THE EFFECT OF NUCLEOSIDE/NUCLEOTIDE REVERSE TRANSCRIPTASE INHIBITORS AND PROTEASE INHIBITORS ON LIPOPROTEIN LIPASE

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ABSTRACT

In the treatment of HIV/AIDS, protease inhibitors (PIs) and nucleoside/nucleotide analog reverse transcriptase inhibitors (NRTIs) are the major components of highly active antiretroviral therapy (HAART). The side effects of these drugs include various metabolic disorders, including insulin resistance, dyslipidemia, and lipodystrophy. The precise mechanistic basis of these remains mostly unknown.

Objectives: In this study, we aimed to understand the enzymatic activity of lipoprotein lipase (LPL) under the influence of NRTIs and PIs.

Material and Methods: Chinese hamster ovary cells transfected with the human insulin receptor (CHO-IR) were used for the first time to study the effects of NRTIs and PIs on the synthesis and release of LPL. The high level of expression of insulin receptor facilitated sensitive detection of any alteration in the phosphorylation of signaling proteins as compared to 3T3-L1 adipocytes.

LPL activity in the supernatant and cell lysate was measured using a colorimetric method employing para-nitrophenyl butyrate(pNPB) as a substrate. The most commonly used ARVs were tested. These included four PIs and six NRTIs.

Results: The results showed that NRTIs stavudine and emtricitabine significantly inhibited the LPL activity from the CHO-IR cells. PIs indinavir and nelfinavir were also found to decrease LPL activity extracellularly when added to the assay reaction in vitro. Similarly, nelfinavir and atazanavir sulfate inhibited the activity of the LPL from the CHO-IR cells after 16-hour treatment. This suggested that these drugs may interfere with the enzyme activity intracellularly either at the level of its synthesis or its transportation from the cytoplasm to the cell surface.

Conclusion: This finding suggests that protease inhibitors may play a role in inhibiting lipoprotein lipase activity in vivo, and may thereby induce metabolic disorders in HIV-positive patients being treated with protease inhibitors.

Key Word: Lipoprotein lipase, Nucleoside/nucleotide reverse transcriptase inhibitors, Protease inhibitors

Introduction

Lipoprotein lipase (LPL) is a central enzyme in lipid metabolism and transport. It is produced in numerous cell types, but the skeletal muscle and the...
parenchyma of adipose tissue are the significant sites of synthesis and physiological actions.

The physiological location of LPL-mediated hydrolysis of lipoproteins is at the capillary endothelial cell, but functional LPL is first synthesized by the parenchymal cells and then translocated to its site of action.  

There are four main levels of regulation, depending upon the location of LPL synthesis in different tissues. These are at the transcriptional, posttranscriptional, translational, and post-translational levels. These four levels are locally controlled by different factors in different tissues. This local control on LPL synthesis enables tissues to maintain a balance in lipid metabolism, and it also modulates other cellular functions and hormonal effects on the local tissues.

LPL plays an important role in lipid metabolism and transport. It catalyzes the hydrolysis of Triacylglycerol (TAG) attached to circulating chylomicrons and Very Low-Density Lipoprotein (VLDL). The resulting reaction produces non-esterified fatty acids (NEFA) and 2-monoacylglycerol for tissue utilization. NEFAs in white adipose tissue are re-esterified for energy storage as TAG. Additionally, fatty acids are oxidized to provide an energy source in the heart and to regulate thermogenesis in brown adipose tissue.

Role of insulin, heparin, and glucose in lipoprotein lipase regulation

As discussed above, the regulation of LPL synthesis is essential for normal homeostasis of lipid metabolism, and insulin is the primary regulator of LPL synthesis in adipocytes and the translocation from there to the luminal domain of the endothelial cells. Generally insulin increases LPL activity but synthesis of LPL involves many steps including LPL gene transcription, mRNA processing, transport and translation, posttranslational modification (glycosylation) “activation” or “inactivation”, and finally secretion. Insulin affects different steps of LPL synthesis in adipocytes such as during the process of differentiation by enhancing gene transcription while in fully differentiated adipocytes it regulates LPL synthesis by increasing mRNA levels and enzyme activity by both posttranscriptional and post-translational modifications of LPL.

