphsophorylated NRTI may play a role, and help explain why only a certain proportion of patients experience lipo-atrophy (Anderson, Kakuda, and Lichtenstein 2004).

Another component to this story includes inhibition of adipogenesis. Peroxisome proliferator-activated receptor-γ (PPARγ) is an essential transcription factor required for maturation from a pre-adipocyte to a mature adipocyte. It has been shown that thymidine analogues can downregulate this nuclear receptor, inhibiting adipogenesis. Later, it was found that mitochondrial toxicity is related to this inhibition, and may be directly linked. In other words, the thymidine analogues are perhaps not inhibiting PPARγ directly, but inducing mitochondrial toxicity that results in crosstalk to the nucleus to stop differentiation of cells (Viengchareun et al. 2007; Stankov et al. 2007).

Importantly, the atrophy induced by RTIs may in fact contribute to HIV PI-induced NAFLD discussed previously. With the increase of cell death or inhibition of differentiation, there is a decrease in uptake of fats plus an increased release during cell death, increasing NEFA and contributing about 60% of the fats depositing in hepatocytes. Therefore, inhibition of RTI-induced cell death would not only aid a patient in an aesthetic discomfort, but also decrease the risk of NAFLD.

5.2 HIV protease inhibitors

As stated above, PIs appear to increase central adiposity, which is a major risk factor for CV disease. In addition, there are direct links of PIs and insulin resistance. The clinical significance of this has led to more research into PI-induced effects in adipose tissue than RTIs. Unfortunately, in the past decade we have only come a few steps closer to elucidating the exact mechanism of PI-induced adipose pathology.

At the start of investigating these drugs, many researchers focused on the possibility that HIV PIs can inhibit adipogenesis and induce apoptosis, which may help explain lipo-atrophy. However, depot-specific differences could not be explained and the conclusion that it was the NRTIs inducing the atrophy and not the PIs altered the focus of the field. In addition, the findings from these early investigations are still not conclusive. This is

explained by inherent difficulties in handling and processing adipose tissue, diversity in cell lines, and different subjective techniques used between laboratories. For example, some investigators found that RITV does not inhibit differentiation of adipocytes, while others stated that it significantly inhibits this pathway. Importantly, most laboratories focused on inhibition of differentiation, not the mechanism of this phenomenon nor how this effect in cell culture could be well correlated in the clinic.

An important turning point in this field came through parallel research conducted in obesity, diabetes, and endocrinology. Major conclusions demonstrated that adipose tissue was a significant player in the inflammatory state and insulin resistance. HIV PI-investigators began to turn from focusing on which drugs inhibited differentiation to the mechanism of HIV PI-induced lipid metabolism dysregulation, cytokine secretions, and connections to the metabolic syndromes seen in this patient population. Ensuing hypotheses included induction of ER stress, mitochondrial toxicity, inhibition of GLUT4, and autophagy induction.

5.2.1 HIV PI-induced ER stress, inflammation, and insulin resistance in adipocytes

As discussed in the previous section, ER stress activation is directly linked to dysregulation of hepatic lipid metabolism. In adipocytes, this is no different, and may be even more complex as cell maturation relies on lipids. In the preadipocyte state, the cell has more characteristics of a fibroblast than an adipocyte, in which it is elongated and located close to the blood vessel. At this stage, it releases minimal cytokines, and is relatively lipid droplet (LD) free. Upon induction of differentiation, the cell rearranges, begins to round when next to other adipocytes, and forms LDs. Although the complete understanding of LD formation is yet to be elucidated, it is known that they originate in some manner from the ER. It is also hypothesized that the two organelles stay connected in a manner that allows free exchange of lipids, but this hypothesis is not completely supported by evidence at this time.

