CELLULAR AND BIOCHEMICAL EFFECTS INDUCED BY ANTIRETROVIRAL DRUGS

BY

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DECLARATION

The work contained in this thesis is original, except where indicated and acknowledged. No portion of this work has been submitted for another degree at this or any other university.

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Khawar Anwar

28 January 2013

DEDICATION

This work is dedicated to the Man who has helped me through all ups and down, he is the one always there for me and praying for me. He made me dream and gave me strength to make it true. He taught me significance of hard work and not to give up no matter how hard you try, I must go on.

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Khawar

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ABBREVIATIONS

aPKC	a typical PKC
AIDS	acquired immune deficiency syndrome
APS	adapter proteins with a pleckstrin homology domain and an SH2 domain
APS	ammonium persulphate
ARV	antiretroviral
BBR	berberine chloride
BCAA	branch chain amino acid
BPB	bromophenol blue
BSA	bovine serum albumin
cPKC	classical PKC
cAMP	cyclic adenosine monophosphate
CCR5	chemokine co-receptor 5
CIM	coefficient-based importance measure
CHO-IR	Chinese hamster ovary transfected with high levels of human insulin receptor
CO_2	carbon dioxide
CXCR4 CXC	chemokine co-receptor 4

dH$_2$O	deionised water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
FC	fold change
FCS	fetal calf serum
FDA	food and drug administration
FFA	free fatty acid
G418-sulphate	geneticin
GC-MS	gas chromatography mass spectrometry
GLUT4	glucose transporter 4
gp41	glycoprotein 41
gp120	glycoprotein 120
HAART	highly active antiretroviral therapy
HDL	high density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
IC$_{50}$	50 % inhibition concentration
IGF	insulin growth factor
IκB	inhibitor κB

IKKβ	IκB kinase β
IRS-1	insulin receptor substrate-1
IRS-2	insulin receptor substrate-2
JNK	Jun kinase
LPL	lipoprotein lipase
MAPK	mitogen-activated protein kinase
mTOR	mammalian target of rapamycin
nPKC	novel PKC
NaCl	sodium chloride
NCS	newborn calf serum
NFκB	nuclear factor kappa B
NNRTIs	non-nucleotide reverse transcriptase inhibitors
NRTIs	nucleoside reverse transcriptase inhibitors
pIRS	phosphorylated IRS
PBS	phosphate buffered saline
PDK	PIP3-dependent protein kinase
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-biphosphate
PIP3	phosphatidylinositol 3,4,5-triphosphate
PKB	protein kinase B
PKC	protein kinase C
PLS-DA	partial-least squares discriminant analysis

PMSF	phenylmethylsulphonyl fluoride
PPAR-γ	peroxisome proliferator-activated receptor gamma
PTP1B	protein tyrosine phosphatase 1B rabbit
PY20	purified mouse anti-phosphotyrosine antibody
RNS	reactive nitrogen species
ROS	reactive oxygen species
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2B	Src homology 2B
S6K1	ribosomal S6 kinase 1
SOCS-1	suppressor of cytokine signaling 1
SOCS-3	suppressor of cytokine signaling 3
SREBP-1	sterol regulatory element binding protein 1
TBST	tris buffered saline + 0.1% tween 20
TEMED	N, N, N', N',-tetra-methyl-ethylenediamine
TNF-α	tumour necrosis factor alpha
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris-hydrochloride
Trypsin-EDTA	trypsin-ethylenediaminetetraacetic acid
Tween 20	polyoxyethylenesorbitan monooleate
TDZ	troglitazone

UPR   unfolded protein response

VLDL   very-low-density lipoprotein

pNPB   para-nitrophenyl butyrate

PUBLICATIONS

1. **Cellular and biochemical effects induced by ARVs in CHO-IR cells**
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   (In press)
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POSTER AND PRESENTATIONS

1. **College of Health Sciences Research Symposium, 2012. University of KwaZulu-Natal, South Africa.**

   Oral Presentation "Cellular and biochemical effects induced by ARVs" in the College of health sciences research symposium in September 2012

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   Title of poster: *Cellular and biochemical effects induced by ARVs in CHO-IR cells.*

3. **College of Health Sciences Research Symposium, 2011. University of KwaZulu-Natal, South Africa.**

   Title of poster: *Inhibition of Insulin receptor Kinase by Antiretroviral Protease Inhibitor*" in the College of health sciences research symposium in September 2011.

ABSTRACT

In the treatment of HIV/AIDS, protease inhibitors (PIs) and nucleoside/nucleotide analogue 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, dyslipidaemia and lipodystrophy. The precise mechanistic basis of these remains largely unknown. In this study we aimed to understand the molecular basis of these metabolic effects by analysing the effects on lipoprotein lipase (LPL) activity, insulin signaling and the cellular metabolic profile. It was previously shown by this group that indinavir inhibits insulin signaling at a proximal level. The study was extended to a wider range of ARVs and in particular, the effects of sodium salicylate (NaSal) and berberine chloride (BBR) were analysed to determine if they could reverse the effects of the drugs on insulin signalling. In addition, Chinese hamster ovary cells transfected with the human insulin receptor (CHO-IR) were used for the first time to study the effects of NRTIs on the insulin signaling pathways. 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.

Three PIs, indinavir, nelfinavir and ritonavir were used in this study. Indinavir and nelfinavir treatment significantly reduced the insulin-stimulated phosphorylation of the IRβ, IRS-1, Akt and MAPK in CHO-IR cells. However phosphorylation of GSK-3α\β was not affected by the PIs. Ritonavir also decreased (not statistically significant) the phosphorylation of IR-β and IRS-1 but its inhibitory effect

on MAPK was the same as by the other PIs. NRTI's did not inhibit insulin-stimulated tyrosine phosphorylation of IRβ and IRS-1 but reduced phosphorylation at MAPK and Akt. .

In order to understand the role of NFκ-B pathway in blocking insulin-stimulated tyrosine phosphorylation, IKK-16, a selective inhibitor of IkB kinase (IKK) was used but no significant involvement of this pathway was found in blocking tyrosine phosphorylation at IRS-1. Similarly, NaSal and BBR were also used to reverse the effects induced by PIs and NRTIs in CHO-IR cells but no significant change was observed on Akt and MAPK. NaSal and BBR reduced (but not significantly) the effects of PIs (indinavir and nelfinavir) on IR-β and IRS-1. These findings suggest that PIs induce insulin resistance by affecting multiple steps in the signaling pathway. At the proximal end of the insulin signalling pathway, protease inhibitors affect IR-β and IRS-1 while at the distal end they affect phosphorylation of Akt and MAPK.

CHO-IR cells were also used to measure LPL activity using a colorimetric method employing pNPB as substrate. The most commonly used ARVs were tested. These included four PIs and six NRTIs .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 cytoplasm to the cell surface. These 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.

Metabolomic analysis was performed on the supernatant of cells treated with PIs and NRTIs, with and without insulin stimulation. Many significant alterations and trends in amino acids and organic acids levels in CHO-IR cells supernatants (treated with PIs) were recorded using ^{1}H-NMR. For example, PIs

decreased the synthesis of threonine, phenylalanine, lysine, arginine, isoleucine butyrate, glutamate, histidine and 2-oxo-isovalerate. Furthermore overproduction of lactate and ketones were observed in the nelfinavir treated cells. This may be a consequence of the secondary effects of insulin resistance induced by the PIs. Similarly NRTIs (stavudine and tenofovir) treatment also induced changes in the levels of many amino acids and organic acids .NRTIs decreased the synthesis of acetate, acetoacetate, histidine, methionine, phenylalanine and tryptophan while significant increase in the acetone was observed in stavudine-treated cells.

Taken together, the results of this study suggest that PIs and NRTIs, inhibits LPL activity and synthesis, affects insulin signalling pathways at different levels and alters the synthesis of different cellular metabolites which may affect the signalling pathways of insulin .

CHAPTER I: GENERAL INTRODUCTION

1.1 Acquired Immune Deficiency Syndrome

The Acquired Immune Deficiency Syndrome (AIDS) a slow progressive degenerative disease which affects the immune system of the host and the infection is caused by the Human Immunodeficiency Virus (HIV). AIDS and HIV were first discovered in 1981 by the United States Center for Disease Control and Prevention ("Pneumocystis pneumonia--Los Angeles," 1981; Report, 1981b). Following the discovery of AIDS and its causative agent it has had huge impact demographically especially in low income and resource poor countries. It has also affected social and economic conditions of the high epidemic areas. The World Health Organization (WHO) has confirmed 25 million HIV-related deaths in 60 million people infected with the virus. In 2011,the WHO reported approximately 34 million people were living with HIV/AIDS and 2.5 million new cases in 2011 (UNAIDS, 2011).

As a result of HIV/AIDS being a leading cause of death worldwide, it has become one of the biggest care challenges today in terms of the prolonged treatment regimen and prevention. As a result of being an infectious disease with a number of complications both from the disease and from treatment, it is a disease that is difficult to treat. Vast numbers of untreated HIV positive individuals progress to the development of clinical AIDS in 7-8 years (Anuurad, Bremer, & Berglund, 2010). There are two distinct types of HIV based on serotypes, type I (HIV-I) and type 2 (HIV-II). However the majority of infections worldwide are reported to have HIV-I serotype (Hahn, Shaw, De Cock, & Sharp, 2000).
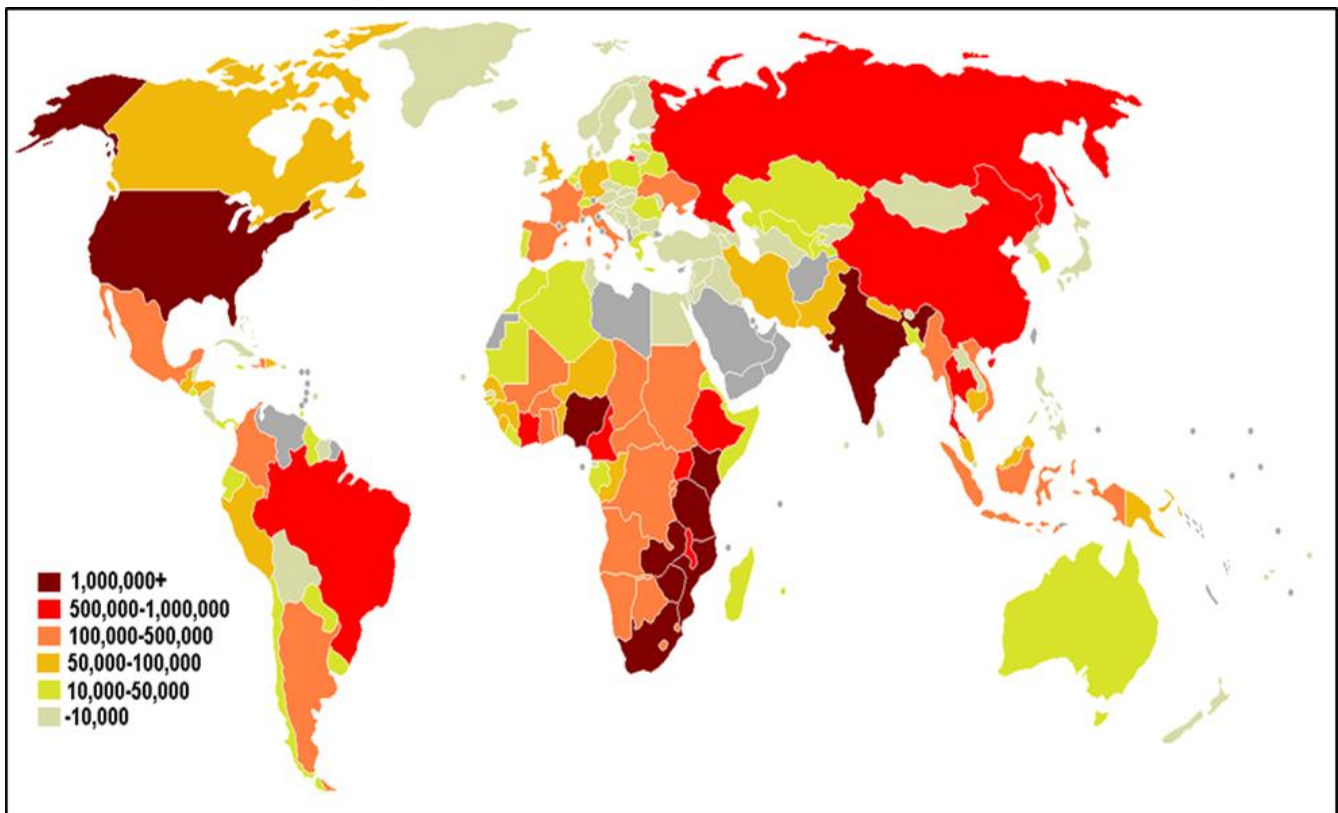
Figure1.1: HIV AIDS Data and Statistics – People living with HIV AIDS world map in 2008.

Source UNAIDS 2008 global report,

The prevalence of HIV infection is highest in Sub-Saharan Africa which accounts for approximately 67% of HIV infections worldwide (WHO., 2009).

The HIV prevalence rate in South Africa was approximately 10.6% higher than the any other country (Shisana, 2009), with 5.38 million people reported to be living with HIV and AIDS. Among adults, 16.6% of the total population is estimated to be HIV positive. Approximately 316,900 adults (age 15 years or older) were newly infected in 2011, while 63,600 children (age 0-14 years) were estimated to be infected with HIV. In the same year 257,910 South Africans were estimated to have died of AIDS, accounting for approximately 43.6% of total deaths in South Africa (Africa, 2011)

1.2 Human Immunodeficiency Virus

The Human Immunodeficiency Virus is a retrovirus that primarily targets the host immune system and makes it weaker and incompetent. HIV-1 has been classified as a member of the lentivirus subfamily of retroviruses, based on its morphology, genomic and pathogenic features. HIV-I has a complex structure compared to other viral families such as oncoretroviruses (Weiss, 1993) (Figure 1.2).

1.2.1 HIV Morphology

The HIV virion has a spherical morphology (100-120nm) and is covered by a lipid bilayer called an envelope which is a derivative of the host cell membrane. It also has the glycoprotein 120 (gp120) within the outer lipid layer. It is attached to the viral envelope by glycoprotein 41 (gp41) (Freed & Martin, 1995; Sierra, Kupfer, & Kaiser, 2005; Turner & Summers, 1999). Under the envelope of HIV there are two layers of protein shells, the outer layer is composed of MA protein called the matrix and inner layer composed CA protein called the capsid. The inner part of the HIV contains two copies of viral genomic material known as the viral core that is surrounded by the capsid (Fig1.2). The viral core contains two copies of RNA with the reverse transcriptase (RT), integrase and protease enzymes required for viral replication in the host.
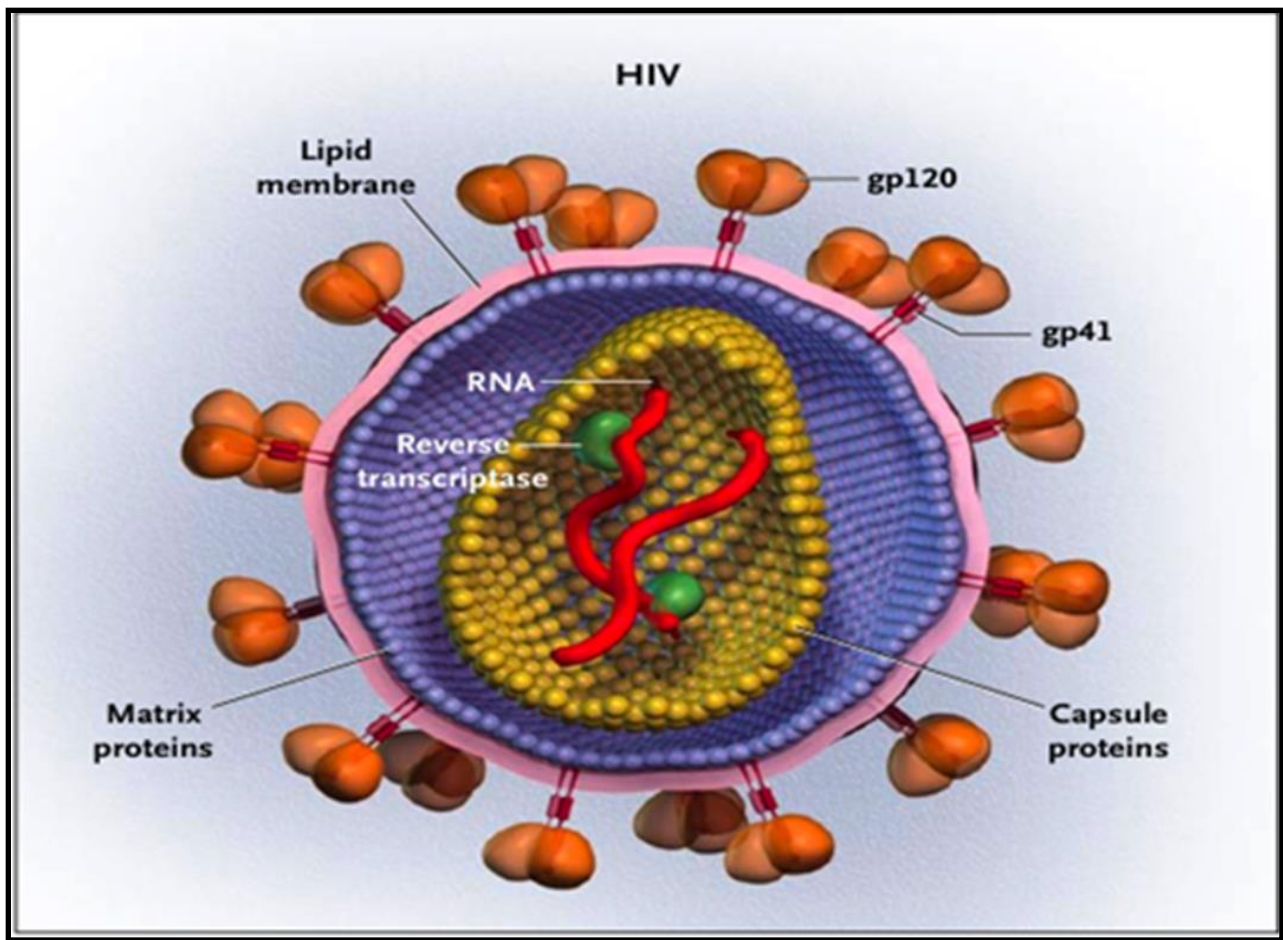
Figure 1.2: Structure of HIV.
[www.nejm.org]

1.2.2 Life cycle of HIV

The life cycle of HIV begins upon entry of virus to host cell. It mainly enters through host CD4 T lymphocyte cells and macrophages via glycoprotein adsorption to the interacting host cell. Adsorption causes the merging of the cell membrane of the host cell and the viral envelope facilitating the release of viral capsid into host cell.

HIV replication comprises a series of steps that starts with the attachment of the viral envelope proteins to the specific CD4 receptor of the targeted host cell. For the entry into the CD4 T lymphocyte, HIV

requires co-receptors called CXCR4 and CCR5 along with CD4 receptors (Berger, Murphy, & Farber, 1999; Carrington, Dean, Martin, & O'Brien, 1999; Schols, 2004).

In the subsequent step to the attachment of HIV to the co-receptors there is a conformational change in the envelope proteins which ultimately results in the fusion of the viral envelope and the host cell membrane. Fusion results in the generation of an opening and the contents of the capsid transfer to the cells through this opening. Subsequently in the cytoplasm the single stranded RNA of the virus is converted in to double stranded-complementary DNA with the help of the reverse transcriptase. (Gotte, Li, & Wainberg, 1999). After the completion of reverse transcription, viral genome and associated proteins enter the nucleus after passing through nuclear membrane and integrate with the host DNA (chromosomal DNA) with the help of enzyme HIV integrase (Craigie, Fujiwara, & Bushman, 1990; Katz, Merkel, Kulkosky, Leis, & Skalka, 1990).