LPL release from cells is increased by both insulin and heparin in a dose and time-dependent manner. The mechanisms by which insulin and heparin release LPL from the cells are not precisely known. Some researchers have observed that heparin acts as a secretagogue, thereby suggesting that the released LPL is at first present in intracellular secretory vesicles, which fuse with the plasma membrane upon stimulation with heparin. Other data indicate that there is a heparin-binding site on the LPL dimer, which binds to the heparin sulphate-proteoglycan extracellular matrix. Heparin treatment of cells shifts the plasma membrane-bound LPL by competition with the HSP, resulting in the release of LPL into the cell culture medium.

Intracellular glucose levels also accelerate LPL synthesis in adipocytes, and its boosting effect on LPL is mainly associated with glycosylation of LPL, which is very important for the regular LPL enzymatic activity and its release. Glucose also enhances the effects of insulin to increase LPL synthesis, but there is no evidence that glucose affects the transcriptional level (mRNA) of LPL synthesis, as observed with insulin. Different studies associate insulin resistance with decreased LPL activity, hypertriglyceridaemia, elevated cholesterol and chylomicronaemia.

A recent study in insulin-resistant offspring of type 2 diabetes parents revealed a link between insulin resistance and decreased mitochondrial content, as evidenced by decreased mRNA and protein expression of LPL in muscle biopsies. Usually, free fatty acid delivery into skeletal muscle results in mitochondrial biogenesis through activation of peroxisome proliferator-activated receptor (PPAR)-δ. The primary role of LPL here is to hydrolyze the serum triglycerides and deliver free fatty acids to the muscle cells, as described above.

In many disorders such as diabetes, atherosclerosis, and obesity, hypertriglyceridaemia is a characteristic feature. A well-known cause of genetic hypertriglyceridaemia is the deficiency of lipoprotein lipase. In humans, type I hyperlipoproteinaemia, resulting from LPL deficiency, is a rare autosomal recessive disease. It is characterized by low or no LPL activity resulting in hypertriglyceridaemia, decreased levels of HDL cholesterol, lipaemiatinalis, and pancreatitis. There are three naturally occurring...
mutations in LPL that affect lipid transport and metabolism and result in hypertriglyceridaemia and obesity in mice 22,23.

Disturbances in body fat distribution, dyslipidemia and insulin resistance, diabetes, and atherosclerosis in HIV patients are associated with ARV. However, the exact mechanisms of these changes have not been fully elucidated. HIV infection with acute and chronic effects of some antiretroviral drugs on regional fat distribution correlates with these side effects 24-26. It has already been reported that HIV infection itself affects triglyceride metabolism and lipoprotein lipase activity 27.

Hypertriglyceridaemia and impaired insulin sensitivity can be observed even in HIV-negative subjects treated for short periods of time using protease inhibitors but fat redistribution, on the other hand, becomes apparent only after several months of treatment and this is most often related to dyslipidemia and insulin resistance 26,28,29.

With the advent of HAART (Highly Activated Anti-Retroviral Therapy), it has become well known that the two-drug families; the NRTI (Nucleoside Reverse Transcriptase Inhibitors) and the PIs (Protease Inhibitors) are often associated with lipid abnormalities and body fat distribution, yet the cellular and biochemical mechanisms underlying these effects are not well understood 26,30. The NNRTI component of HAART also contributes to dyslipidemia, although the relationship to lipodystrophy is unclear 31-33.

**Materials and Methods**

The rationale for measuring lipoprotein lipase in CHO cells

In CHO cells, LPL is consistently produced independently of the cell differentiation stage 16. The increased level of expression of insulin receptors also makes it easy to modulate the insulin response. Moreover, since this model system also used for the study of insulin signaling, it appeared prudent to use this cell line to quantify the regulation of LPL activity by insulin and ARVs. We, therefore, analyzed the effect of HPIs and NRTIs on LPL activity in CHO cells, transfected with insulin receptors to understand whether ARVs could potentially modulate lipid metabolism through LPL and modulate the LPL response to insulin.