Either way, the close proximity of the ER to LDs, as well as the dependence of the ER on the production of neutral lipids, demonstrates how much the UPR can affect an adipocyte. In addition, adipocytes need constitutive activation of the UPR as the IRE1-XBP-1 pathway is essential in adipogenesis (Sha et al. 2009; Basseri et al. 2009), while overstimulation of the UPR can inhibit differentiation altogether (Shimada et al. 2007). This dependence on the UPR has not been demonstrated in hepatocytes and, in addition, another difference is observed with SREBP-1c. In adipocytes, SREBP-1c is actually downregulated in times of ER stress (Gregor and Hotamisligil 2007), as it is also a major player in adipogenesis.

However, in addition to these differences, both cell types respond with some degree of insulin resistance upon UPR activation (Djedaini et al. 2009). In the fields of obesity and diabetes, we have come to realize that DMII is directly related to the inflammation and malfunction of overloaded adipose tissue. With increasing overload, adipocytes begin to hypertrophy, becoming stressed and signaling this with a release of proinflammatory cytokines. These cytokines cause an infiltration of circulating macrophages, which engulf over-stressed or dying cells, forming characteristic crown-like structures. Interestingly, HIV patients on HAART therapy appear to be in the same state as an obese individual in terms of inflammatory and dysfunctional adipose tissue. Patients often have a decrease in insulin sensitivity, as well as having dyslipidemia and liver disease as previously discussed. These factors can also be teased apart from over-nutrition, as shown in the D.A.D. studies, among other clinical investigations.

HIV PIs have been repeatedly shown to induce ER stress, and the results in our laboratory support others' work. This activation has been shown to be directly linked to alterations in SREBP-1c processing in adipocytes, as well as suggested to be the cause of inhibition of differentiation observed with these drugs. In addition, the inflammatory cascade is induced in adipocytes treated with HIV PIs (Jones et al. 2005; Kim et al. 2006; Leroyer et al. 2011). The inflammatory state is highly predictive of onset of insulin resistance, and once again this induction is also directly linked to ER stress. In fact, activation of the UPR can induce inflammatory activation through the JNK pathway as stated previously. Additionally, cytokine release from neighboring cells can induce ER stress in the host cell, causing a vicious cycle.

A key study in this field was done by Djedaini and colleagues, in which they treated human adipocyte cells with LOPV. They noted both increased activation of ER stress and decreased IRS1 phosphorylation, as well as reduced glucose uptake (Djedaini et al. 2009). What linked these phenomena was eIF2-α phosphorylation – a key protein activated during the UPR. When these investigators inhibited this phosphorylation with a specific molecule, salubrinal, they were able to decrease IRS1 phosphorylation slightly. However, when done in conjunction with minimal concentrations of LOPV, there was a significant decrease of IRS1 phosphorylation, demonstrating a synergistic effect of the two drugs.

Activation of the UPR leading to a decrease in insulin signaling may only be part of the story. Others have shown that PIs can actually inhibit the glucose transporter directly (Hertel et al. 2004). It has been proposed that this inhibition induces a starvation-like state in the cell with the decrease of intracellular glucose, causing activation of ER stress. This would lead to a decrease in insulin signaling, propagating insulin resistance further. At this point, adipocytes would rely heavily on lipids, hydrolyzing triglyceride stores and thus increasing NEFA release, causing lipotoxicity and insulin resistance at the physiological level. Despite these findings, more research is obviously needed to determine which pathway or proteins HIV PIs induce/inhibit when they first come into contact with an adipocyte. Only then can we be certain of the consequences of a given structure on the drugs currently on the market.

5.2.2 Adiponectin and autophagy

Adipocytes release not only pro-inflammatory cytokines, but also the anti-inflammatory cytokine adiponectin. Adiponectin is unique in a number of ways. It is exclusively secreted by adipose tissue and is negatively correlated with diseases such as atherosclerosis and insulin resistance. In overexpanded or stressed tissue, there is a decrease of adiponectin secretion (Kern et al. 2003), and an increase of macrophage-inducing TNF-α, IL-6, and MCP-1, implicating inflammatory diseases such as atherosclerosis and insulin resistance.