After the incorporation of viral genome, expression of the viral genes generates precursor strands of viral polyproteins (Gag (p55) and Gag-Pol (p160)). These polyproteins are processed by a protease (aspartyl endopeptidase) which cleaves the polyprotein strands and new HIV virions are produced (Debouck, 1995). In the final steps of the life cycle, the assembly of the new virus particles take place at the surface of cells. The viral RNA and proteins combine and new viral particles leave the host cells by budding off (B. K. Chen, Gandhi, & Baltimore, 1996; Simon et al., 1997). During the course of budding the virion also uses the outer layer of the host cell to form a viral envelope. These virions are able to grow and become mature virions that infect other healthy human host cells within 1.5 days (Sierra et al., 2005; Turner & Summers, 1999).
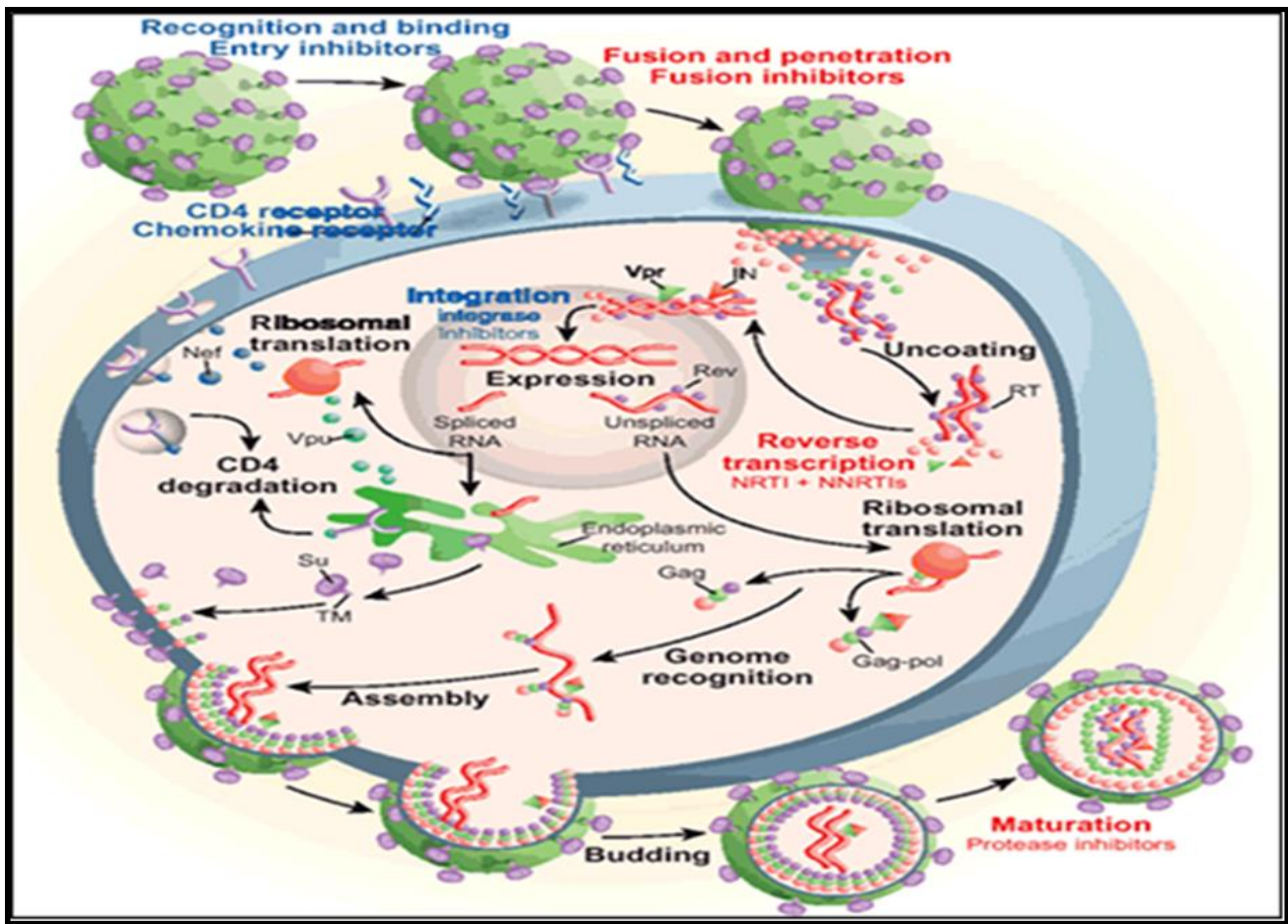
Figure 1.3: HIV life cycle.(Turner & Summers, 1999).

1.2.3 HIV infection and treatment

Initially HIV infection is followed by an asymptomatic period which does not require any antiviral therapy immediately. In the asymptomatic period the host immune system halts the progression of HIV infection but over the period of time rapid replication of virus weakens the immune system and this leads to AIDS. At this stage, proper treatment is required to manage the disease progression and associated metabolic challenges (USAID Health Policy Initiative, 2009).

Since maternal antibodies are present in newborn blood, the status of HIV-infected children cannot be determined until they reach 18 months. Therefore, WHO recommends cotrimoxazole to be provided to all children born to HIV positive mothers until their status is known. For HIV-positive children,

cotrimoxazole should be prescribed for up to five years. After the age of 5 years, cotrimoxazole should be continued if the infection has progressed to stage III (USAID Health Policy Initiative, 2009).

1.3 Highly active antiretroviral therapy

Highly active antiretroviral therapy (HAART) improves the life expectancy and prognosis of HIV patients, by reducing viral load along with parallel remedies to increase life span of patients by minimizing opportunistic infections and increase CD4+ T-cell count, thus enabling the host immune system to counter active viral replication (Hammer et al., 1997; Piacenti, 2006).

Table 1.1: Estimated number of adults receiving ART and percentage of children receiving ART and cotrimoxazole in South Africa

	Adults (15+ years)	Children	
	Estimated number receiving ART*	Estimated number receiving ART	Estimated % receiving cotrimoxazole
2010	1 058 399	105 123	38,0 %

*Source: Health Information Epidemiology Evaluation and Research, Department of Health (November 2010 / Report) (Africa, 2011).

Currently HAART is the only treatment which became available for HIV/AIDS in 1995 (Arvind et al., 2008). HAART had a positive health impact with successful outcomes for HIV-infected individuals as the number of HIV cases started to fall (Powderly, 2004). Despite new control efforts, 5.8 million cases were reported along with 2.3 million AIDS-related deaths (WHO., 2009).

HAART is a treatment regime that usually combines multiple antiviral drugs to maximize its effectiveness. These antiviral drugs are derived from five broad classes aiming to target different parts of virus and thus attenuate rapid replication. These are: 1) Entry inhibitors which inhibit entry of virus; 2) reverse transcriptase inhibitors to inhibit core enzyme for viral replication; 3) nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleotide based reverse transcriptase

inhibitors (NNRTIs), to inhibit integration of viral RNA in host genome; 4) integrase inhibitors, and 5) protease inhibitors (Hammer et al., 2008).

Although HAART has improved life span effectively, it comes with many adverse effects as the combination of antiviral drugs affects the metabolism leading to a number of metabolic disorders (Table 1.2).

HIV is an RNA-based virus which are known to carry mutations during replication due to lack of proof reading activity by RNA polymerase (Flint, 2004). HAART requires an optimal combination of drugs with better human tolerance and has also been considered effective in reducing the risk of viral mutations (Hammer et al., 2008).
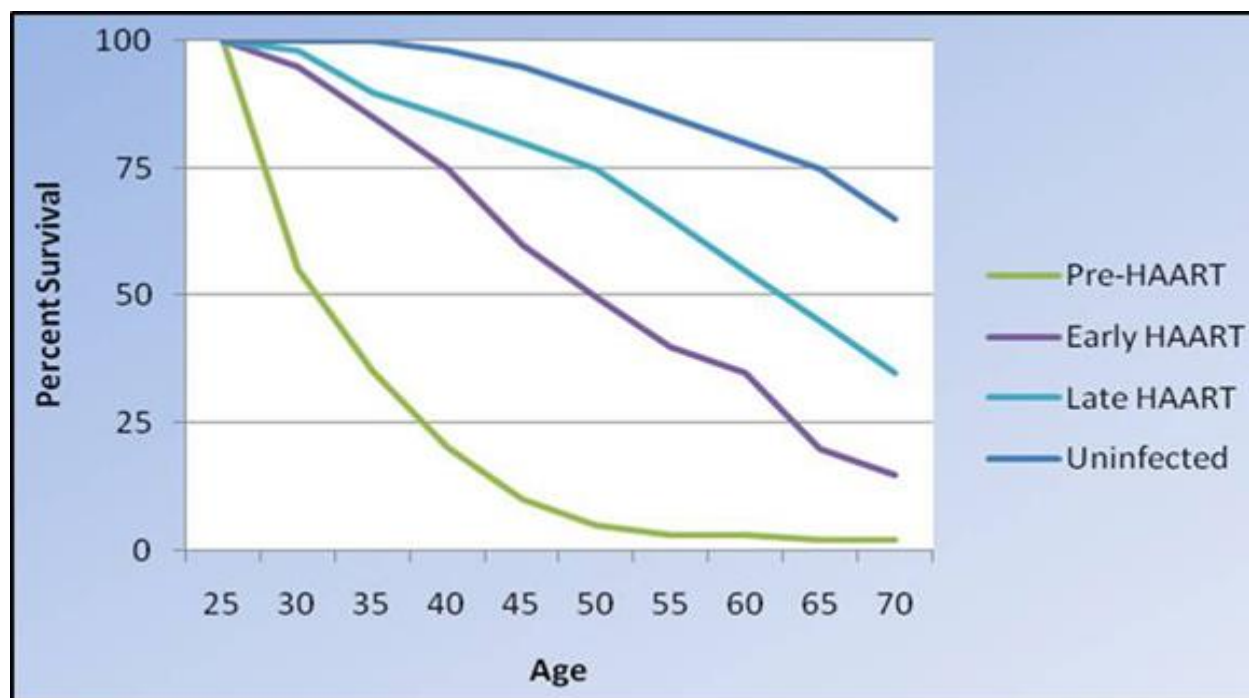
Figure 1.4: Changes in survival of people infected with HIV. Figure adapted with permission from (Lohse et al., 2007).

While approximately twenty five antiretroviral drugs have been approved for clinical use in the treatment of AIDS, the search for novel drugs and their subsequent development is continuously in progress (De Clercq, 2009; Hughes, Robinson, Tseng, & MacArthur, 2009).

HAART was implemented in clinical practice in 1995.However continuous efforts from the past two decades to improve HIV treatment via our understanding of infection at the molecular level has helped in advancement of treatment options. In the present era, because of these efforts HIV epidemic has transformed into a manageable complex infection (Chene et al., 2003; Egger et al., 2002; Opravil et al., 2002).

A brief description of the FDA approved drug classes that are currently in use for the treatment of HIV are presented in Table 1.2.

Table 1.2 FDA-approved antiretroviral drugs .

NRTIs	NNRTIs	PIs	FI
Abacavir (ABC, Ziagen)	Nevirapine	Atazanavir (Reyataz)	Enfuvirtide
Didanosine (ddI, Videx)	Delavirdine (Rescriptor) 1st Generation	Darunavir (Prezista)	
Emtricitabine (FTC, Emtriva)	Efavirenz (Sustiva) 1st Generation	Fosamprenavir (Lexiva)	
Lamivudine (3TC, Epivir)	Nevirapine (Viramune) 1st Generation	Indinavir (Crixivan)	
Stavudine (d4T, Zerit)	Etravirine (Intelence) 2nd Generation	Lopinavir/ritonavir (Kaletra)	
Tenofovir (TDF, Viread)	Rilpivirine (Edurant) 2nd Generation	Nelfinavir (Viracept)	
Zalcitabine (ddC, Hivid)		Saquinavir (Invirase)	
Zidovudine (ZDV, Retrovir; formerly azidothymidine [AZT]		Tipranavir (Aptivus)	

1.3.1 Nucleoside/nucleotide analogue reverse transcriptase inhibitors

The nucleoside/nucleotide reverse transcriptase inhibitor (NRTIs) was the first drug class approved for the treatment of HIV infection. Although NRTIs were less effective against HIV than non-nucleoside

reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs), they had a central role in antiretroviral treatment and remain part of the current standard of care. NRTIs are equally effective against HIV-I and HIV-II (Cox, Aperia, Albert, & Wahren, 1994).

NRTIs are competitive inhibitors of reverse transcriptase (RT) enzyme substrate. NRTIs bear high structural similarity with DNA nucleoside bases. NRTIs block the activity of RT by competing against natural substrate and incorporate into viral DNA and act as chain terminators (Mitsuya & Broder, 1986). Phosphorylation at active 5' end is required in the cell for its activity (W. Y. Gao, Agbaria, Driscoll, & Mitsuya, 1994). Tenofovir is the only NRTI that requires phosphorylation by cellular enzyme to form a diphosphate which is necessary for its antiviral activity.

1.3.2 Non-nucleoside analogue reverse transcriptase inhibitors

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) were introduced in 1996. Nevirapine was the first FDA-approved NNRTI. NNRTIs exhibit potent activity against HIV-I and are preferred in the treatment regimens (Table1.2) (Shen et al., 2008).

NNRTIs bind with HIV-RT in a non-competitive fashion (Grob et al., 1992; Merluzzi et al., 1990). All NNRTIs bind at the p66 subunit, a hydrophobic pocket that is distinct from the substrate site and block DNA polymerase by conformational changes that result in the disruption of the catalytic site of enzyme. NNRTIs do require phosphorylation and subsequent incorporation into viral DNA for their optimum anti-viral activity (Witvrouw et al., 2004).

1.3.3 Protease inhibitors

HIV PIs were first introduced in 1995 and are an integral part of the HIV treatment regimen (AIDSinfo, 2011). A total of 8 compounds have been approved so far (Table 1.2).

HIV-I protease is a complex enzyme. Protease inhibitors specifically target the HIV protease enzyme resulting in the release of dysfunctional viral particles into the cell devoid of infectious activity (Navia et al., 1989; Pillay, Bryant, Getman, & Richman, 1995).
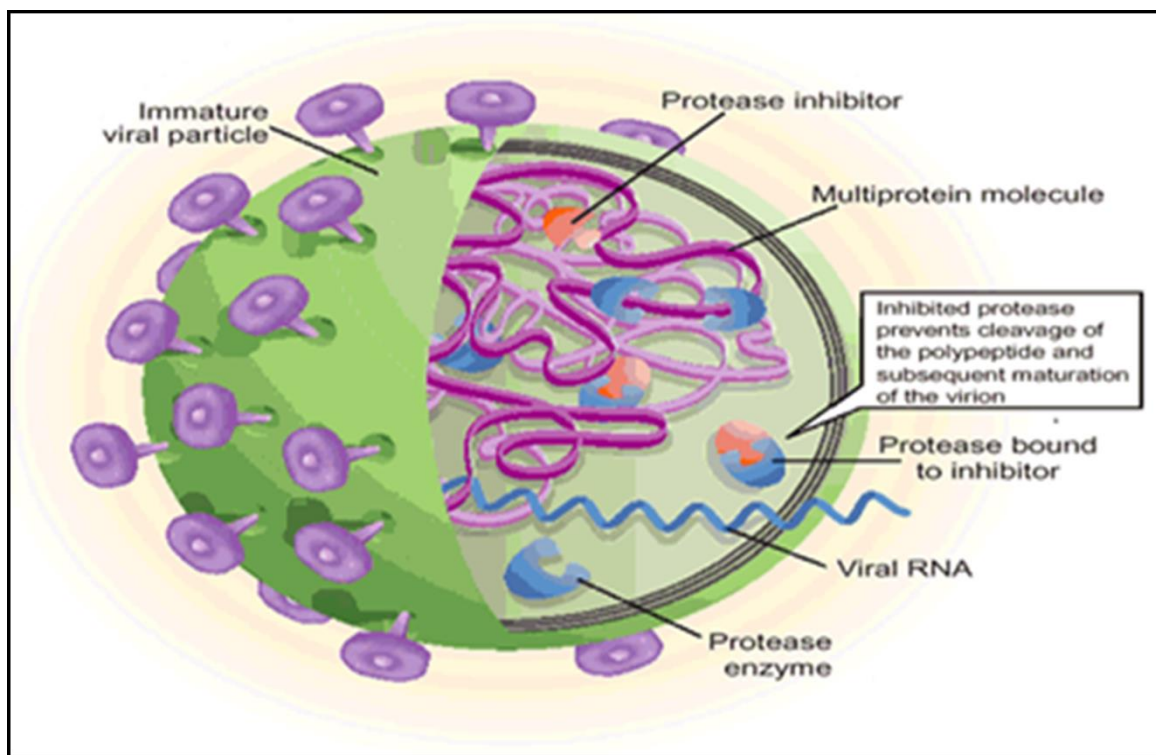
Figure 1.5: Mechanism of action of a protease inhibitor, Figure adapted from (Richman et al., 2001).

1.3.4 Fusion Inhibitor

Enfuvirtide is the only FDA-approved fusion inhibitor (FI), which is a 36 amino acid peptide and homologous to the heptad-repeat 2(HR2). After binding to the heptad-repeat 1(HR1) motif, it inhibits the formation of the six helix bundle that is required for fusion (Lalezari et al., 2003). Heptade-repeat (HR) 1 and 2 are the two different motifs of gp 41 that unite to form a six helix bundle hairpin structure. This hairpin structure then pulls the membrane of the virus and cell closer and result in membrane fusion (Lalezari et al., 2003; Lazzarin et al., 2003).

1.4 Cellular and biochemical alterations induced by highly active antiretroviral therapy

Since HAART has been successful in decreasing morbidity and mortality rates significantly (Paik & Kotler, 2011), the Southern African HIV Clinician's Society recommends maximally suppressive ART regimen to obtain effective outcomes for anti-HIV treatment and to avoid resistance problem (Meintjes G, Conradie F, Osih R, & D., 2012). Non-suppressive regimens *e.g.,* mono/dual NRTI therapy have a role in the prevention of mother-to-child transmission (PMTCT), but these treatment regimens are strongly discouraged ((SAHCS), 2008).

In southern Africa, to reduce pill burden and to provide improved treatment compliance, different fixed-dose drug combinations have been recommended. Ritonavir is usually used in combination with lopinavir and saquinavir and is strongly recommended to be taken with other PIs (Meintjes G et al., 2012).

Table 1.3: Common Adverse Drug Reactions of ARV Agents Available in Southern Africa.