Several methods have been used to measure LPL activity in adipocytes and serum or plasma. However, these methods are either expensive or laborious and cumbersome. By contrast, an earlier method for assaying LPL in milk established that LPL-catalysed hydrolysis of p-nitrophenyl butyrate (pNPB) could be measured relatively easily and cheaply using the appearance of p-nitrophenyl phosphate as an end product 34.

Preparation of CHO-IR cells for the assay of released lipoprotein lipase

CHO-IR cells were cultured and serum-starved, as described in sections 2.2.1 and 2.2.2. The cells were washed with PBS (2 ml) twice and left in an additional fresh PBS (500 μl) containing insulin (100ng/ml) (17nM) for one h at 37 °C in an incubator, to stimulate LPL activity. After one h, heparin sodium-Fresenius (final concentration 100 U/ml) was added for a further two h to release the enzyme from the cell surface 35-38. The cell culture supernatants were then collected in order to assay for LPL activity. The remaining cells in the well were lysed using lysis buffer and measured for total protein concentration, as described in section 2.2.3.

The purified LPL standard was derived from *Pseudomonas* sp (Fluka, Buschs, Switzerland) in a range of concentrations from 2.13 U/mg to 213 U/mg. Enzyme activity was also measured after the addition of phenylmethylsulphonyl fluoride (PMSF) (1 mM), to confirm that the assessed enzyme activity was attributed to the LPL, as PMSF is known to inhibit LPL activity 39. PMSF (10 mM) was prepared in ethanol.

Assay of lipoprotein lipase activity

LPL activity in supernatants from CHO-IR cells was determined as follows 34:

Cell culture supernatants (100 μl) were mixed with 900 μl buffer (0.1 M sodium phosphate, monobasic, anhydrous, pH 7.2; 0.9 % sodium chloride; 0.5 % (v/v) Triton X-100 (900 μl)) 34,40. pNPB (10 μl of 50 mM in acetonitrile at 1 % (v/v) final concentration) was then added. The reactions were incubated in cuvettes at 37 °C in a water bath 34 for specific periods. Absorbance was then measured using a visible light spectrophotometer at 400 nm.

Enzyme activity was calculated using the following formula 39:
1.01 = volume (in ml) of incubation mixture

df = dilution factor (0.1)

0.0148 = μM extinction coefficient of \( p \)-nitrophenol at 400 nm

0.1 = volume (in ml) of enzyme used

Units/mg protein = (units/ml supernatant) ÷ (mg protein/ml supernatant)

Unit definition:

One unit will release 1.0 mole of \( p \)-nitrophenol phosphate per minute at pH 7.2 and 37°C when using \( p \)-nitrophenyl butyrate as the substrate.

For this experiment, six NRTIs and four PIs that are commonly used in the treatment regimes in South Africa were examined for their effects on lipoprotein lipase secretion. CHO-IR cells were serum-starved and treated with HIV protease inhibitors and NRTIs for 16 h. The drugs were used at concentrations close to their peak serum concentration (Cmax) values: Zidovudine (AZT) 10 μM, efavirenz 20μM, emtricitabine 20μM, atazanavir sulphate 20μM, stavudine 10μM, lamivudine 10μM, tenofovir 1 μM.

The cells were then stimulated with insulin to increase LPL activity and treated with heparin to release LPL. The LPL activity in 100μl of the cell culture supernatants was measured after 30 min of incubation in a water bath at 37°C.

In addition to measuring whether these drugs influence LPL activity in cells, the same concentration of the drugs was added directly to the assay reaction after recovering the untreated (no drug) cell culture supernatants from cells stimulated with both insulin and heparin. This was done to determine if the drugs inhibited LPL activity directly. These were then incubated at 37 °C in a water bath for 30 min.