HIV PIs have been repeatedly reported to decrease adiponectin, from the RNA level to secretion. Our laboratory has also noted this phenomenon, as well as found a substantial increase in IL-6 secretion in mouse adipocytes treated with HIV PIs for 48 hours, in a dose-dependent manner. MCP-1 and TNF-α mRNA levels also increase. This is just one more piece of evidence that HIV PIs induce a pro-inflammatory state in adipose.

Interestingly, adiponectin can alleviate ER stress (Zhou et al. 2010). Zhou and colleagues found in their cellular studies that ER stress initiation is sufficient to decrease adiponectin release. In animal models, they reported stabilization of adiponectin further decreased obesity-induced ER stress in adipose tissue. Moreover, induction of autophagy could alleviate ER stress responses in the cell, stabilizing adiponectin secretions.

As discussed previously, increasing evidence points to autophagic dysregulation as a component of altered lipid metabolism in cells. In adipocytes, there is a constitutive base of autophagy aiding in the recycling of lipid stores. Indeed, with a decrease in autophagy in pre-adipocytes, there is a resulting inhibition of adipogenesis (Singh, Xiang et al. 2009). *In vivo* animal studies from the same group found a complete reduction of adipose mass when they induced a decrease in the autophagy pathway, resulting in smaller adipocytes and altered LD morphology. This is in seeming opposition to what occurs in the liver with a decrease in autophagy and may be best explained best by Singh and colleagues. They detail the need for autophagy to control adipose mass so lipids can be continually stored in adipose tissue, while in the liver, autophagy attempts to decrease TG accumulations (Singh 2010).

5.3 Other HIV PI effects in adipocytes

Although ER stress activation represents an important cellular mechanism underlying HIV PI-induced side effects in adiopocytes, HIV PIs may also influence cellular lipid metabolism through other mechanisms, such as mitochondrial toxicity. In this realm, most investigators who are proponents of this mechanism found concurrent inhibition of adipocyte differentiation with increased markers of mitochondrial dysfunction (Viengchareun et al. 2007). When considering this hypothesis, however, the close tie of ER and mitochondrial homeostasis must be contemplated, especially in the case of the apoptosis cascade. Indeed, some have shown the same close connection when mitochondrial dysfunction leads to the UPR (Burkart et al. 2011), and likewise the UPR has been shown to induce mitochondrial dysfunction (Lee et al. 2010). This tight interplay often makes it difficult to discern where the initial damage occurs, but it is irrefutable that ER stress activation plays a major role.

Another mechanism proposed is inhibition of PPARγ activity that leads to inhibition of differentiation. As stated before, many investigators discovered that RTIs and PIs were able to decrease the capability of adipogenesis. In these studies, it was found that PIs could inhibit PPARγ activity, and this was not through direct interaction[reviewed in (Caron et al. 2009)]. Affecting PPARγ will alter the cell physiology, such as a decrease of adiponectin secretion and alteration of lipid metabolism. However, without demonstrating a direct pathway of HIV PI-induced PPARγ downregulation, it is difficult to pursue this mechanism when there are also other promising pathways in the works.

6. HIV PIs increase inflammation at the vasculature and alter macrophage lipid metabolism

Macrophages are key cells involved in atherosclerotic plaques. Initiation of a plaque can occur very early, when the endothelial lining becomes damaged or inflamed, inducing leukocyte infiltration. The damage is often attributed to oxidized lipoproteins in the bloodstream, occurring with high LDL levels and decreased anti-oxidants. After initial damage, macrophages localize to the area. In addition to their innate role to induce inflammation in the process of healing, macrophages will also take up circulating lipids that are not being properly processed by peripheral tissues. Macrophages will keep accumulating lipids, expanding and releasing pro-inflammatory cytokines that call in more macrophages, all the while not 'fixing' the initial problem of damaged endothelium. As the plaque builds, more lipids tend to accumulate in the given area and stability decreases, having the potential of completely restricting flow or dislodging.