Generic name	Class of drug*	Common or severe adverse drug reactions
Zidovudine (AZT)	NRTI	Bone marrow suppression, gastro-intestinal (GI) upset, headache, myopathy, hyperlactataemia /steatohepatitis (medium potential), lipoatrophy
Didanosine (ddI)	NRTI	Peripheral neuropathy, pancreatitis, nausea, diarrhoea hyperlactataemia/steatohepatitis (high potential
Lamivudine (3TC)	NRTI	Anaemia (pure red cell aplasia) (rare), hyperlactataemia/steatohepatitis (very low potential)
Stavudine (D4T)	NRTI	Peripheral neuropathy, lipoatrophy, hyper lactataemia/steatohepatitis (high potential), pancreatitis, HIV associated neuromuscular weakness syndrome (HANWS) (rare), dyslipidaemia
Abacavir (ABC)	NRTI	Hypersensitivity reaction, hyperlactataemia/steatohepatitis (very low potential)
Tenofovir (TDF)	NRTI	Renal failure, tubular wasting syndrome, reduced bone mineral density, hyperlactataemia/steatohepatitis (very low potential)
Emtricitabine (FTC)	NRTI	Palmar hyperpigmentation, hyperlactataemia/ steatohepatitis (very low potential)
Nevirapine (NVP)	NNRTI	Rash, hepatitis
Efavirenz (EFV)	NNRTI	Central nervous system symptoms (vivid dreams, problems with concentration, confusion, mood disturbance, psychosis), rash, hepatitis, gynaecomastia
Etravirine (ETV)	NNRTI	Rash, hepatitis
Indinavir (IDV) (rar Atazanavir (ATV)rarely used)	PI	Kidney stones, unconjugated hyperbilirubinaemia (visible jaundice in minority of patients), GI disturbances, hair loss, hyperglycaemia, headache, dyslipidaemia
Atazanavir (ATV)	PI	Unconjugated hyperbilirubinaemia (visible jaundice in minority of patients), dyslipidaemia (low potential), renal stones (rare), hepatitis
Lopinavir/ritonavir (LPV/r)	PI	GI upset, dyslipidaemia, hepatitis
Darunavir (DRV)	PI	GI upset, rash, dyslipidaemia, hepatitis Contains sulphonamide moiety (use with caution in patients with sulpha allergy)
Saquinavir (SQV) (hard gel formulation, rarely used	PI	GI disturbance (mild), hepatitis, hyperglycaemia, dyslipidaemia

*All protease inhibitors (PIs) may be associated with cardiac conduction abnormalities (especially PR prolongation). This seldom results in clinically significant effects, but caution should be taken when co-prescribing other drugs that cause delayed cardiac conduction, such as acrolides.
NRTI = nucleoside reverse transcriptase inhibitor; NtRTI = nucleotide reverse transcriptase inhibitor; NNRTI = non-nucleoside reverse transcriptase inhibitor; PI = protease inhibitor; InSTI = integrase inhibitor (integrase strand transfer inhibitor)

A number of side effects have been reported with HAART. These include occurrence of high levels of cholesterol, triglycerides and total serum low-density lipoprotein (LDL). This has been reported in approximately 70% of HIV-infected patients in association with the lipodystrophy syndrome. 8-10 % of HIV infected patients have insulin resistance with elevated levels of C-peptide and insulin leading to type II diabetes mellitus (Carr, Samaras, Burton, et al., 1998; Grinspoon & Carr, 2005; Wand et al., 2007), (see section 1.4.1 for detail).

These biochemical effects increase the increase risk of developing cardiovascular and metabolic disorders in HIV-infected individuals (Friis-Moller, Weber, et al., 2003). Previous studies showed that the protease inhibitors, NRTIs, and NNRTIs play a major role in inducing these metabolic disorders in HAART-treated patients. However, not much is known about the mechanisms of these adverse effects (Gougeon et al., 2004).
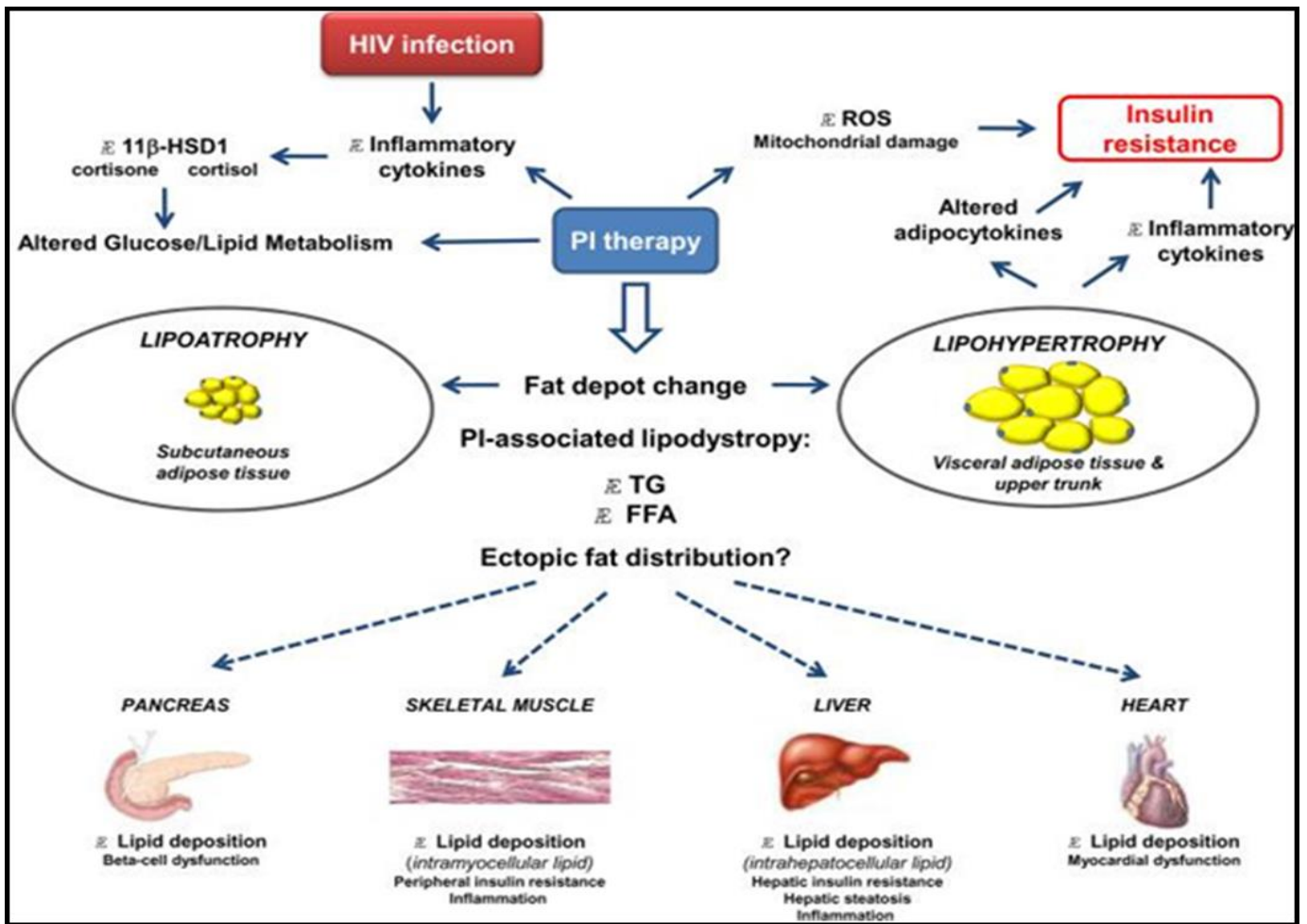
Fig 1.6: A scheme for the development of HIV/Protease inhibitor-associated lipodystrophy and associated adverse effects. (Anuurad et al., 2010).

11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; FFA, free fatty acids;HIV, human immunodeficiency virus; PI, protease inhibitor; ROS, reactive oxygen species.TG, triglyceride) (11β-HSD1,11β-hydroxysteroid dehydrogenase type 1.

1.4.1 Insulin resistance

In 1996, when PIs were first used to treat HIV infected individuals, the very first metabolic alteration or abnormality observed was insulin resistance (Carr, Samaras, Burton, et al., 1998; Carr, Samaras, Chisholm, & Cooper, 1998). An understanding of the molecular mechanism of this induced insulin resistance presents a challenge towards making antiviral drugs safer for use in HIV patients. It is absolutely critical to understand the molecular pathway to improve the quality of life of patients and minimize the associated metabolic abnormalities (Hruz, 2011).

A number of models have been used to explore ARVs and to understand how they induce insulin resistance and other associated cellular and biochemical alterations. These have included human and animal studies. Cellular models have been used to provide useful insight about ARVs and associated metabolic alterations. It has been reported that PIs can block glucose uptake by cells and association of NRTIs with mitochondrial toxicity is well described (Hertel, Struthers, Horj, & Hruz, 2004; Mallal, John, Moore, James, & McKinnon, 2000). Therefore detailed evaluation of cellular compartments and mechanisms will enable us to learn about involvement of various organelles e.g. endoplasmic reticulum which may play a role in the metabolic disturbances. Oxidative stress in the endoplasmic reticulum is linked to inducing insulin resistance. Furthermore, alteration in adipokine secretion and alterations in lipid metabolism may also play a part (Hruz, 2011).

Short or long term exposure to PIs causes insulin resistance. Clinical studies have shown induction of insulin resistance even after a single therapeutic dose of certain PIs in healthy volunteers (Lee GA, 2004; Noor et al., 2001; Noor et al., 2002). The acute response to PIs is inhibition of peripheral glucose uptake (G. A. Lee et al., 2006; Noor, Flint, Maa, & Parker, 2006; Noor et al., 2004). Similarly prolonged or chronic treatment has an effect on hepatic glucose metabolism (Haugaard et al., 2005; G. A. Lee et al., 2004). These findings provide some understanding of the toxicity induced by PIs (GA

Lee, 2005; Noor et al., 2006; Noor et al., 2004; van der Valk et al., 2001). In addition HIV-I infection leads to a reduction of insulin sensitivity via activity of the protein (Vpr). Vpr is a HIV- I accessory protein which is reported to increase sensitivity to glucocorticoids by up regulating glucocorticoid-responsive promoters *in vitro* (Kino et al., 2002). Vpr also causes the reduction of peroxisome-proliferator-activated receptor-γ (PPAR-γ) activity and obstructs the suppressive effects of downstream insulin-regulated transcription factors, which results in tissue-selective insulin resistance (Kino et al., 2005; Shrivastav, 2000). Therefore it is clear that both viral infection and PI associated changes during the course of treatment with PIs on a short term or long term basis alters cellular mechanism and this may directly or indirectly lead to insulin resistance.

1.4.1.1 Insulin receptor and post-receptor signaling

Insulin signalling begins with binding of ligand to its receptor, resulting in activation of the intrinsic tyrosine kinase moiety which is an integral part of insulin receptor (IR). The IR has two extracellular and two trans-membrane subunits called alpha (*α1 and α2*) and beta (*β1 and β2*) subunits respectively. Two alpha subunits and beta subunits form a heterotetrameric complex linked by disulphide bonds. The tyrosine residues that are phosphorylated are present in the *β* subunit of IR. Upon binding of insulin to the extracellular *α* subunit a conformational change is transmitted through the membrane to the intracellular tyrosine kinase C terminal domain of the *β* subunit and thus auto-phosphorylation of tyrosine residues occurs in *β* subunit (Pessin & Saltiel, 2000; Van Obberghen et al., 2001). Auto phosphorylation activates the catalytic activity of the tyrosine kinase of IR towards other substrates. The phosphotyrosine-binding (PTB) domains of the insulin receptor substrate family (IRS) interact with the juxtamembrane phosphorylation sites and facilitate interaction and phosphorylation of IRS with the insulin receptor (Saltiel & Kahn, 2001). IRS proteins are ubiquitous but they are expressed in

the target cells for insulin, including hepatocytes, myocytes and adipocytes (Brunetti, Manfioletti, Chiefari, Goldfine, & Foti, 2001).

The subsequent phosphorylation of important tyrosine residues on IRS proteins lead to binding of the Src homology 2 (SH2) domains of the p85 regulatory subunit of phosphatidylinositol PI 3-kinase. The catalytic subunit of PI 3-kinase, p110 on activation, then phosphorylates phosphatidylinositol (4, 5) bisphosphate (PIP$_2$) forming phosphatidylinositol (3, 4, 5) triphosphate (PIP$_3$). PIP3 in the next step activates the phosphoinositide–dependent kinase-1 (PDK1). PDK1 phosphorylate serine/threonine kinase Akt (also known as PKB). PIP3 also interacts directly with the PH domain of the Akt to recruit Akt to the plasma membrane. The activation of PKB/Akt is required for the steps leading to glucose transport, which involves the migration of glucose transporter 4 (GLUT4) from the cytoplasm to the cell membrane, in order to facilitate the uptake of extracellular glucose. Akt activation also leads to phosphorylation of glycogen synthase kinase 3 (GSK3) in cytoplasm. GSK-3 was initially identified as a glycogen regulating enzyme in response to insulin (Welsh, Wilson, & Proud, 1996). GSK-3 is a serine/threonine protein kinase that phosphorylates and inactivates glycogen synthase and is an important downstream member of the PI3K/Akt cell survival pathway. Its activity is inhibited by Akt-mediated phosphorylation at Ser21 of GSK-3α and Ser9 of GSK-3β (Srivastava & Pandey, 1998).
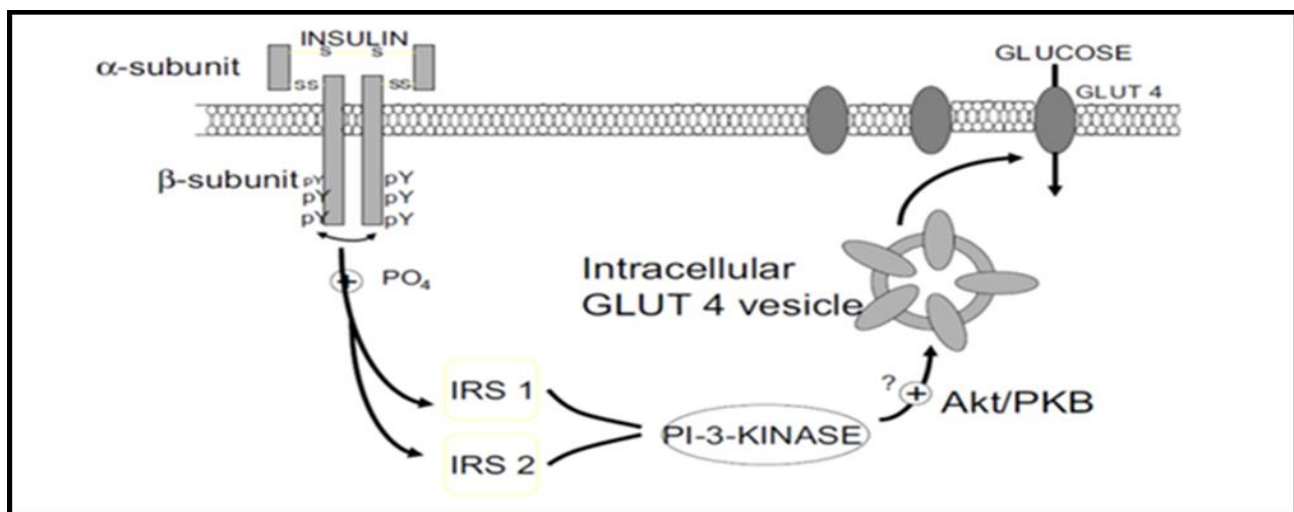
Figure1.7: Insulin signaling pathway showing binding of insulin with the IR leading to activation of Glut4 which imports glucose in to the cell (Bhattacharya, Dey, & Roy, 2007).

Insulin also causes mitogenic cellular response in the adipocyte through MAP kinases ERK1/2 pathway. ERK1/2 phosphorylates and regulates a large number of substrates including transcription factors such as Elk-1 and c-Fos (Cheng, Tseng, & White, 2010; Plotnikov, Zehorai, Procaccia, & Seger, 2011).ERK1/2 also increase the mRNA translation in response to insulin through the phosphorylation and inactivation of the TSC1/2 complex (Ma, Chen, Erdjument-Bromage, Tempst, & Pandolfi, 2005) and through phosphorylation of raptor in mTORC1(Carriere et al., 2011; Langlais, Yi, & Mandarino, 2011) . Besides it , in response to stimulation with growth factors, also phosphorylation of S6 was shown to be regulated by the ERK1/2 pathway acting in parallel to mTORC1 regulation of S6 (Roux et al., 2007).So translation of S6 is regulated both by the PI3K/PKB/mTORC1 and the ERK1/2 pathway.

1.4.1.2 Mechanismsof insulin resistance induced by ARVs at cellular level

As a result of the alteration in metabolism and cellular pathways associated with HIV infection and treatment regimen using anti-retroviral therapy (ART), huge attention has been devoted to the link between insulin resistance and changes in cellular pathway due to antiviral drugs. These drug dependent cellular and metabolic changes affect glucose uptake and eventually contributes to the development of insulin resistance (Hruz, 2011). Insulin resistance is considered to be the primary metabolic complication of type 2 diabetes, (Meyer, Levin, Grimmsmann, Beck-Nielsen, & Klein, 2002; Nolan, Freidenberg, Henry, Reichart, & Olefsky, 1994; Savage, Petersen, & Shulman, 2005).

Insulin resistance is a significant clinical challenge in the treatment of HIV patients as they prolong exposure of patients to various combinations of drugs has contributed to the side effects seen. These are influenced by factors such as genetic predisposition, age, environmental factors and various disease related factors (Butt et al., 2009).

Glucose homeostasis is one of the main metabolic targets affected by ART. The organs and tissues involved in regulation of glucose homeostasis provide a link to monitor changes caused by ART and

HIV infection. However the cross communication between these tissues also regulates the insulin sensitivity. In order to gain a better understanding of glucose homeostasis and metabolic syndrome in terms of using ART, we can take advantage of findings of the effects of ART in HIV negative patients and vice versa (Hruz, 2011).

1.4.1.3 Protease inhibitors effect on glucose transport

The association of PI-based therapy with hyperglycaemia and insulin resistance, in HIV-infected patients, has been reported numerous times (G. Behrens et al., 1999; Dube, Johnson, Currier, & Leedom, 1997; Walli et al., 1998). Cultured 3T3-L1 adipocytes have widely been used to analyse the effects on glucose transport and have proved useful in the investigation of insulin signalling, glucose transport and insulin resistance caused by anti-retroviral therapy.

1.4.1.3.1 Inhibition of GLUT4

The binding of insulin to the insulin receptor of the adipocyte eventually results into the translocation of GLUT 4. Similarly fatty acids (FA) metabolized by the adipocyte are mainly produced by the action of LPL on the triglyceride (TG) transporting lipoproteins (chylomicrons and VLDL (very low density lipoproteins)) (Figure 1.8) (Large, Peroni, Letexier, Ray, & Beylot, 2004). Studies have shown that antiviral drugs such as PIs affect glucose uptake or transport. The mechanism by which PIs block glucose uptake has been reported to be via direct inhibition of GLUT4 i.e. glucose transporter (Isoform 4) (Murata, Hruz, & Mueckler, 2000). PIs have been shown to block insulin-stimulated glucose uptake in 3T3-L1 adipocytes via inhibition of the insulin-mediated GLUT4 translocation (Hertel et al., 2004; Noor et al., 2006). Interestingly, therapeutically relevant drug concentrations of PIs cause reversible inhibition of the glucose uptake while acute drug exposure has no effects on insulin signalling transduction and GLUT4 translocation *in vivo* (Murata, Hruz, & Mueckler, 2002). Inhibition of glucose

uptake will result in decreased synthesis of TG (Figure 1.8) (Parker et al., 2005). The decreased TG production in the adipocytes will result in decreased cell size and lipoatrophy. Similarly, proteasome inhibitions by PIs results in the deposition of unfolded proteins and induce ER stress (Parker et al., 2005; Ron & Walter, 2007).
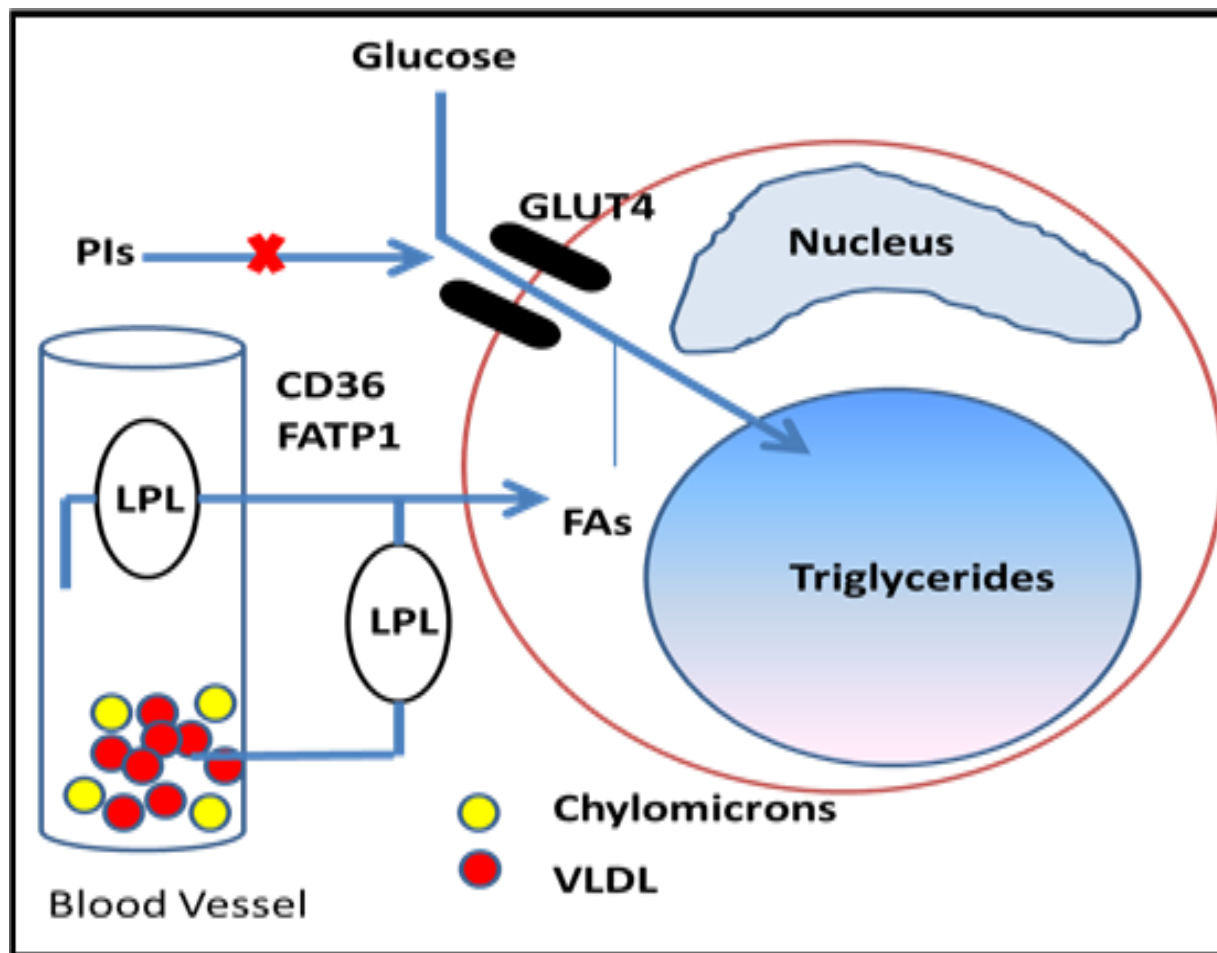
Figure 1.8: Protease inhibitors inhibit adipocyte glucose uptake.