**RESULTS**

Changes in LPL activity measured in CHO-IR cells after insulin and heparin. The LPL-catalysed hydrolysis of pNPB was successfully measured in supernatants of CHO-IR cells stimulated with both insulin and heparin (Figure 4.2.a). Cells which were stimulated with both 100 ng/ml insulin and heparin sodium-Fresenius (final concentration 100U/ml) had significantly increased LPL activity almost 4 fold, with detectable enzyme release from the cell surface from 5 min (\( p = 0.0009 \)) up to 30 min (\( p = 0.05 \) using unpaired t test (*) indicate
The effect of nucleoside/nucleotide reverse transcriptase inhibitors on lipoprotein lipase activity in supernatant from the CHO-IR cells. LPL activity was measured in CHO-IR treated with Zidovudine (AZT), Efavirenz (EFV), Emtricitabine (FTC), Atazanavir-sulfate (ATV), Stavudine(d4T), Lamivudine(3TC) and, Tenofovir (TDF) for 16 h using the optimized assay conditions. LPL activity increased threefold (p=0.0009) when cells were exposed to both 100 ng/ml (17nM) insulin and heparin, compared to that of the unstimulated cell culture supernatants with heparin or insulin. The effect showed by NRTIs on LPL activity compared to the enzyme activity in the untreated cells exposed to both insulin and heparin in two different experiments is discussed below.

In the first experiment, the NRTIs were added directly to the supernatant of the untreated cell, those treated with insulin and heparin to observe the effects of the drugs on the lipoprotein lipase activity directly after secretion. NRTIs did not alter LPL activity significantly in the supernatants. A small decrease (3TC* p=0.130, d4T*p=0.141, AZT* p=0.394, FTC* p=0.127, EFV* p=0.378, TDF* p=0.126) in the LPL activity was observed, although these differences were not statistically significant as shown...
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in Figure 4.3.

In order to examine the effect of the NRTIs in the synthesis and secretion of LPL, CHO-IR cells were treated for 16 hours with the NRTIs in the serum-free medium. After treatment, two drugs stavudine (p=0.0191) and emtricitabine (p=0.0173) resulted in significant decreases in the measured LPL activity from the cells. The remaining NRTIs, zidovudine (10µM), lamivudine, tenofovir (1µM), and efavirenz (20 µM) resulted in decreased LPL activity, but this was not statistically significant as shown in Figure 4.4.

Effect of protease inhibitors on lipoprotein lipase activity in supernatant from the CHO-IR cells

CHO-IR cells were serum-starved for 16 h (without drugs), and supernatants were collected after exposure with insulin (100ng/ml) and heparin. LPL activity in these supernatants was measured after adding the HPIs directly to the assays reaction before the incubation. Four HPIs indinavir (50µM), nelfinavir (30µM), atazanavir (20µm) and ritonavir (30µM) were used in this experiment, and the resulting differences in LPL activity were compared against heparin and insulin-induced cell supernatants without any addition of drugs, as shown in Figure 4.5 below. A significant decrease in LPL activity was observed in the presence of nelfinavir (p=.0115) and indinavir (p=.0221), while a small but insignificant decreased in LPL activity was observed in the presence of atazanavir and ritonavir.

In order to evaluate the effect of PIs on the intracellular synthesis and secretion of the LPL, CHO-IR cells were treated with the drugs for 16 h; then, the supernatant was collected, followed by exposure to the insulin and heparin. A high concentration of insulin (100ng/ml) was used in this experiment to exercise the maximum capacity of the cells to secrete enzyme. LPL activity was measured and then compared with the untreated cell supernatant (only heparin and insulin exposed). A significant decrease in LPL activity of nelfinavir (p=.0193) and atazanavir sulfate (p=0.069) was observed while smaller (statistically insignificant) decreases in LPL were observed with indinavir and ritonavir treated cell supernatants (Figure4.6).

Discussion

In this study, for the first time, CHO-IR cells were used to measure LPL activity using a colorimetric method employing pNPB as a substrate. LPL has been shown to catalyze the hydrolysis of short-chain fatty acyl esters such as tributyrin, p-nitrophenyl acetate, and pNPB in Vivo. This substrate is more convenient to use than lipid-soluble substrates, as the product of LPL-catalysed hydrolysis of pNPB, p-nitrophenol, absorbs light strongly at 400 nm.

CHO cells provide a convenient model to study intracellular synthesis and translocation of lipoprotein lipase. A significant advantage of CHO cells is that the synthesis of LPL is constant in these cells and is not associated with the differentiation processes of the cells. Another advantage compared with adipocyte cell lines, which have been used in previous studies, is that the CHO cells make the cell fractionation process easy because they do not contain large fat-droplets. Moreover, since the cell expresses large number of receptors, it is very easy to modulate the insulin dose-response.