Patients taking HIV PIs have been found to have accelerated atherosclerotic lesions. An important causative factor is most likely the dyslipidemia discussed previously, potentially inducing initial damage, although direct influence on the endothelial layer could also be at fault. In fact, numerous clinical studies have noted decreased endothelial function in HAART-treated patients. This decrease has been directly attributed to the PI component (Teixeira et al. 2009). In cell culture investigations, it has been found that PIs can directly induce apoptosis of vascular smooth muscle cells, which could contribute to both endothelium dysfunction as well as plaque instability, maybe through increased reactive oxygen species (Rudich et al. 2005).

Once the initial damage ensues at the endothelium, macrophages can migrate through the endothelial layer into the intima and begin secreting inflammatory cytokines as well as developing scavenger receptors. These receptors are directly responsible for LDL uptake in macrophages, leading to foam cell formation. In regard to macrophages, HIV PIs actually have multiple affects. One of the more important of these was discovered by Dressman et al. who found that PIs actively induce CD36 (Dressman et al. 2003). CD36 is a fatty acid transporter in metabolically active tissues and, in macrophages, it binds and endocytoses oxidized LDLs. This directly leads to the formation of foam cells, which are at the core of plaques.

In addition to inducing CD36, HIV PIs can increase foam cell formation via other pathways. HIV PIs have been shown by several investigators to increase active SREBPs in macrophages. Our laboratory has demonstrated that HIV PI-induced upregulation of SREBPs occurs through the activation of ER stress (Zhou, Pandak et al. 2005). In addition, PIs were found to decrease cholesterol efflux in foam cells by inhibiting scavenger receptor B1 and caveolins (Wang et al. 2007). Thus, PIs can both induce the intake and inhibit the export of the ingredients of a foam cell.

Previous findings in our laboratory demonstrate that inner-membranes are overloaded with cholesterol in HIV PI-treated macrophages, and ER calcium stores depleted. Taking the above together, we hypothesize that HIV PIs induce cholesterol overload in macrophages, leading to an alteration in cholesterol composition of the ER membrane. As stated before, a slight alteration in membrane composition can induce the UPR, which would lead to an increase of SREBP-1 in macrophages, causing lipid overload and increasing foam cell formation of these cells. In addition, a proportion of these cells would die through the apoptotic pathway, inducing an unstable plaque, and a basis for the drastic side effects seen with these drugs.

7. Metabolic disease, cardiovascular dysfunction, and concluding remarks

As demonstrated throughout this chapter, PIs have drastic effects in multiple cell types and tissues. In addition to those listed above, PIs have also been shown to increase pro-insulin secretions from β-cells, demonstrating their altered functions (Behrens et al. 1999). It is postulated that PIs fist induce a transition from normal to impaired glucose tolerance due to insulin resistance induction in the liver and adipose tissues. With worsening glucose resistance, more insulin must be secreted, and over time this leads to β-cell dysfunction, which can be demonstrated by the release of inactive pro-insulin. However, as some investigators have shown direct effects of HIV PIs on the β-cell, it is possible these mechanisms are occurring simultaneously, accounting for the more rapid induction of insulin resistance seen in this patient population (Schutt et al. 2004).

In any case, there is no doubt that HAART has decreased total mortality in HIV-infected persons, but determining the mechanism behind life-altering side effects could help improve therapies. Also, not all HIV PIs have the same degree of metabolic disease-inductions; such as AMPV, which has the least effect on lipid metabolism and does not need a RITV booster. As mentioned previously, some have actually proposed to switch to NNRTIs and leave out the PIs for favorable lipid profiles in patients. Even more, we have recently shown that Raltegravir can actually mitigate HIV PI-induced ER stress, relieving lipid metabolism dysregulation in hepatocytes (Cao et al. 2010).