1.4.1.3.2 Role of core peptidomimetic structure in insulin resistance

Detailed structural analysis of the first generation of PIs has revealed the presence of phenylalanine-like core structures surrounded by hydrophobic moieties called peptidomimetic which establishes direct interaction with GLUT4 to inhibit the glucose uptake (Hertel et al., 2004). Furthermore, this peptidomimetic class of PIs are more potent inhibitors on GLUT4 translocation. PIs, having peptidomimetic structures (ritonavir and indinavir) were compared with a non-peptidomimetic class

such "tipranavir". Tipranavir showed no inhibition of GLUT4 activity explaining the involvement of peptidomimetic structure in the mechanism of inhibition (Hruz & Yan, 2006). This is one aspect of the inhibition of glucose uptake by PIs. However it is interesting that atazanavir (ATV) which has a peptidomimetic core does not act the same way and shows no effect when used in therapeutic concentration refuting the idea that peptidomimetic core is required for exerting such an effect *in vitro* or *in vivo* (Noor et al., 2004; Yan & Hruz, 2005).

Earlier structural analysis of PIs also revealed the hydrophobic interaction around phenylalanine –like structure and presence of an additional pyridine ring in ATV can have steric interference or hindrance and it may prevent the binding to GLUT4. However, this is one of the hypotheses based on hydrophobicity but further studies are required to learn the structural differences and mode of actions among PIs (Hertel et al., 2004).
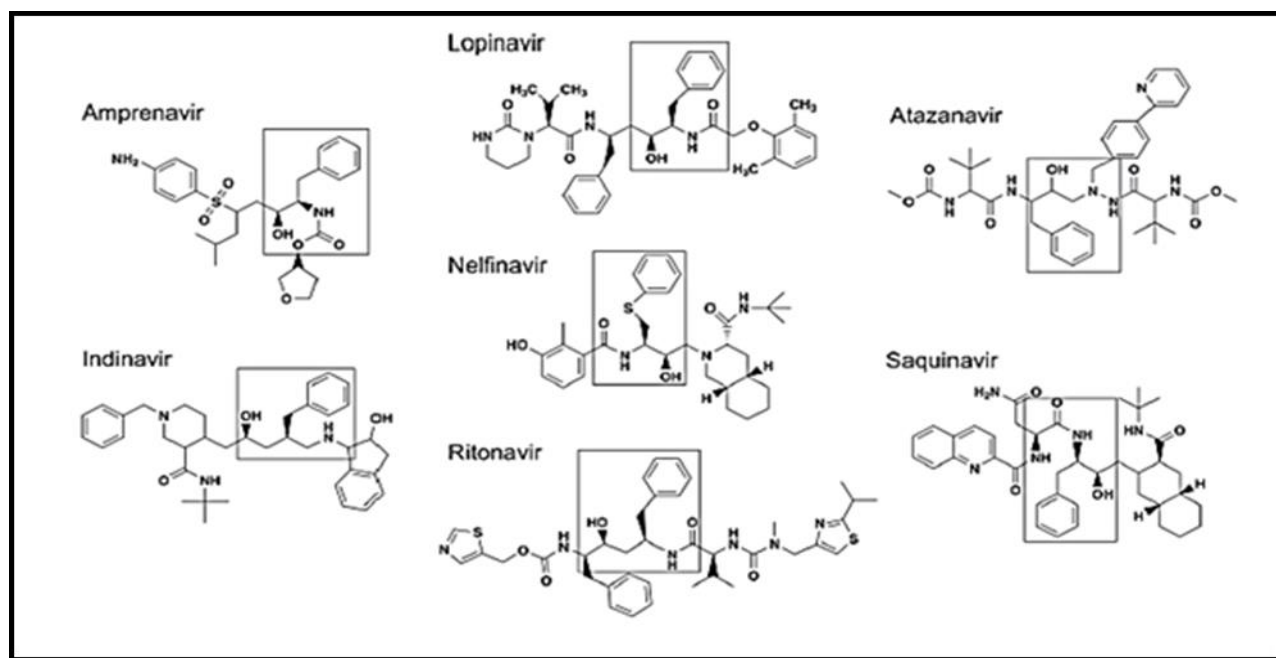
Figure 1.9: Shared structural features of the HIV protease inhibitors. Squares represent the core peptidomimetic structure found within all HIV PIs.(Oliver P. Flint, PhD4, & Bellamine, 2009).

1.4.1.3.3 Effect of protease inhibitors on proximal end of inulin signalling

The previous studies described have attributed the major mechanism of insulin resistance due to the inhibition of glucose uptake by PIs suggesting that the major effect is at the distal end of signaling, via GLUT 4 (Ben-Romano et al., 2003). However, this mechanism may vary amongst PIs when used for long periods of time and PIs may also affect the proximal steps in insulin signalling.

For example a previous study showed that saquinavir inhibited insulin receptor substrate-1 (IRS-1) phosphorylation in cultured adipocytes after 48 hours exposure. However, this only occurred at relatively high toxic drug concentrations (Algenstaedt et al., 2003). Exposure of 3T3-L1 adipocytes to nelfinavir for 18 hours led to inhibition of phosphorylation of Akt without affecting activation of PI3 kinase (Ben-Romano et al., 2004).

Oxidative stress is one of the possible underlying causes for these effects (Ben-Romano et al., 2006). Prolonged exposure (3 weeks) to indinavir has been associated with activation of the suppressor of cytokine signaling-1 (SOCS-1) signalling cascade, as well as increased levels of the sterol response element binding protein-1 (SREBP-1) and the inflammatory cytokine tumour necrosis factor-α (TNF-α) in diabetes-prone rats (Carper et al., 2008).

1.4.1.4 Effect of ARVs on Cellular organelles and cytokines and mechanism of insulin resistance

(a) Mitochondrial dysfunction

Human and animal studies have shown that insulin resistance occurs independently of HIV infection (Hruz, Murata, Qiu, & Mueckler, 2002; Hruz & Yan, 2006; Noor et al., 2006). In these studies, the results have shown that most of the PIs cause abrupt peripheral insulin resistance which correlates with the ability of PIs to block GLUT4 translocation in *vitro.*

The dysregulation in glucose levels amongst HIV-infected patients receiving thymidine NRTIs suggests a strong association between insulin sensitivity and lipodystrophic changes (Shlay et al., 2005). Zidovudine/lamivudine treatment has also been shown to be correlated with insulin résistance.

However, this occurs without the alteration in body fat composition as compared to patients receiving NRTI-sparing regimens (Blumer et al., 2008). In another study the association between NRTIs use and insulin resistance was validated, HIV-negative human volunteers receiving stavudine for 4 weeks had decreased mitochondrial function and increased insulin resistance (Fleischman et al., 2007). The exact molecular mechanisms involved in inducing insulin resistance have yet to be established. There is mounting evidence suggesting the involvement of mitochondrial dysfunction mediating the insulin resistance in non-HIV patients with type 2 diabetes (Kelley, He, Menshikova, & Ritov, 2002).

Thymidine containing NTRIs (zidovudine and stavudine) are associated with severe mitochondrial toxicity and lipoatrophy. (McComsey & Walker, 2004). The precise mechanisms underlying mitochondrial toxicity are still unclear. Mitochondrial DNA depletion is a consequence of inhibition of DNA polymerase γ by NRTIs, is one possible mechanism, however other factors may contribute (Feeney & Mallon, 2010).

(b) Endoplasmic reticulum stress

Lipoprotein production is regulated by intracellular lipid sensors (Horton, Goldstein, & Brown, 2002) known SREBPs (Riddle, Kuhel, Woollett, Fichtenbaum, & Hui, 2001). The SREBPs sensor regulatory mechanism works when intracellular lipid levels decreases. It activates the sensor followed by transportation from endoplasmic reticulum (ER) to the nucleus. In the nucleus the sensor up-regulates the genes involved in synthesis and transport of cholesterol, triglyceride and fatty acid synthesis. Once the genes are activated the sensor itself undergoes degradation by the proteasomes (Hirano, Yoshida, Shimizu, & Sato, 2001).

PIs influence activation of the intracellular lipid sensor. In hepatocytes indinavir and ritonavir activate SREBP-1 and SREBP-2 (Riddle et al., 2001; Williams, Rao, Natarajan, Pandak, & Hylemon, 2004) or in some cases these PIs are linked to SREBP migration into the nucleus (Facilitated by lamins A and C), resulting in SREBP-1 dislocation (Caron, Auclair, Sterlingot, Kornprobst, & Capeau, 2003; Caron

et al., 2001; Coffinier et al., 2007). Excessive accumulation of cholesterol in the ER membranes occurs as a result of the activation of SREBP, resulting in the alteration of intracellular homeostasis. SREBP is also involved in stress sensing, regulation of cell growth, differentiation and apoptosis. This mechanism of SREBP activation and involvement in intracellular activities is described as the unfolded protein response (UPR) (Xu, Bailly-Maitre, & Reed, 2005; K. Zhang & Kaufman, 2004).

In order to restore cellular homeostasis due to higher levels of misfolded or unfolded proteins in ER, cellular signalling pathways are activated to regulate translation, attenuation of unfolded proteins, up-regulation of ER chaperones and the degradation of unfolded proteins. However, during the restoration of cellular homeostasis cells can undergo apoptosis.

ER stress is said to induce unfolded protein response. This has helped in the understanding of its association with inflammation and insulin resistance in the associated metabolic syndrome not associated with HIV infection (Hotamisligil, 2010). A few *in vitro* studies have indicated that proteasome inhibition by some PIs can result in ER stress and the unfolded protein response in adipocytes (Parker et al., 2005). Excess calorie intake and lack of physical activity can lead to obesity. Obesity-related ER stress can further exacerbate visceral adiposity.

Endoplasmic reticulum stress related activation of c-Jun N-terminal kinase (JNK) or IKK either directly or via increased generation of reactive oxygen species (ROS), induce serine phosphorylation of IRS-1. NRTI-induced mitochondrial dysfunction can also induce ROS production, which further enhances the inhibitory effects on insulin signaling pathways, but similar to PI-mediated toxicities, mitochondrial dysfunction varies depending on the class of the drug (Maagaard & Kvale, 2009).

(c) Inflammatory cytokines

There is strong association of inflammation with adipose tissues and it was investigated in non HIV-infected patients (Cawthorn & Sethi, 2008). Inflammatory cytokines are secreted by macrophages resident in adipose tissues and elevated levels of these cytokines are somehow associated with insulin

resistance in HIV positive patients. TNF-α and IL-6 are among the inflammatory cytokines that mediate insulin resistance (Brown, Tassiopoulos, Bosch, Shikuma, & McComsey, 2010).

The mechanism of induction of insulin resistance via TNF- α is channelled via activation of mitogen activated protein kinase (MAPK) which leads to the inhibition of interferon kappa B kinase (IKK) which eventually phosphorylates serine residues of IRS-1. Protein tyrosine phosphorylase b (PTP1b) plays role in an inactivation of insulin receptor and IRS-1 via tyrosine dephosphorylation. TNF-α influences the activation of PTP1b, which can dephosphorylate and deactivate insulin receptors and IRS proteins (Tilg & Moschen, 2008). Accumulation of fatty acids in skeletal muscles due to elevated hepatic triglyceride is yet another indirect route to insulin resistance mediated by TNF-α (Qin, Anderson, & Adeli, 2008).

1.4.1.5 Effect of ARVs on skeletal muscle and mechanism of insulin resistance

Skeletal muscle is responsible for the majority of peripheral glucose uptake upon insulin activation. Insulin activation brings about the translocation of GLUT4 from intracellular storage vesicles to the plasma membrane. Since the insulin signalling pathways are the same in adipocytes and myocytes, it is not surprising that PIs also play a role in the inhibition of glucose uptake in these tissues and can directly cause a decrease in glucose disposal in muscle.

On the basis of the gold standard test used to measure glucose uptake in peripheral blood (clamp studies), it was concluded the impaired skeletal muscle glucose uptake is associated with HAART in HIV infected patients (G. M. Behrens et al., 2002; DeFronzo, Tobin, & Andres, 1979).

Other cellular mechanisms may be involved in contributing towards impaired skeletal muscle insulin sensitivity. Long term exposure of ARVs to patients, can lead to ectopic skeletal muscle accumulation which can be associated with adipose tissue lipolysis. This compromises adipocyte storage capacity ultimately leading to severe lipoatrophy in patients on HAART (Gan et al., 2002; Luzi et al., 2003).

Another study has indicated the involvement of PIs in abnormal fatty acid oxidation in cultured C2C12 myotubes (Richmond et al., 2010).

The effects of abnormal fat partitioning in skeletal muscles among uninfected patients has provided new insights among those infected with HIV (Unger, Clark, Scherer, & Orci, 2010). Many lipid derivatives have been identified as being involved in insulin resistance via the activation of protein kinase θ, JNK and IKK, which cause phosphorylation at serine 307 residue of IRS-1 resulting in IRS-1 inactivation (Z. Gao et al., 2004). A Few studies have recently demonstrated that an excess in diaglycerol can cause IRS-1 inactivation (Samuel, Petersen, & Shulman, 2010). Similarly, another study characterized the insulin signalling pathway in muscle harvested from lipodystrophic HIV-infected patients under hyperinsulinaemic clamp conditions. The results showed dysregulation of the insulin signaling pathway downstream of IRS, at the level of Akt phosphorylation (Haugaard et al., 2005). An *in vivo* study investigated the extent to which nelfinavir impaired Akt phosphorylation using cultured adipocytes. Similar findings were observed in insulin signalling pathways in skeletal muscle exposed to PIs (Ben-Romano et al., 2004).

1.4.2 Effect of ARVs on fat redistribution in HIV infected patients

It is not clear if lipodystrophy is induced due to a single mechanism. It has been proposed that multiple different disorders in HIV-infected patients contribute to the development of lipodystrophy. Furthermore, it is considered that the mechanism of peripheral lipoatrophy is different from the mechanism associated with central lipohypertrophy (Mulligan et al., 2006). Similarly in the early stage of PI treatment, the associated accumulation of trunk fat (buffalo hump) indicates that visceral fat is relatively more resistant to the side effects of PIs and NRTIs but the impact of hyperlipidaemia is same (Dube et al., 2005; Mallon, Miller, Cooper, & Carr, 2003).

A study of HIV patients with lipoatrophy, having been treated for more than three months with ARVs (PIs and NRTIs) showed increased glucose uptake in subcutaneous fat. This was not seen in patients treated similarly, but without the evidence of fat wasting (Hadigan et al., 2006). It has been hypothesized that the counteractive action of PIs causes the inhibition of GLUT4. However, no up-regulation of GLUT1 expression was observed to account for the slow increase in glucose uptake (Hadigan et al., 2006). NRTIs also play an important role in the development of lipoatrophy. However, only a link between PIs and the wasting of white adipose tissue has been established in human (Dube et al., 2005; Heath et al., 2002) and animal models (Prot et al., 2006).

Visceral fat is functionally and metabolically different from subcutaneous fat (DiGirolamo, Newby, & Lovejoy, 1992; Katzmarzyk, Perusse, & Bouchard, 1999). Irrespective of the cause, the pathophysiologic signals that stimulate alterations in the body fat distribution in HIV, affect cellular metabolism and at the same time inhibit normal storage and increase the accumulation of ectopic visceral fat (Unger, 2003).

The clinical picture depends on the amount of body fat at baseline and total energy balance (lipoatrophic or lipohypertrophic). Patients with higher total body fat and positive energy balance, after the exposure of the ARVs result in further increases in trunk, visceral, breast, and dorsocervical fat accumulation. However those with lower total body fat and neutral or negative energy balance, present with decrease in fat from arms , face and missing ectopic fat deposition (He, Engelson, & Kotler, 2005). Interestingly the final picture irrespective of the initial level of adipose tissue, consistently results in central adiposity (Bacchetti et al., 2005).

This hypothesis seems consistent with many group studies, that peripheral lipodystrophy and central adiposity results from separate and independent metabolic pathways (Bacchetti et al., 2005; Wand H, 2005). The role of the drugs in this scenario appears pivotal because some body fat changes may improve after switching to newer therapies (McComsey et al., 2004; Shlay et al., 2005). Thus, it

appears that these aforementioned findings support independent mechanisms and an individual role of each drug in affecting metabolic pathways involved in lipid formation and distribution.

The molecular and genetic basis for non-HIV lipodystrophy syndromes has helped efforts to explain the metabolic variations that occur in ARV-treated HIV infected individuals (Garg & Misra, 2004; Simha & Garg, 2006). It is believed that abnormal storage of triglycerides as fat results in irregular lipid deposition amongst the insulin targeted tissues (such as liver and skeletal muscles) and this eventually leads to impaired insulin signaling (Samuel et al., 2010).

As described earlier, visceral and subcutaneous fat deposits, are metabolically and functionally different from each other (Wajchenberg, 2000) and these differences must be considered while analysing insulin resistance in HIV-infected patients. Visceral adipose tissue (VAT) appears to be closely linked with insulin resistance (Wajchenberg, 2000) and varies in nature through the effects of glucose level regulation. However in HIV-infected patients, omental-mesenteric fat is more closely associated with insulin resistance rather than VAT (He, Engelson, Albu, Heymsfield, & Kotler, 2003). The molecular basis of the metabolic differences between fat depots is still not well understood. Factors such as regional blood flow and cellular composition of different tissues (adipocytes, fibroblasts, endothelial cells, macrophages), and relative metabolic activity may explain these metabolic differences (Hruz, 2011).