CHO-IR cells treated with heparin and insulin showed a measurable 3-fold increase in LPL activity. Insulin plays a crucial dose-dependent role in LPL synthesis and significantly affects the posttranscriptional and posttranslational levels with minimal effects at mRNA level during differentiation of adipocytes. These results indicate that the insulin-induced synthesis of LPL is dose-dependent, and it is significant (p=0.0135) increases in the LPL activity in the presence of high concentration (17nM) of insulin while no significant (p=0.0628) increase is observed with low insulin concentrations 10ng/ml (1.7nM). Insulin does not cause the release of LPL from the cell surface. However, cells stimulated with insulin did show significant increases in LPL activity in CHO-IR cells, even in the absence of heparin.

The results also show that NRTIs did not change LPL activity in the supernatant. Stavudine and emtricitabine significantly inhibited the LPL activity from the CHO-IR cells after treatment. Stavudine, saquinavir, indinavir, efavirenz, and tenofovir are the ARVs most likely to cause lipodystrophy (buffalo hump), and for this reason, stavudine is no longer considered an appropriate treatment for most patients in developed countries and is no longer recommended by the WHO. However, due to its low price, it is still widely used in the developing world. Moreover, fat loss on long term exposure, one of the major
side-effects of stavudine, is associated with buffalo hump.47

Major PIs such as indinavir, nelfinavir, atazanavir sulfate, and ritonavir are still being used in South Africa, and these were used in this study as well.48 Indinavir and nelfinavir were also found to decrease LPL activity when added to the assay reaction in vitro. This indicates that the drugs also inhibit LPL activity extracellularly. Similarly nelfinavir and atazanavir sulfate inhibited the activity of the LPL from the CHO-IR cells after 16 h treatment. This suggests that these drugs may interfere with the enzyme activity intracellularly either at the level of its synthesis or its transportation from cytoplasm to the cell surface. Nelfinavir was found to be the only drug that inhibited LPL activity intracellularly as well as extracellularly. This finding suggests that protease inhibitors may play a role in inhibiting LPL activity in vivo, and may thereby induce metabolic disorders in HIV-positive patients being treated with PIs.

A previous study elucidating the possible mechanism of severe hypertriglyceridaemia caused by ritonavir described the decreased LPL activity responsible for the decrease in TG clearance and decreased fatty acid uptake from VLDL and albumin in adipocyte tissue. Also, ritonavir decreased the total LPL activity in the plasma.49

Similarly, few other studies describe a relationship between the intracellular lipid content and insulin resistance in muscle cells.50,51 A recent study revealed that decreased expression of LPL in insulin-resistant muscle cells results in decreased free fatty acid influx in the cells, which is a potent stimulator of mitochondrial biogenesis through activation of PPAR-δ.52 Similarly, Tissue-specific overexpression of LPL plays a role in development of tissue-specific insulin resistance like in skeletal muscle and liver. Increased intracellular lipid (diacylglycerol and ceramides) which activate the novel and conventional PKCś (PKCθ, PKCδ, and PKCβ) responsible of insulin resistance by affecting the insulin signalling pathway in to the cells.53-56

LPL deficiency or overexpression is critical in the regulation of lipid metabolism, and changes may induce metabolic disorders such as hypertriglyceridaemia, chylomicronaemia, pancreatitis, atherosclerosis, coronary artery disease and lipodystrophy associated with insulin resistance and diabetes mellitus.

The hydrolysis and release of lipids for storage from triglyceride-rich lipoproteins such as chylomicrons and VLDLs, and their subsequent storage in adipose tissue, cannot take place without lipoprotein lipase. Therefore regulation of LPL expression is critically important for normal lipid-lipoprotein homeostasis.

Conclusion

This study indicates that LPL activity can be readily assayed in CHO-IR cells using pNPB as a substrate. Furthermore, this assay and CHO-IR cells can be used to analyze the effects of PIs and NRTIs on LPL activity. This is the first study to report that stavudine, emtricitabine, indinaviratazanavir sulfate, and nelfinavir significantly decrease LPL activity.

References