This leaves the question of cost versus benefit. HIV-specialists are sure to argue that some of the PIs that are controversial are keeping their patients' morbidity drastically down. Without these particular drugs, the chance of increased viremia and viral mutations is too large. Switching to AMPV, for instance, is not always feasible. In addition, although we have shown the potential of Raltegravir against the cellular side effects, this might not feasibly translate into the clinic due to pill-burden and expense. Therefore, physicians continue to push exercise and lipid-lowering drugs. Development of better therapeutic strategies for HIV infection and HAART-induced metabolic syndrome requires more extensive studies and efforts.
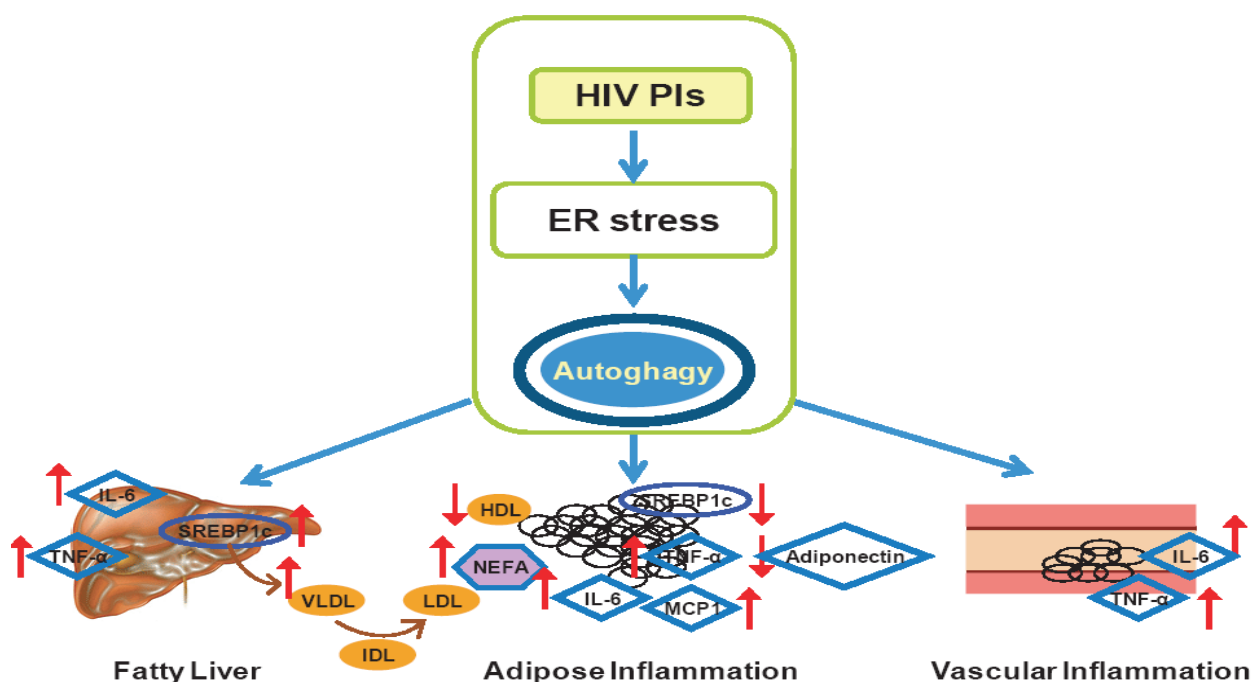

Fig. 2. Summary of HIV PI-induced pathological effects.

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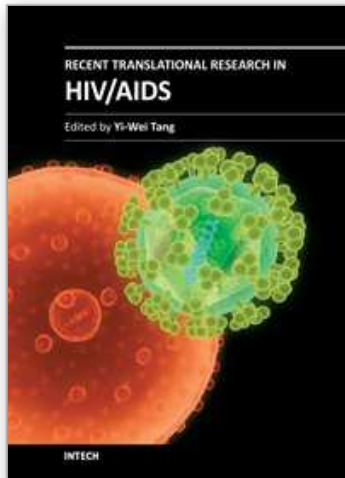
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