1.4.2.1 Adipose tissue

Adipose tissue is the largest endocrine organ and source of energy, and hence plays an important role in the maintenance of normal glucose levels in the body (Galic, Oakhill, & Steinberg, 2010). Any dysregulation in the body fat and adipocyte function is directly related to the non-HIV metabolic syndrome (Bruce & Byrne, 2009). The development of lipodystrophy in HIV-infected patients (increased visceral adiposity and peripheral lipoatrophy) has been directly associated with the use of ART and affected pathways have been explored using both *in vitro* and *in vivo* models (D. Chen,

Misra, & Garg, 2002).The link between lipid and insulin resistance has previously been described. Association between free fatty acids and blockage of insulin stimulated glucose uptake in muscle through inhibition of pyruvate dehydrogenase was studied previously (Randle, 1963). Many *in vivo* and *in vitro* experiments elucidated that lipid-induced insulin resistance occurred via impaired insulin signalling pathways and through decreased glucose uptake rather than by a decreased glycolytic pathway (Dresner et al., 1999; Griffin et al., 1999)**.**

Recent studies have shown that HIV PIs affect adipocyte differentiation (Dowell, Flexner, Kwiterovich, & Lane, 2000; R. J. Kim, Wilson, Wabitsch, Lazar, & Steppan, 2006; Lenhard et al., 2000; B. Zhang et al., 1999). Moreover, other researchers have revealed that PIs alter the expression and location of SREBP-1, which affects the expression of adipogenic factors (Bastard et al., 2002; Caron et al., 2001).

Metalloproteinase ZMPSTE24 is essential for lamin A synthesis. Abnormality in ZMPSTE24 has been linked to hereditary lipodystrophy (Agarwal, Fryns, Auchus, & Garg, 2003). Some PIs such as indinavir and nelfinavir target ZMPSTE24 directly in adipocytes (Caron et al., 2003; Coffinier et al., 2007; Goulbourne & Vaux, 2010).

(a) Role of Adipocytokines

The role of adipose tissue goes further than what was initially understood as being only for energy storage, in that its role includes being involved in maintaining the level of glucose in the body by secreting adipocytokines such as adiponectin, leptin, and resistin (Waki & Tontonoz, 2007). The need to further understand the role of HAART and its effects on adipocytokine levels were realized when altered levels of adipocytokines were observed among HAART treated HIV-infected patients (Tsiodras, Perelas, Wanke, & Mantzoros, 2010).

Abnormal secretion of leptin, adiponectin and resistin by adipocytes can result in insulin resistance. This hypothesis is supported by the fact that an increase in insulin sensitivity was observed following leptin administration to patients suffering with severe hereditary lipodystrophy (Oral & Chan, 2010). Leptin administration has also been shown to improve lipid profiles and insulin sensitivity among leptin deficient lipodystrophic HIV-infected subjects (Mulligan et al., 2009).

Adipokines (adiponectin and leptin) have direct effects on muscle insulin sensitivity (Rabe, Lehrke, Parhofer, & Broedl, 2008). Studies conducted in HIV-negative human volunteers have highlighted the possible importance of PI-induced changes in adipokine levels. Single doses of lopinavir in healthy human volunteers reduced insulin sensitivity (measured by hyperinsulinaemic euglycaemic clamps) without affecting adiponectin levels (G. A. Lee et al., 2006). However, after 4 weeks of lopinavir treatment, no insulin resistance was noticed but adiponectin levels were elevated suggesting that compensatory changes may have been provoked (G. A. Lee et al., 2004).

Adiponectin is secreted in large quantities, and can elicit fatty acid oxidation .It also enhance insulin-dependent reduction in gluconeogenesis in liver (Berg, Combs, Du, Brownlee, & Scherer, 2001). A study has shown that decreased adiponectin concentrations is linked to insulin resistance and impaired glucose homeostasis in ARV treated HIV-infected patients with lipoatrophy and metabolic disorders (Vigouroux et al., 2003) or HIV seronegative patients (Weyer et al., 2001).

The causes appear to be multifaceted and affect both, adipocyte mass and function. It remains unclear whether HAART plays a primary role in affecting insulin signalling and glucose transport in adipocytes or whether it directly affects the adipokine secretion. 3T3-F442A adipocytes treated with nelfinavir, ritonavir and saquinavir all demonstrated decreased adiponectin secretion (Jones, Janneh, Back, & Pirmohamed, 2005). Similarly, in another study cultured human adipocytes exposed to various PIs have shown that a change in adipokine expression levels is associated with the production of reactive oxygen species (ROS) (Lagathu et al., 2007). A study to evaluate the role of NRTIs in HIV

infected subjects also suggests an association with stavudine and zidovudine (NRTIs) with decreased adiponectin and SREBP1c (Jones, Qazi, et al., 2005).

1.4.3 Dyslipidaemia

It is well established fact now that HAART is associated with abnormal levels of lipids in body. HIV infected individuals are most susceptible to metabolic syndrome and about 20-30% have low levels of HDL cholesterol. Furthermore, approximately 10-30% and 20-40% HIV infected patients have elevated cholesterol and triglycerides in blood respectively (Grinspoon & Carr, 2005). Abnormal redistribution of fats is a consequence of HAART and is either characterized by fat deposition in localized areas mainly under the skin called lipohypertrophy or localized loss of fat tissue and the condition is known as lipoatrophy. This abnormal fat redistribution (mixed lipodystrophy) is one of the main effects linked with HAART (Grinspoon & Carr, 2005; Lugassy, Farmer, & Nelson, 2010; Nagy et al., 2003). However, the highest prevalence of lipid abnormalities occurs among those with visible lipodystrophy (Grinspoon & Carr, 2005).

1.4.3.1 Protease inhibitors and nucleoside reverse ttranscriptase inhibitors associated dyslipidaemia

There is high percentage of nearly 10-85% patients affected by dyslipidaemia as a result of NRTIs and PIs. Such a high incidence of dyslipidaemia due to HAART depends on length of exposure to drug. Time is the important factor in terms of HAART associated risk factors such as lipodystrophy. It is also worth noticing that about 35% of the patients on HAART develop lipodystrophy in association with hyperglycaemia (Leow, Addy, & Mantzoros, 2003; Lugassy et al., 2010; Mantzoros, 2009).There is

also an elevation in the levels of total cholesterol and low density lipoproteins at the commencement of HAART, suggesting a return to pre-seroconversion levels (Riddler et al., 2003).

Abnormalities associated with HAART are not limited to adult patients but have been evident in young patients and children. Length of exposure to these drugs has similar effects/ consequences in younger patients and in children as discussed in adult patients (Aldrovandi et al., 2009; Carter et al., 2006; Vigano et al., 2003). HIV-infected children and adolescent experiencing lipodystrophy can range from 5% (Carter et al., 2006) to 85% (Vigano et al., 2003) of the cases.

A recent study suggests that the effects of treatment among naïve HIV-infected patients demonstrate that a pattern in fat redistribution can occur during the course of a two-year treatment. From the first few months up to a year, there is an increase in the peripheral fat, however after such time there appears to be a gradual decline. On the other hand central fat accumulation occurs in a steady manner during the six month of therapy (Calmy et al., 2008; Dube et al., 2007; Mallon et al., 2003).

Clinical findings indicate that during the two years of treatment hypercholesterolaemia develops early (after 4 months) while hypertriglyceridaemia and hyperinsulinaemia usually develop later (Mallon et al., 2003). The percentages of therapy induced hyperglycaemia, hypercholesterolaemia, hypertriglyceridaemia and lipodystrophy were; 5%, 25%, 20% and 15% respectively during the 5 years of receiving HAART (Tsiodras, Mantzoros, Hammer, & Samore, 2000).

HIV infection related abnormalities in lipid and lipoproteins levels were observed among both patients with or without AIDS. Before the era of HAART, HIV-infection was linked with acute phase response and inflammatory mediators such as interferon-α (Grunfeld et al., 1991) or TNFα. These responses were thought to promote dyslipidaemia (Feingold et al., 1993).

In the early phases of HIV infection, levels in both low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) are lower as compared to controls but with progression

of the disease to AIDS, triglycerides become significantly higher and LDL particles become less dense (Feingold et al., 1993).

1.4.3.2 Possible mechanisms for protease inhibitors -associated dyslipidaemia

Several mechanisms which link PIs to dyslipidaemia have been suggested include inhibition of SREBP-1, breakdown of Apo lipoprotein -B (Liang et al., 2001) increase in production of very low-density lipoproteins (VLDLs) (Purnell et al., 2000) and a resultant reduction in the lipoprotein lipase activity (Ranganathan & Kern, 2002) and changes in distribution of lipid stores (Reeds et al., 2003).

The effect of PIs on serum lipid levels appear to be independent from HIV infection itself but not all PIs are involved in causing dyslipidaemia in HIV-negative and positive subjects (Jemsek et al., 2006; Noor et al., 2001).Similarly lipid associated abnormalities also seen in treatment regimen without PIs (Dube et al., 2003; van Leth et al., 2004).

1.5 Objective

1.5.1 Background of study

This work continued on work done previously with   the 3T3 LI Adipocyte (the most commonly used cellular model for insulin resistance) treated with PIs. This previous work was conducted in University of Cape Town as part of another PhD thesis and has been published in the Journal of Cellular Biochemistry (2013).

Protease inhibitors and NRTIs, are the major components of HAART, and cause various metabolic disorders in HIV patients (section 1.4). The primary side-effects induced by these drugs are insulin resistance and lipodystrophy. The understanding of the insulin resistance and lipodystrophy induced by PIs and NRTIs is still at its initial stages. Existing knowledge does not precisely describe the

mechanisms of insulin resistance and lipodystrophy because most studies have been performed using different models and thus providing different conclusions.

1.5.2 Aims of study

The objectives were therefore:

1. To analyse the effects of PIs and NRTIs on the insulin signalling pathway of Chinese hamster ovarian cells transfected with high levels of human insulin receptor (CHO-IR). The study will focus on the proximal steps in the cascade as well as some of the more distal steps and regulatory pathways such as nuclear factor k B (NFκB).

2. To determine the potency of therapeutic compounds sodium salicylate and berberine chloride to reverse the effects induced by PIs and NRTIs.

3. To evaluate the effect of PIs and NRTIs on metabolic pathways in CHO-IR cells by analysing metabolites in the cell culture medium, particularly amino acids and organic acids.

4. To analyse and compare the effects of PIs and NRTIs on lipoprotein lipase activity, an important step in the insulin-activated anabolic functions. This will be achieved by establishing a rapid method for analysing LPL activity in supernatants from CHO-IR cells.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Materials

2.1.1 Antibodies

Actin goat polyclonal IgG (SC1615)	Santa Cruz Biotechnology, CA, USA
Goat anti-mouse horseradish peroxidase-conjugated (HRP) (31430)	Pierce, IL, USA
Goat anti-rabbit HRP (SC2004)	Santa Cruz Biotechnology, CA, USA
IR β-subunit rabbit polyclonal IgG (SC711)	Santa Cruz Biotechnology, CA, USA
Mouse anti-goat HRP (#31400)	Pierce, IL, USA
Mouse monoclonal IgG of PY20 (610000)	BD Transduction Laboratories, NJ, USA
P-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Mouse mAb #9106	Cell signaling Technology
p44/42 MAPK (Erk1/2) Rabbit mAb #9102	Cell signaling Technology
P-Akt rabbit(Ser 473)(9271)	Cell signaling Technology
Akt anti-rabbit (#9272)	Cell signaling Technology
P-GSK-3alpha/beta(ser21/9)(#9331)	Cell signaling Technology

2.1.2 Cell line

CHO-IR cells	Dr. Gus Gustafson, University of Maryland, USA (Wilcox et al., 2004)

2.1.3 Chemicals/Reagents

Acetic acid (2789)	Merck, South Africa
Acetonitrile (1525162)	BDH laboratory supplies UK
Acrylamide/bis (A3449)	Sigma, MO, USA
Antibiotic/antimycotic solution (17-745E)	Lonza, MA, USA
Ammonium per sulphate (APS) (A3678)	Sigma, MO, USA
Bromophenol blue (B5525)	Sigma, MO, USA
Bicinchoninic acid solution(B9643)	Sigma, MO, USA
Bovine serum albumin (BSA) (A3059)	Sigma, MO, USA
Copper (II) Sulphate solution C2284	Sigma, MO, USA
LumiGlo Chemiluminescent substrate system (54-60-00)	KPL, Maryland, USA
Dulbecco's modified Eagle's medium (DMEM) (D5671)	Sigma, MO, USA
Dimethyl sulphoxide (DMSO) (802912-500)	Merck, South Africa
Ethanol(2233540LP)	Merck, South Africa
Fetal calf serum (FCS) (DE14-801FI)	Lonza, MA, USA
G418-sulphate (11811-064)	Gibco, CA, USA
Gentamicin (G1272)	Sigma, MO, USA
Glycerol (LC2676520)	Merck, South Africa
Glycine (2676600EM)	Merck, South Africa
Ham's F12 medium (12-615F)	Lonza, MA, USA
Heparin sodium-fresenius 1000 i.u/ml	Bodene, Port Elizabeth, South Africa
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (IM)	Lonza, MA, USA
Hydrogen per oxide H_2O_2 (3063820LP)	Merck, South Africa
Insulin Human (12643)	Sigma, MO, USA
Isopropanol (5075040LC)	Merck, South Africa
L-glutamine (17-605E)	Lonza, MA, USA
Lipoprotein lipase (LPL) from *Pseudomonas* sp. (62336)	Fluka, Buschs, Switzerland

Methanol (4164080LC)	Merck, South Africa
Phosphate buffered saline (PBS) (4417)	Sigma, MO, USA
Phosphatase inhibitors for cell culture (04906837001)	Roche, Basel, Switzerland
Protease inhibitors	Merck, Darmstadt, Germany and NIH, MD, USA
Ponceau S (p3504)	Sigma, MO, USA
Precision plus protein dual colour standard (marker) (161-0374)	Bio-Rad, CA, USA
Protease inhibitors for cell culture (04693124001)	Roche, Basel, Switzerland
Skimmed milk powder (92964)	Marvel, London, UK
Sodium dodecyl sulphate (SDS) (3017542)	BDH laboratory supplies UK
Sodium chloride (5822320EM)	Merck, South Africa
Sodium phosphate, monobasic, anhydrous (6346)	Merck, South Africa
Sodium salicylate (S2679)	Sigma, MO, USA
N, N, N', N',-tetra-methyl-ethylenediamine (TEMED) (1.10732.100)	Merck, South Africa
Tris base(1.08382.500)	Merck, South Africa
Triton x-100 (UN3082)	Sigma, MO, USA
3-Trimethylsilypropionic acid (TSP)(180335)	Sigma, MO, USA
Trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) (17-161E)	Lonza, MA, USA
Tween 20 (63158)	Honeywell Riedel-de haen USA
2-mercaptoethanol (4161200)	Merck, South Africa
p-nitrophenyl butyrate (pNPB)(N9876)	Sigma, MO, USA

2.1.4 Consumables

6 well plate	Corning, NY, USA 31.1
6-well plate (140675)	Techno Plastic Products, Switzerland
15 ml centrifuge tubes	Corning, NY, USA 31
25 cm^2 flask (430639)	Corning, NY, USA 31

50 ml centrifuge tube	BD Biosciences, USA
75 cm^2 flask (156499)	Corning, NY, USA 31
Cryotube	Corning, NY, USA 31
Disposable cuvette	Lasec, Cape Town, South Africa
Eppendorf tubes and tip	QSP, CA, USA
Filter paper (3030-861)	Whatman, Kent, UK
Nitrocellulose membrane (RPN303P) Hybond ECL	Amersham Biosciences, NJ, USA
Syringe	Pall, NY, USA
Syringe filter (0.2μm)	Pall, NY, USA

2.1.5 Instruments, software and web server

ChemiImager Alliance 2.7	UVI tech limited UK
Cary 60 spectrophotometer	Agilent Technologies, CA, USA
BIO-RAD Turbo transfer	Bio-Rad, CA, USA
H^1-NMR Bruker Advance III spectrometer	Bruker Biospin, Karlsruhe, Germany
AMIX program, version 3.9	Bruker Biospin, Rheinstetten, Germany
Graph Pad Prism	GraphPad Software, CA, USA
SPSS	IBM SPSS Data Collection
(http://www.hmdb.ca/)	

2.2 Methods

This study was designed to investigate the molecular basis of insulin resistance induced by protease inhibitors and NRTIs *in vitro,* using a cell culture system. The study of insulin signaling was performed using CHO-IR cells (Ahmed & Pillay, 2001) . Advantages such as short doubling time and easy handling make CHO-IR cell line a useful cell culture model for the study of insulin signaling and make the planning of experiments relatively straightforward. CHO-IR cells have been transfected with high levels of human insulin receptor at a level of approximately 1×10^6 receptors / cell. High protein expression in these cells allows sensitive detection of changes in signalling proteins involved in the insulin receptor signalling pathway.

The cells were serum-starved and then stimulated with insulin, both in the presence and absence of protease inhibitors; nelfinavir, indinavir, ritonavir and nucleoside reverse transcriptase inhibitors, tenofovir and stavudine. The level of insulin stimulation was compared with that of the basal unstimulated cells (the control). The cell lysates were then subjected to immunoblotting with the appropriate antibodies. The CHO-IR cells were also pre-treated with therapeutic compounds sodium salicylate and berberine chloride, prior to treatment with PIs and NRTIs. The results were analysed using Alliance 2.7 imaging system (ChemiImager).

In addition, the cell culture supernatants of CHO-IR cells treated with PIs and NRTIs were used to assay for lipoprotein lipase activity and for metabolomic analysis.

2.2.1 Preparation of culture medium and maintenance of cell lines

The CHO-IR cells were cultured in Ham's F12 mixture culture medium in 75 cm^2 flasks, and incubated at 37 °C in a 5 % CO_2 incubator. The medium was supplemented with 10 % FCS, 2 mM L-glutamine, 1 % of 100 x antibiotic / antimycotic (combination of penicillin, streptomycin and amphotericin), 0.5 %

gentamicin and 400 µg/ml G418-sulphate solution. Ham's F12 medium was used as a base medium for the CHO-IR cells as the cells require L-proline, which is supplied by the medium. FCS has a high level of insulin growth factor (IGF) to stimulate the growth of cells. L-glutamine is an essential amino acid for supporting the growth of cells, and is an alternative energy source for rapidly dividing cells and cells that use glucose inefficiently. Antibiotic / antimycotic solution is effective against the most common forms of cell culture contamination, including gram-positive and gram-negative bacteria, yeast and fungi. Gentamicin was used to prevent contamination of the mycoplasma in the cell culture medium. As mentioned earlier in this chapter, CHO-IR cells are transfected cells with insulin receptor. The transfected insulin receptor is on a plasmid that contains the G418 resistance gene. G418 is an antibiotic to ensure that only transfected cells are present in a flask while any cells that lose the plasmid will die.

Within 3-4 days, after the cells had reached semi-confluence in a $75cm^2$ flask, they were split for subculture in the following way: the culture medium was discarded and the cells were rinsed, first with 10 ml of sterile phosphate buffered saline (PBS) followed by the addition of 2ml 1x trypsin-ethylenedinitrilotetraacetic acid (Trypsin-EDTA) to coat the surface. The excess was removed immediately and the flask was incubated at 37 °C for 2 min to allow the cells to become de-attached from the flask. The trypsin reaction was terminated using 5 ml of culture medium, and 1 ml of the culture medium was then transferred for every new $75mm^2$ flask containing 20 ml of fresh culture medium.

Stocks of frozen CHO-IR cells were routinely prepared for storage in -80 ultra-freezers and then in liquid nitrogen. The freezing medium for CHO-IR cells was prepared using 20 % dimethyl sulfoxide (DMSO) in FCS solution. Following trypsinization and suspension in culture medium, the cells were mixed in a cryotube, with a volume ratio of 1:1 to freezing medium. The tubes were then placed in an isopropanol cryo-freezing container (Mr. Frosty) (Nalgene) at -80 °C. Isopropanol was added to the

container to freeze the cells gradually. Isopropanol facilitated the temperature in the container to drop at a rate of about 1°C per minute.

Cells were thawed by placing the cryotubes in 37 °C in an incubator for approximately 2 min, or until thawed. The cells/freezing medium solution was then added to 5 ml of the culture medium (i.e. Ham's F12 mixture culture medium) in a 25 cm^2 flask, and resuspended. The culture medium was changed after 12 h, grown as above, and split into a 75 cm^2 flask when the cells were in a state of semi-confluence.

2.2.2 Serum starvation procedure

1 x 10^5 cells/ml of CHO-IR cells were plated into 6-well plates until they were in a state of semi-confluence. CHO-IR cells were serum-starved for 16 h in a serum-free medium (2 mM L-glutamine, 1 % of 100 x antibiotic/antimycotic and 0.1 % gentamicin in DMEM). The cells were incubated at 37 ºC, with 10 % CO_2.

The cells were stimulated with 10 ng/ml (1.7 nM) insulin for 5 min before aspirating the serum-free medium. The serum-free medium from the CHO-IR cells were stored at -80 ºC until subsequent analysis.

2.2.3 Preparation of cell lysates

After serum-starvation for 16 h, followed by insulin stimulation for 5 min, the serum-free medium was discarded. The 6-well plates were put on ice. Subsequently, 200 µl lysis buffer (consisting of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 1 % phenoxylpolyethoxyethanol (Tritron X 100), 10 % glycerol, 50 mM sodium chloride (NaCl), protease inhibitor and phosphatase inhibitor) was added evenly to the plates, which were left on ice for 10 min. Protease and phosphatase

inhibitor tablets had been previously added to the lysis buffer (1 tablet each/10ml of lysis buffer) to protect proteins of the cells against dephosphorylation. Specifically, protease inhibitor inhibits a broad spectrum of serine, cysteine, metalloproteases and calpains, while phosphatase inhibitor inhibits a broad spectrum of phosphatases such as acid, alkaline, serine/threonine and tyrosine protein phosphatase.

The cells were scraped with a scraper and transferred into a micro-centrifuge tube. Tubes were then centrifuged at 4 °C and 10 000 rpm for 10 min. 120 μl of the supernatant were mixed with 30 μl of 5 x Laemmli buffer (25 nM Tris, pH 6.8, 2 % SDS, 0.002 % bromophenol blue, 10 % glycerol and 5 % 2-mercaptoethanol) prior to boiling at 100 ºC for 5 min, and brief spinning to settle all the evaporation from the lid of the micro-centrifuge tube. The cell lysate was stored at -20 °C prior to use, or at -80 °C for extended maintenance.

2.2.4 Measurement of protein concentration

A bicinchoninic acid (BCA) assay was used to measure the concentration of protein in the cell lysate. The principle of assay relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the protein present. It has been shown that cysteine, cystine, tryptophan, tyrosine, and the peptide bonds are able to reduce Cu^{2+} to Cu^{1+}. BCA forms a purple-blue complex with Cu^{1+} in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins at absorbance maximum 562 nm. (BSA) was used as a protein standard.

Protein detection reagent was prepared by adding 1 mL of copper sulphate pent hydrate 4% Solution to 49 mL of the bicinchoninic Acid Solution. 1 mg of bovine serum albumin (BSA) was used to generate the standard curve by diluting to 0.8mg, 0.6mg, 0.4mg and 0.2 mg. Twenty five microliters of the sample was mixed with 200 μl of detecting reagent in each well and incubated for 30 min at 37°C.

The absorbance of the mixture in the 96 wells plate was measured at 562 nm, with the reagent as a blank.

2.2.5 Stock preparation of drugs and therapeutic compounds

All drugs and compounds were made in different stock concentrations in dimethyl sulphoxide (DMSO) except nelfinavir which was prepared in ethanol as described in Table 2.1. The drugs 0.5 M sodium salicylate and 2.6 mM berberine chloride (BBR) were prepared fresh on the day of experiment in 1 M Tris, pH 7.4 and DMSO respectively(Yuan et al., 2001). CHO-IR cells were pre-treated with sodium salicylate (5 mM) and berberine chloride (27μM) at 37 °C for 1 h prior to treatment with ARVs for 16 h. Selective inhibitor of IκB kinase (IKK) IKK-16 (4mM) was prepared in H_2O. Cell lysates were prepared as described in section 2.2.3.

Table 2.1 ARVs stock concentrations and solvent

Drug	Class	Stock Concentration	Solvent	Drug concentration	References
Indinavir	PI	5mM	DMSO	100 μM	(Murata et al., 2002)
Nelfinavir	PI	5mM	100 % Ethanol	30 μM	(Ben-Romano et al., 2004)
Ritonavir	PI	5mM	DMSO	10μM	(Cammalleri & Germinario, 2003)
Atazanavir sulfate	PI	5mM	DMSO	20 μM	(Minami et al., 2011)
Lamivudine	NRTI	10mM	DMSO	10 μM	(Heald et al., 1996)
Stavudine	NRTI	10mM	DMSO	5 μM	(Dudley et al., 1992)
Zidovudine(AZT)	NRTI	10mM	DMSO	10 μM	(Minami et al., 2011)
Emtricitabine	NRTI	10mM	DMSO	20 μM	(Minami et al., 2011)
Efavirenz	NRTI	10mM	DMSO	20 μM	(Minami et al., 2011)
Tenofovir	NRTI	1mM	DMSO	5 μM	(Cihlar, Birkus, Greenwalt, & Hitchcock, 2002)

2.2.6 Statistical analysis

Statistical comparisons were made using Student's t-test or factorial-ANOVA using Microsoft Excel 2003, SPSS, GraphPad Prism and Sigma plotting software.

CHAPTER 3: ANALYSIS OF THE INSULIN SIGNALING PATHWAYS FOLLOWING EXPOSURE TO ANTIRETROVIRAL DRUGS

3.1 Introduction

The advancement in HIV treatment has increased the life span of HIV-infected individuals yet the treatment regime i.e. HAART comes with a package of side effects which affects major organs causing cardiovascular disease (Friis-Moller, Sabin, et al., 2003), chronic kidney disease (Wyatt et al., 2007) and neurocognitive impairment(Suarez et al., 2001). Although safer ARVs aiming to minimize antiretroviral toxicities have been developed, these still lead to metabolic complications resulting in insulin resistance. Insulin resistance is one of the first metabolic side-effects with high incidence observed following the implementation of HAART.

There are a number of potential mechanisms whereby antiretroviral drugs can induce insulin resistance. This can occur by direct effects on the insulin signaling cascade in the cell or indirectly by interfering with lipid metabolism or with the actions of adipocytokines (Hruz, 2011). Insulin resistance observed in HIV-associated lipodystrophy is classical example of inducing insulin resistance by affecting the lipid metabolism. There are a lot of common effects exhibited by the different antiretroviral drugs in their tendency to induce insulin resistance and *in vivo* this makes it more difficult to attribute the insulin resistance to a particular antiretroviral. HIV-infected patients are generally more susceptible to the consequences of antiretroviral-induced insulin resistance and diabetes mellitus. Optimum treatment plans for treating the insulin resistance during HIV therapy have not been established as yet (Feeney & Mallon, 2011).

Initially it was believed that only PIs are responsible for most of the metabolic complications described in the previous chapter. In 1999, Brinkman reported for the first time that certain NRTIs (Thymidine analogues which basically inhibit the replication of HIV reverse transcriptase) have possible

involvement in the inhibition of DNA polymerase-g which plays an important role in the mitochondrial replication (Brinkman, Smeitink, Romijn, & Reiss, 1999; Lewis, Day, & Copeland, 2003). Up to 15-20 % patients treated with NRTIs develop several types of mitochondrial toxicities leading to lipodystrophy in adipose tissue, insulin resistance in skeletal muscles and lactic acidosis in liver and neuropathy in nerves. This tends to depend on the specificity of NRTIs and the nature of the tissue (Gerschenson & Brinkman, 2004; Lewis et al., 2003). Two recent studies further clarified the involvement of different NRTIs (stavudine, zidovudine, lamivudine) in insulin resistance and mitochondrial dysfunction (Fleischman et al., 2007; van Vonderen et al., 2010).
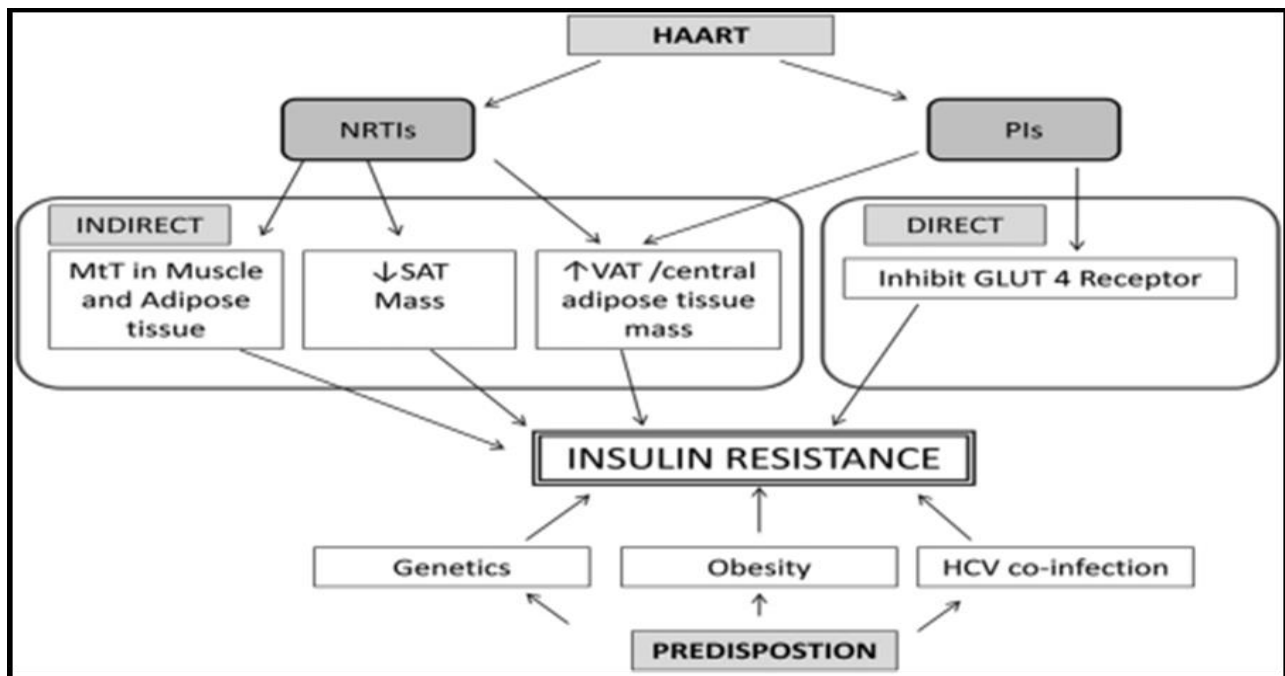
Fig.3.1 Potential causes of insulin resistance in HAART-treated HIV-infected patients. HAART = highly active antiretroviral therapy. NRTIs = nucleoside reverse transcriptase inhibitors. PIs = protease inhibitors. HCV = hepatitis C virus. MtT = mitochondrial toxicity.SAT = subcutaneous adipose tissue. VAT = visceral adipose tissue(Feeney ER, 2011).

In this study we sought to evaluate the role of protease inhibitors and NRTIs in insulin resistance by examining the effects on insulin signaling pathways. We used the following PIs (nelfinavir indinavir, ritonavir) and NRTIs (tenofovir and stavudine) in the study. CHO-IR cells were treated with various drug concentrations for 16 h, followed by insulin stimulation (10 ng/ml) (1.7 nM) for 5 minutes.

3.1.1 Sodium salicylate, berberine chloride and IKK-16 Treatment

Berberine chloride is contained in a Chinese traditional medicinal compound used for the treatment of type 2 diabetes mellitus, lowering cholesterol and treating diarrhoea (Kong et al., 2004; Lau, Yao, Chen, Ko, & Huang, 2001; C. E. Taylor & Greenough, 1989).

Studies to evaluate the mechanistic basis of berberine to decrease glucose showed that berberine stimulates glucose transport through a mechanism different from insulin in 3T3-L1 adipocytes (Zhou et al., 2007). Berberine activated AMP- activated protein kinase (AMPK) and induced glycolysis in L6, C2C12, and 3T3-L1 cell lines (Yin, Gao, Liu, Liu, & Ye, 2008)]. Two separate studies indicated that berberine also activated extracellular signal-regulated kinase (ERK) (Zhou et al., 2007) and c-jun N-terminal kinase, (JNK) (S. Lee et al., 2007) in HepG2 cells. On the basis of these studies, could hypothesized that berberine acts on several different pathways to moderate insulin sensitivity.

Another potential hypoglycemic compound is Sodium salicylate. The molecular mechanism by which sodium salicylate reduces blood glucose levels is through IKKβ in the NFκB pathway (Yuan et al., 2001). The hypoglyacemic properties of salicylates have been known for many decades. The compound has been reported to reduce glucose levels in fasting state of diabetic patients (Yuan et al., 2001). After it was shown that IKKβ was involved in insulin resistance and that salicylates were found to be strong hypoglycaemic candidates (Fleischman, Shoelson, Bernier, & Goldfine, 2008; Koska et al., 2009), even though they had been superseded by other oral hypoglycaemic compounds such as metformin (Yuan et al., 2001). With this mind, it would be appropriate to test the role of the NFκB pathway using salicylates or alternative IKKB inhibitors. In this study, we used IKK-16 (selective inhibitor of the IκB kinase (IKK)) to evaluate the involvement of the NFκB pathway in the effects of the antiretroviral drugs on the insulin signalling pathway.

As discussed previously, the precise mechanism by which protease inhibitors induce insulin resistance is not clear (Ismail, 2009) . Therefore, the main objective of this study was to investigate possible

mechanisms by which protease inhibitors induce insulin resistance in CHO-IR cells by the analysis of signalling proteins in the insulin signalling pathway.

3.2 Methods

3.2.1 Preparation of acrylamide gels for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-polyacrylamide gels (dimensions 8 cm x 7.3 cm x 1.5mm) were prepared in batches of 12 in a multi gel casting chamber (Bio-Rad) according to the standard protocol. The stacking gel (upper) was a 4 % (0.126 M Tris, pH 6.8, 4 % acrylamide/bis , 0.1 % SDS, 0.05 % ammonium persulphate (APS) and 0.1 % N, N, N', N',-tetramethylethylenediamine (TEMED)) and the resolving gel (lower) was a 7.5 % (0.375 M Tris, pH 8.8, 7.5 % acrylamide/bis , 0.1 % SDS, 0.05 % APS and 0.05 % TEMED) each (Figure 3.2). The stacking gel also contained the 10-wells for sample loading.

3.2.2 Electrophoresis

The protein samples (25 μl) and a molecular weight marker (Precision Plus Protein Dual Colour Standard, Bio-Rad) (5 μl) were loaded into the wells of the gel. The four gels were assembled with the electrode assembly and then placed in the tetra cell mini buffer dam (Bio-Rad) filled with running buffer (0.025 M Tris, 0.192 M glycine and 0.1 % SDS). Electrophoresis was performed at 150 V and 0.06 A for 1 h.
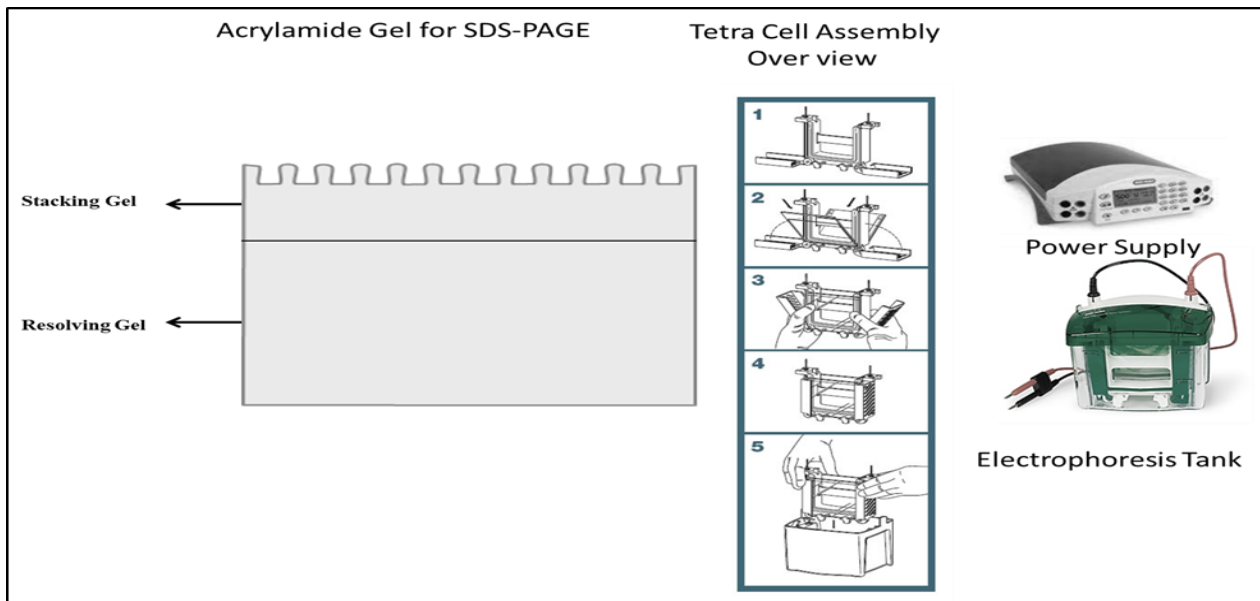
Figure 3.2: SDS-polyacrylamide gel electrophoresis procedure.

3.2.3 Protein transfer

Nitrocellulose membrane and thick filter paper were soaked for 15 min in transfer buffer (0.025 M Tris, 0.192 M glycine and 20 % methanol). Following electrophoresis, the acrylamide gels were placed for 10 min in transfer buffer to equilibrate. The protein transfer sandwich was assembled with the nitrocellulose membrane and gel in the middle and thick filter papers on either side as shown in Figure 3.3, and placed in tray of the Bio-Rad turbo Transfer. The transfer procedure was performed at 0.25 A for 45 min.
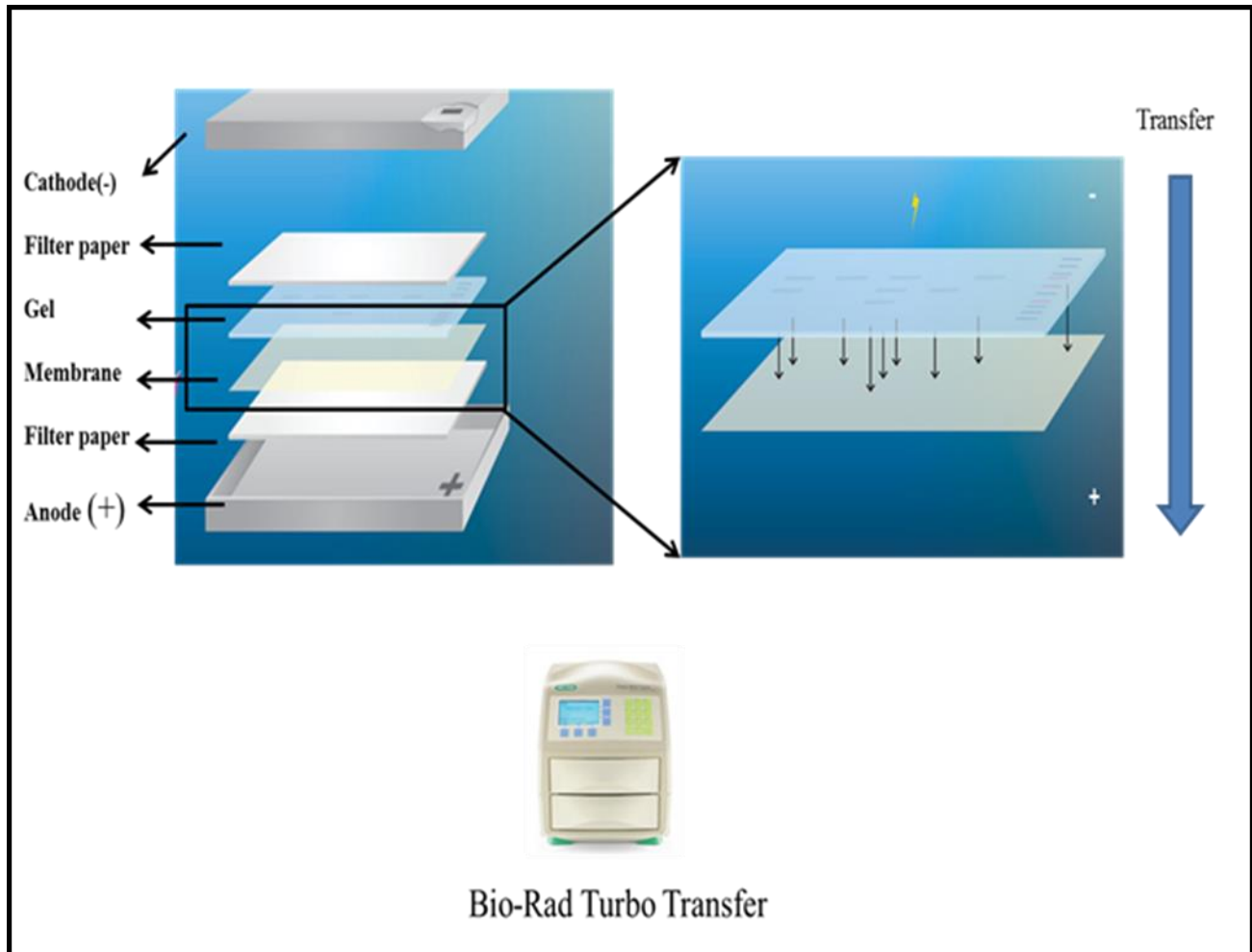
Figure 3.3: Transfer sandwich.

3.2.4 Immunoblotting

The membrane was stained (by rinsing) with ponceau (0.1 % Ponceau S in 5 % acetic acid) to verify the successful and uniform transfer of proteins to the membrane. The membrane was de-stained with deionized water and incubated in a blocking solution (5 % BSA or 5 % skimmed milk solution in Tris-buffered saline (0.05 M Tris, 0.150 M NaCl, pH 7.4)) + 0.1% Tween 20 (TBST) for 1 h at room temperature (18-25 °C) depending upon the nature of the primary antibodies. The membrane was then

incubated with a specific primary antibody   (Table 3.1) for 1 h, and washed with 20 ml TBST for 15 min, 4 times.

The membrane was then incubated with a horseradish peroxidase (HRP)-labelled secondary antibody (Table 3.1) to detect the primary antibody bound to the protein of interest. It was washed three times 10min each with TBST and then once rinsed with deionized water (20 ml). The membrane was then soaked in chemiluminescent reagent (LumiGlo) (600μl) for 1 min, and excess reagent was drained using tissue paper. The membrane was covered with transparent plastic and viewed in the ChemiImager.

Table 3.1: Primary and secondary antibodies with dilution and blocking conditions.

Primary antibody	Concentration (antibody : blocking solution)	Blocking solution	Secondary antibody	Concentration (antibody : blocking solution)	Blocking solution
Mouse anti-PY20(1 mg/ml)	1 in 2000	3 % BSA	Goat anti-mouse (1 mg/ml)	1 in 5000	3 % BSA
IR β-subunit rabbit polyclonal IgG (200 μg/ml)	1 in 1000	5 % NFDM	Goat anti-rabbit (400 μg/ml)	1 in 1000	5 % NFDM
Actin goat polyclonal IgG (200 μg/ml)	1 in 1000	5 % NFDM	Mouse anti- goat (1 mg/ml)	1 in 1000	5 % NFDM
P-p44/42 MAPK (Erk1/2)	1 in 1000	5 % BSA	Goat anti-mouse (1 mg/ml)	1 in 5000	5 % BSA
P-Akt rabbit(Ser 473)	1 in 1000	5 % BSA	Goat anti-rabbit (400 μg/ml)	1 in 1000	5 % BSA
P-GSK-3alpha/beta	1 in 1000	5% BSA	Goat anti-rabbit (400 μg/ml)	1 in 1000	5% BSA
Akt anti-rabbit	1 in 1000	5% NFDM	Goat anti-rabbit (400 μg/ml)	1 in 1000	5% NFDM
p44/42 MAPK (Erk1/2)	1 in 1000	5 % NFDM	Goat anti-rabbit (400 μg/ml)	1 in 1000	5 % NFDM
PY20 = phosphotyrosine, IR = insulin receptor, MAPK=Mitogen-activated protein (MAP) kinases, PKB(Akt)= protein kinase B, GSK-3=Glycogen synthase kinase-3					
* Incubation time is 1 h for primary and secondary antibody at room temperature					

3.2.5 Re-probing of membrane

The membrane was incubated with hydrogen peroxide solution for 30 min at 37 ℃, and 5 min at room temperature for stripping to block the HRP signals (Sennepin et al., 2009). In the next step membrane was put in blocking solution once more and then re-probed with a different primary antibody (Table 3.1) as indicated in Section 3.2.4.

3.2.6 Quantification of chemiluminescence

The membrane was analysed using a real-time imager, ChemiImager (Fluorchem 5500 Programme, Alpha Innotech Corporation, CA, USA) to quantify the chemiluminescent intensity.

3.3 Results

3.3.1 Effects of the HIV protease inhibitors and nucleoside reverse transcriptase inhibitors on insulin-stimulated phosphorylation.

In the first stage of the study, cells were exposed to PIs (indinavir ,nelfinavir and ritonavir) and an NRTI (tenofovir), prior to insulin stimulation to examine the effects on the insulin receptor - β (IR- β) subunit tyrosine phosphorylation, IRS-1 tyrosine phosphorylation and downstream activation of proteins such as protein kinase B (Akt or PKB), MAPK and GSK-3 in CHO-IR cells. The cells were pre-treated with PIs and NRTIs for 16 h followed by insulin stimulation (10 ng/ml) (1.7 nM) for 5 min.

3.3.2 Effects of protease inhibitors

3.3.2.1 Effects of indinavir on insulin receptor β, and insulin receptor substrate-1 phosphorylation and activation of Akt, Map Kinase and GSK-3   (Figure 3.4.)

Indinavir was used in two concentrations (25 and 50 µM).Indinavir at 50 µM significantly inhibited the tyrosine phosphorylation at IR-β (p=.0048) and IRS-1(p=.0037) (Figure 3.4(a)) while phosphorylation of MAPK was inhibited by both concentrations (p=.0021) (Figure 3.4(b). The phosphorylation of Akt was mildly (not significantly) reduced while GSK3 was not affected by either concentration of indinavir (Figure 3.4(c) and (d)). The phosphorylation of Akt was significantly reduced in the later experiments when used new indinavir as shown in figure 3.8(c) and 3.10 (c)

Figure 3.4: Effects of indinavir on insulin-stimulated phosphorylation in CHO-IR cells. CHO-IR cells were treated with different concentrations of indinavir for 16 h followed by (10ng/ml) insulin stimulation. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: **(a)** phosphotyrosine PY20 antibody **(b)** Phospho-p44/42 MAPK (Erk1/2**) (c)** phospho-Akt **(d)** Phsopho-GSK-3α/β. The blots were then stripped and reprobed with anti-IR β-subunit (a) p44/42 MAPK (Erk1/2)(b) total Akt (c) and actin (d) respectively (lower panel). Bound antibodies were visualised by chemiluminescence. Chemiluminescence was quantified by CemiImaging system. The band intensity of the proteins from one experiment was normalised to the band intensity of total protein. Data are shown as mean ± S.E.M. (n=2). pIR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B, P-GSK3= phospho-Glycogen synthase kinase-3 alpha/beta.*p < 0.05, using one way ANOVA."*" statistically significant change in phosphorylation after insulin stimulation as copmared to basel level."#" statistically significant change in the phosphorylation after drug treatment as compared to the insulin treatment alone.

3.3.2.2 Effects of nelfinavir on insulin receptor β, and insulin receptor substrate-1

phosphorylation and activation of Akt, MAPK and GSK-3 (Figure 3.5)

For comparison with indinavir , nelfinavir was used in two concentration (30μM and 60μM).Both low and high concentrations of nelfinavir significantly inhibited the insulin-stimulated tyrosine phosphorylation of the insulin receptor β-subunit (IR- β) (p=.0027), IRS-1(p=.0019), MAPK (p=.001)and Akt (p=.00129) by greater than 50%, (Figure 3.5 (a), (b) and (c) respectively). Nelfinavir did not cause change in the serine phosphorylation of GSK-3 (figure 3.4(d)) detected by the phospho specific antibody which detects phosphorylation of GSK-3alpha/beta (ser21/9). The expression levels of total insulin receptor β-subunit, total Akt, total MAPK and actin were unchanged (Figure 3.5 (a) , (b) (c) and (d)) as demonstrated by blotting for the total protein.
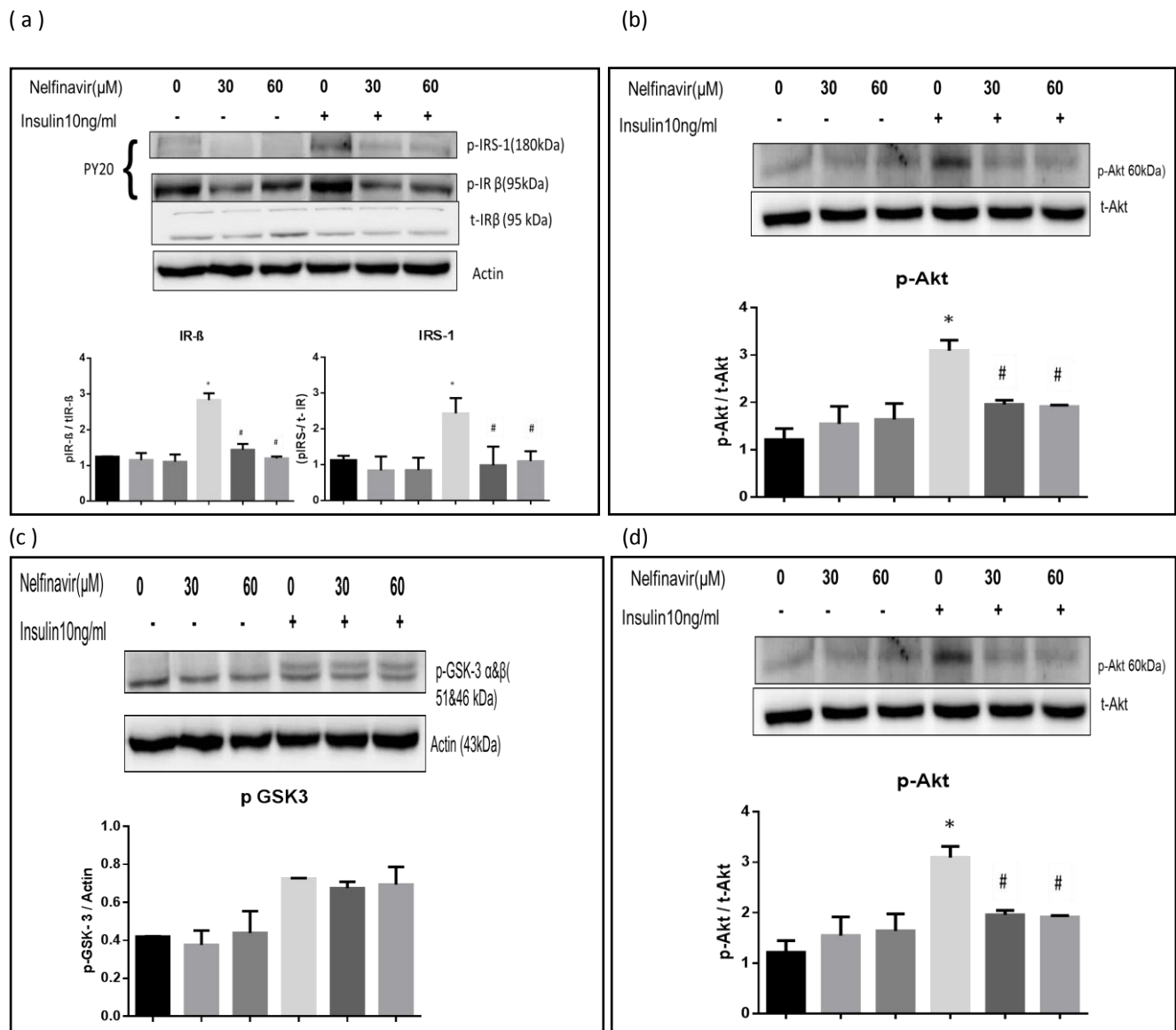
Figure 3.5: Effects of nelfinavir on insulin-stimulated phosphorylation in CHO-IR cells. CHO-IR cells were treated with different concentrations of nelfinavir for 16 h followed by (10ng/ml) insulin stimulation. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: **(a)** phosphotyrosine PY20 antibody **(b)** Phospho-p44/42 MAPK (Erk1/2) **(c)** phosphor-Akt **(d)** Phospho-GSK-3α/β. The blots were then stripped and reprobed with anti-IR β-subunit (a) p44/42 MAPK (Erk1/2)(b) total Akt (c) and actin (d) respectively (lower panel). Bound antibodies were visualised by chemiluminescence. Chemiluminescence was quantified by ChemiImaging system. The band intensity of the proteins from one experiment was normalised to the band intensity of total protein.Data are shown as mean ± S.E.M. (n=2). pIR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B, P-GSK3= phospho-Glycogen synthase kinase-3 alpha/beta.*p < 0.05, using one way ANOVA."*" statistically significant change in phosphorylation after insulin stimulation as copmared to basel level."#" statistically significant change in the phosphorylation after drug treatment as compared to the insulin treatment alone.

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3.3.2.3 Effects of ritonavir (Figure. 3.6)

Ritonavir was the third protease inhibitor investigated at two concentrations. The effects of ritonavir on the insulin signaling pathway were less profound. In particular, there was minimal change in insulin receptor and IRS phosphorylation (Figure 3.6 (a)). However, ritonavir affected the phosphorylation of MAPK at both concentrations. Insulin-stimulated Akt and GSK-3 phosphorylation were unaffected by either concentration of the ritonavir (Figure 3.6 (b) and (c)).
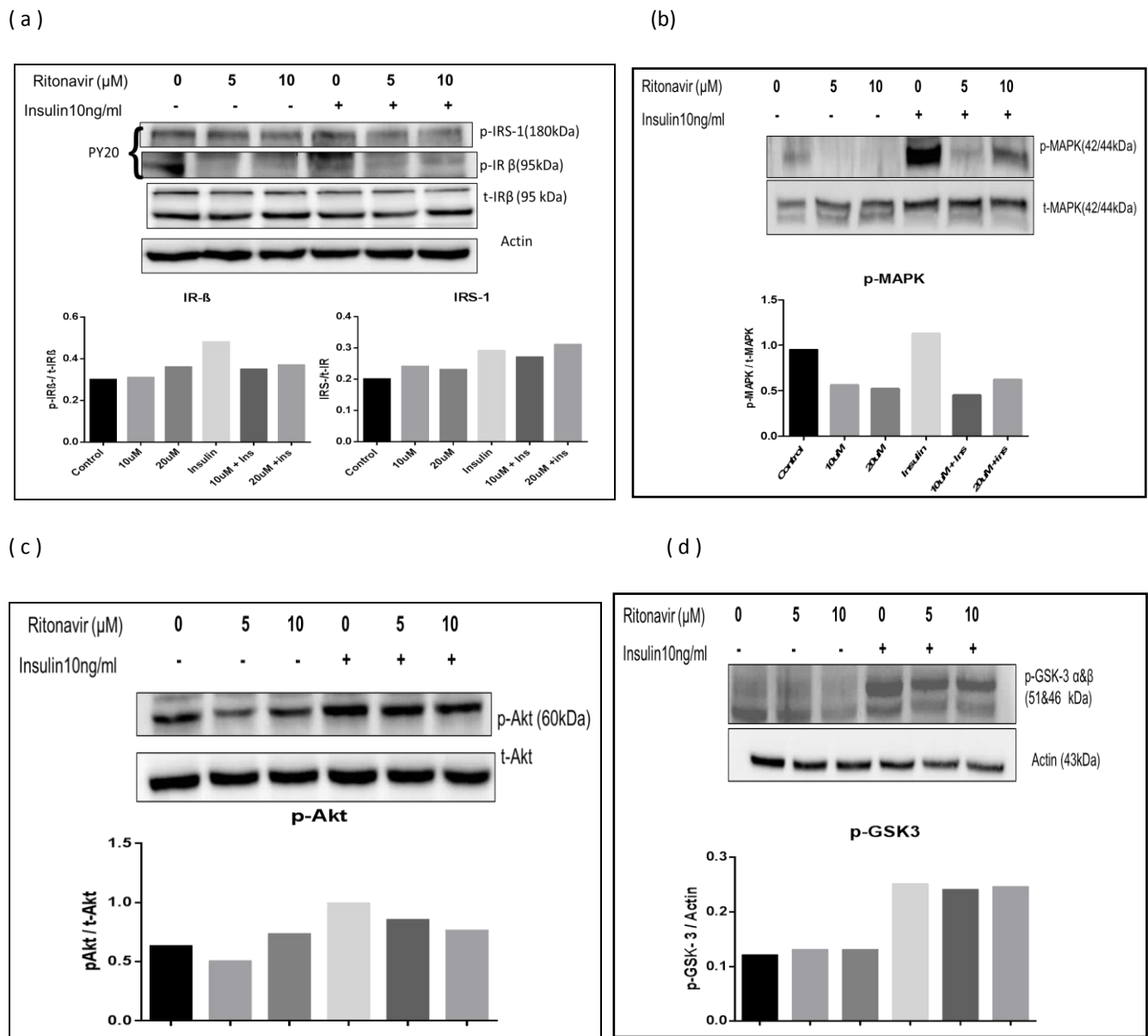
Figure 3.6: Effects of ritonavir on insulin-stimulated phosphorylation in CHO-IR cells. CHO-IR cells were treated with different concentrations of ritonavir for 16 h followed by (10ng/ml) insulin stimulation. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: **(a)** phosphotyrosine PY20 antibody **(b)** Phospho-p44/42 MAPK (Erk1**/**2) **(c)** phosphor-Akt **(d)** Phsopho-GSK-3α/β. The blots were then stripped and reprobed with anti-IR β-subunit (a) p44/42 MAPK (Erk1/2)(b) total Akt (c) and actin (d) respectively (lower panel). Bound antibodies were visualised by chemiluminescence. Chemiluminescence was quantified by CemiImaging system. The band intensity of the proteins from one experiment was normalised to the band intensity of total protein. pIR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B, P-GSK3= phospho-Glycogen synthase kinase-3 alpha/beta.

3.3.3 Effects of NRTIs on insulin signalling pathways

3.3.3.1 Effects of the tenofovir on insulin receptor signaling pathway

Tenofovir did not affect on the proximal part of the insulin signaling pathway e.g. insulin receptor-β sub unit and IRS-1(Figure 3.6(a)) .Similarly no significant decrease in phosphorylation of Akt and GSK-3 with tenofovir (Figure 3.7(c) and (d)) was observed. However, like protease inhibitors, tenofovir also significantly decreased the phosphorylation of MAPK (Figure 3.7(b)).
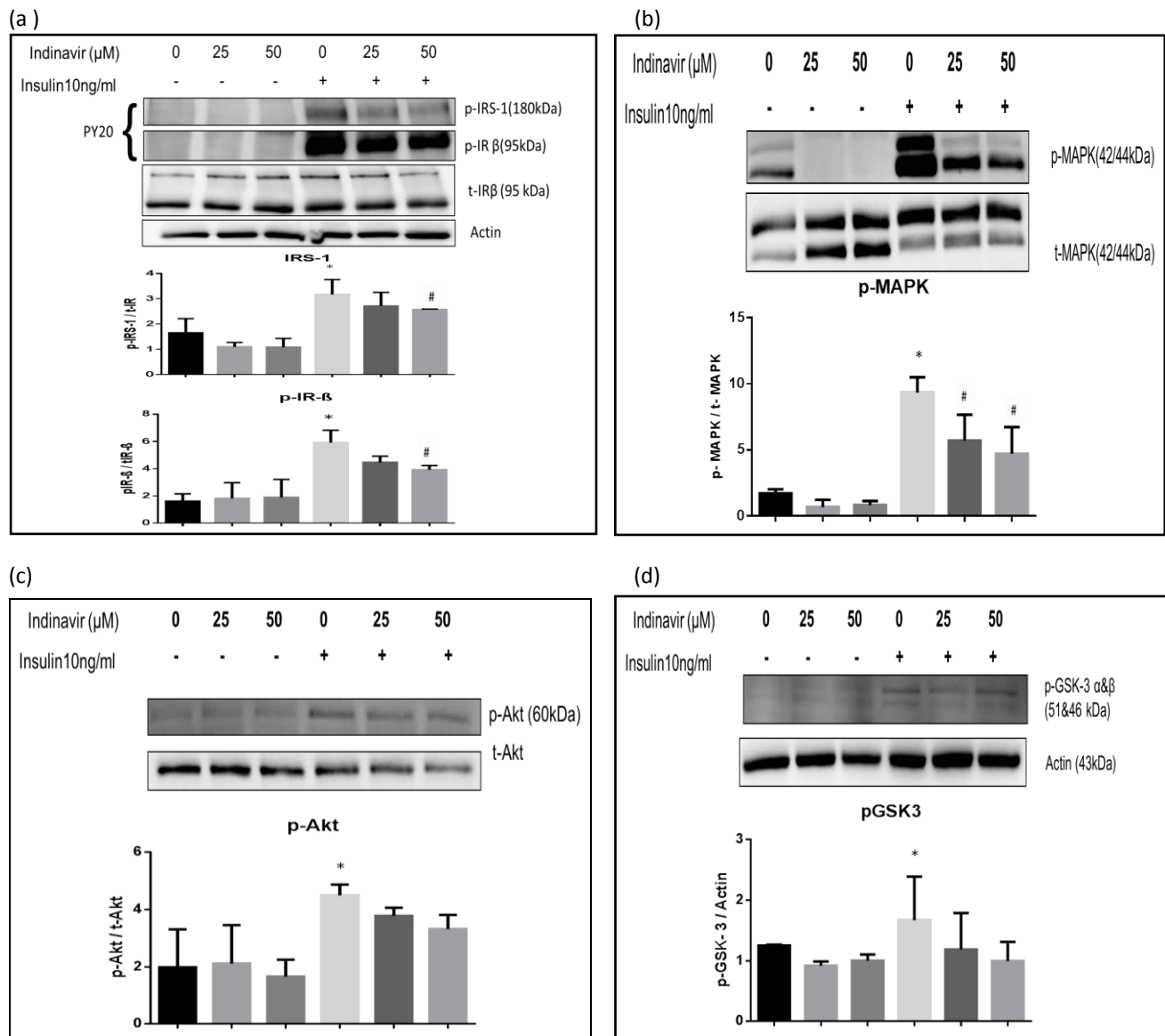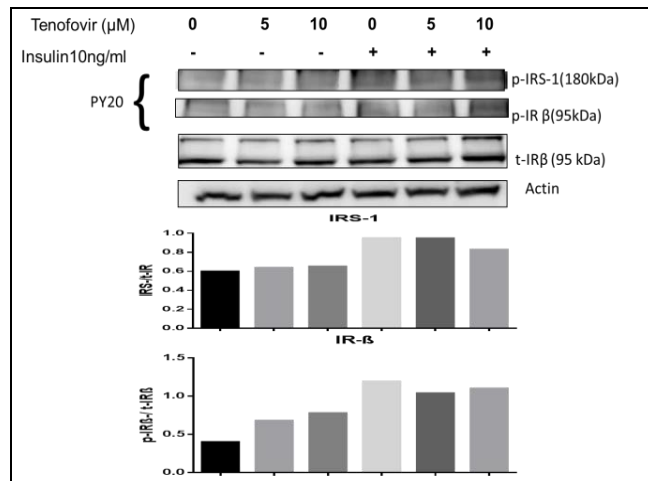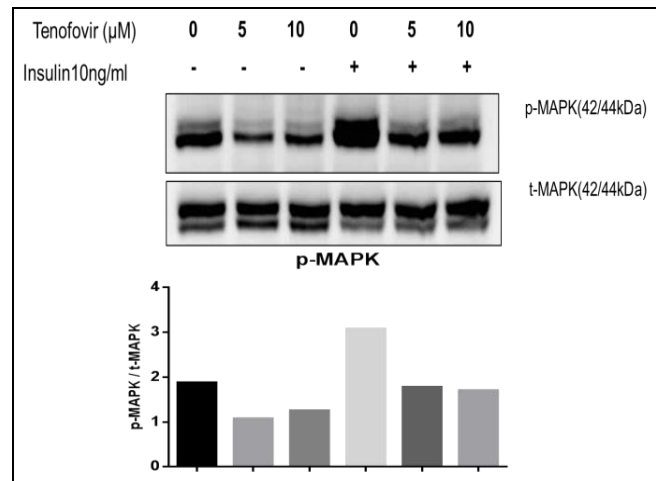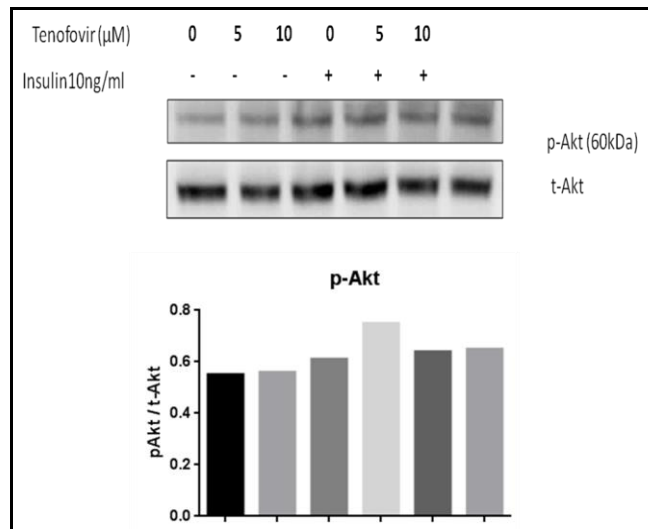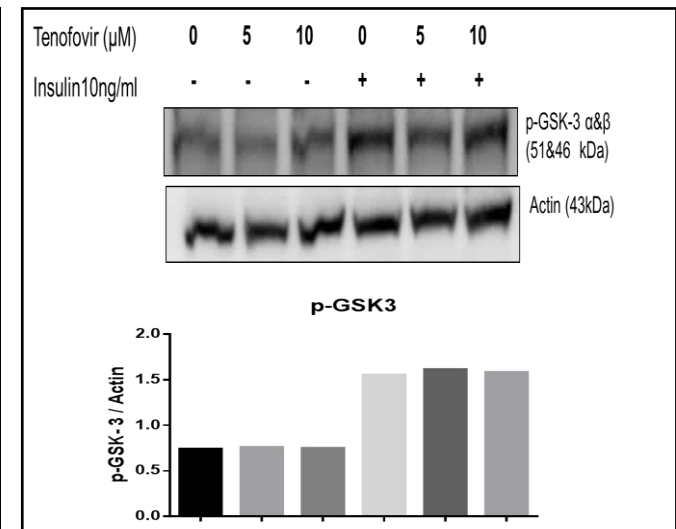
Figure 3.7: Effects of Tenofovir on insulin-stimulated signaling pathways in CHO-IR cells. CHO-IR cells were treated with different concentrations of tenofovir for 16 h followed by (10ng/ml) insulin stimulation. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: **(a)** phosphotyrosine PY20 antibody **(b)** Phospho-p44/42 MAPK (Erk1/2) **(c)** phosphor-Akt **(d)** Phsopho-GSK-3α/β. The blots were then stripped and reprobed with anti-IR β-subunit (a) p44/42 MAPK (Erk1/2)(b) total Akt (c) and actin (d) respectively (lower panel). Bound antibodies were visualised by chemiluminescence. Chemiluminescence was quantified by the ChemiImaging system. The band intensity of the proteins from one experiment was normalised to the band intensity of total protein. p-IR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B, P-GSK3= phospho-Glycogen synthase kinase-3 alpha/beta.

3.3.4 Effect of protease inhibitors after pre-treatment of CHO-IR cells with sodium salicylate, berberine chloride and IKK-16

In order to evaluate the potential role of the NFκB pathway in the effects of protease inhibitors and NRTIs (tenofovir) cells were pre-treated with IKK-16 or sodium salicylate (NaSal). In addition berberine chloride (BBR) also tested as a potential therapeutic compound to determine if it would alter the insulin-desensitizing effects of ARVs.

3.3.4.1 Effects on indinavir

CHO-IR cells were treated with NaSal (5mM), BBR (2.7mM) and IKK-16 (2μM) for 1 h before the addition of indinavir.

In the presence of indinavir phosphorylation of insulin receptor β-subunit (IR-β $p=.0087$) substrate (IRS-1 $p= .0002$) and downstream proteins (Akt $p=.008$ and MAPK $p=.0001$) except GSK-3 were significantly reduced (Figure 3.8 (a), (b), (C) and (d)). In BBR pre-treated CHO-IR cells, a slightly increased (but not statistically significant) phosphorylation at IR-β and Akt was observed (Figure 3.8 (a) and (c)). BBR did not alter the effect of indinavir to inhibit MAPK (Figure 3.8(b)). GSK-3 phosphorylation remained unaltered after indinavir or berberine treatment (Figure 3.8(d)).

Figure 3.8: Effects of BBR on the insulin signaling pathways in CHO-IR cells (Indinavir). CHO-IR cells were pre-treated with berberine chloride (2.7 mM) for 1 h prior to treatment with indinavir (50 μM) for 16 h. The cells were stimulated with insulin for 5min. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: (a) IR-βand IRS-1with anti-phosphotyrosine PY20 antibody (b) Phospho-p44/42 MAPK (Erk1/2) (c) phospho-Akt and (d) Phospho-GSK-3α/β (upper panels). The blots were reprobed with anti t-IR-β (a) ,p44/42 MAPK (Erk1/2)(b) total Akt (c) and actin (d) respectively (lower panel) . Bound antibodies were visualised by chemiluminescence and quantified by ChemiImager. The band intensity of the proteins from 2 independent experiments was normalised to the band intensity of total IR. Data are shown as the mean ± S.E.M. (n=2). p-IR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B, P-GSK3= phospho-Glycogen synthase kinase-3 alpha/beta. $*p < 0.05$, using one way ANOVA."*" statistically significant change in phosphorylation after insulin stimulation as copmared to basel level."#" statistically significant change in the phosphorylation after drug treatment as compared to the insulin alone.

In NaSal-treated CHO-IR cells phosphorylation of IR-β in the presence of indinavir was slightly increased (Figure 3.9 (a)) but NaSal also did not reverse the effects of indinavir on IRS-1, MAPK and Akt (Figure 3.9 (a) (b) and (c)).

(a)

(b)

(C)

(d)
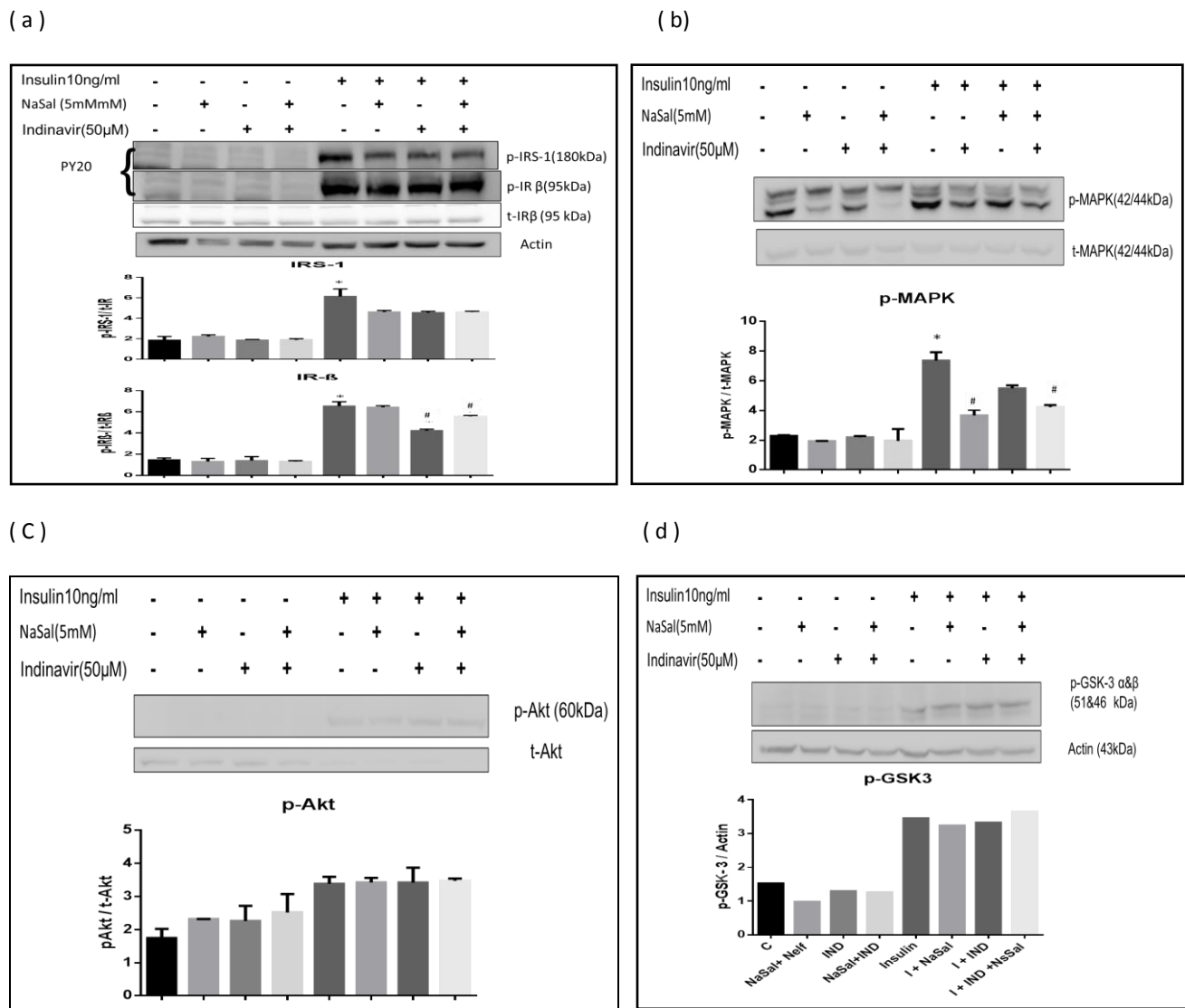
Figure 3.9: Effects of sodium salicylate on CHO-IR cells treated with indinavir. CHO-IR cells were pre-treated with sodium salicylate (5 mM) for 1 h prior to treatment with indinavir (50 μM) for 16 h. The cells were stimulated with insulin for 5min. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: **(a)** IR-βand IRS-1with anti-phosphotyrosine PY20 antibody **(b)** Phospho-p44/42 MAPK (Erk1/2) **(c)** phospho-Akt and **(d)** Phospho-GSK-3α/β (upper panels). The blots were reprobed with anti t-IR-β (a) ,p44/42 MAPK (Erk1/2)(b) total Akt (c) and actin (d) respectively (lower panel) . Bound antibodies were visualised by chemiluminescence and quantified by ChemiImager. The band intensity of the proteins from 2 independent experiments was normalised to the band intensity of total IR. Data are shown as the mean ± S.E.M. (n=2). p-IR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B, P-GSK3= phospho-Glycogen synthase kinase-3 alpha/beta. *p< 0.05, using one way ANOVA. "*"statistically significant change in phosphorylation after insulin stimulation as copmared to basel level."#" statistically significant change in the phosphorylation after drug treatment as compared to the insulin alone.

3.3.4.2 Effects of IKK-16 on cells pretreated with indinavir

In cells pre-treated with IKK-16 there was not no significant change in the phosphorylation of   insulin receptor β, IRS-1, Akt and MAPK (Figure 3.10(a),(b) and (c))or GSK-3 phosphorylation (Figure 3.10(d)).

(a)

(b)

Insulin10ng/ml	-	-	-	-	+	+	+	+
IKK-16(2uM)	-	-	+	+	-	-	+	+
Indinavir(50µM)	-	+	-	+	-	+	-	+

PY20 {

p-IRS-1(180kDa)
p-IR β(95kDa)
t-IRβ (95 kDa)
Actin

IRS-1

p-IRS/t-IR

IR-ß

p-IRG/t-IRB

(b)

Insulin10ng/ml	-	-	-	-	+	+	+	+
IKK-16(2uM)	-	-	+	+	-	-	+	+
Indinavir(50µM)	-	+	-	+	-	+	-	+

p-MAPK(42/44kDa)
t-MAPK(42/44kDa)

p-MAPK

p-MAPK / t-MAPK

(c)

Insulin10ng/ml	-	-	-	-	+	+	+	+
IKK-16(2uM)	-	-	+	+	-	-	+	+
Indinavir(50µM)	-	+	-	+	-	+	-	+

p-Akt (60kDa)
t-Akt

p-Akt

pAkt / t-Akt

(d)

Insulin10ng/ml	-	-	-	-	+	+	+	+
IKK-16(2uM)	-	-	+	+	-	-	+	+
Indinavir(50µM)	-	+	-	+	-	+	-	+

p-GSK-3 α&β
(51&46 kDa)
Actin (43kDa)

p-GSK3

p-GSK-3 / Actin

Figure 3.10: Effects of IKK-16 on the insulin signaling pathway of CHO-IR cells pre-treated with indinavir. CHO-IR cells were pre-treated with IKK-16 (2 µM) for 1 h prior to treatment with indinavir (50 µM) for 16 h. The cells were stimulated with insulin for 5min. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: **(a)** IR-βand IRS-1with anti-phosphotyrosine PY20 antibody **(b)** Phospho-p44/42 MAPK (Erk1/2) **(c)** phospho-Akt and **(d)** Phospho-GSK-3α/β (upper panels). The blots were reprobed with anti t-IR-β (a) ,p44/42 MAPK (Erk1/2)(b) total Akt (c) and actin (d) respectively (lower panel) . Bound antibodies were visualized by chemiluminescence and quantified by ChemiImager. The band intensity of the proteins from 2 independent experiments was normalised to the band intensity of total IR. Data are shown as the mean ± S.E.M. (n=2). p-IR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B, P-GSK3= phospho-Glycogen synthase kinase-3 alpha/beta. *p< 0.05, using one way ANOVA.“*” statistically significant change in phosphorylation after insulin stimulation as copmared to basel level.“#” statistically significant change in the phosphorylation after drug treatment as compared to the insulin alone.

3.3.4.3 Effects of sodium salicylate, BBR and IKK-16 on nelfinavir

Following the same lines as the experiments with indinavir, the effects of NaSal (5mM), BBR (2.7mM) and IKK-16 (5µM) were evaluated on nelfinavir. The CHO-IR cells were treated similarly for one hour before then treating the cells with nelfinavir. Nelfinavir significantly blocked the phosphorylation of IRS-1(p=.0053), Akt (p=.009) and MAPK (p=.0021) (Figure 3.11(a) (c) and (d)) even in the BBR treated cells. Nelfinavir also reduced (but not significantly) the phosphorylation at GSK-3 as shown in Figure 3.11(d).
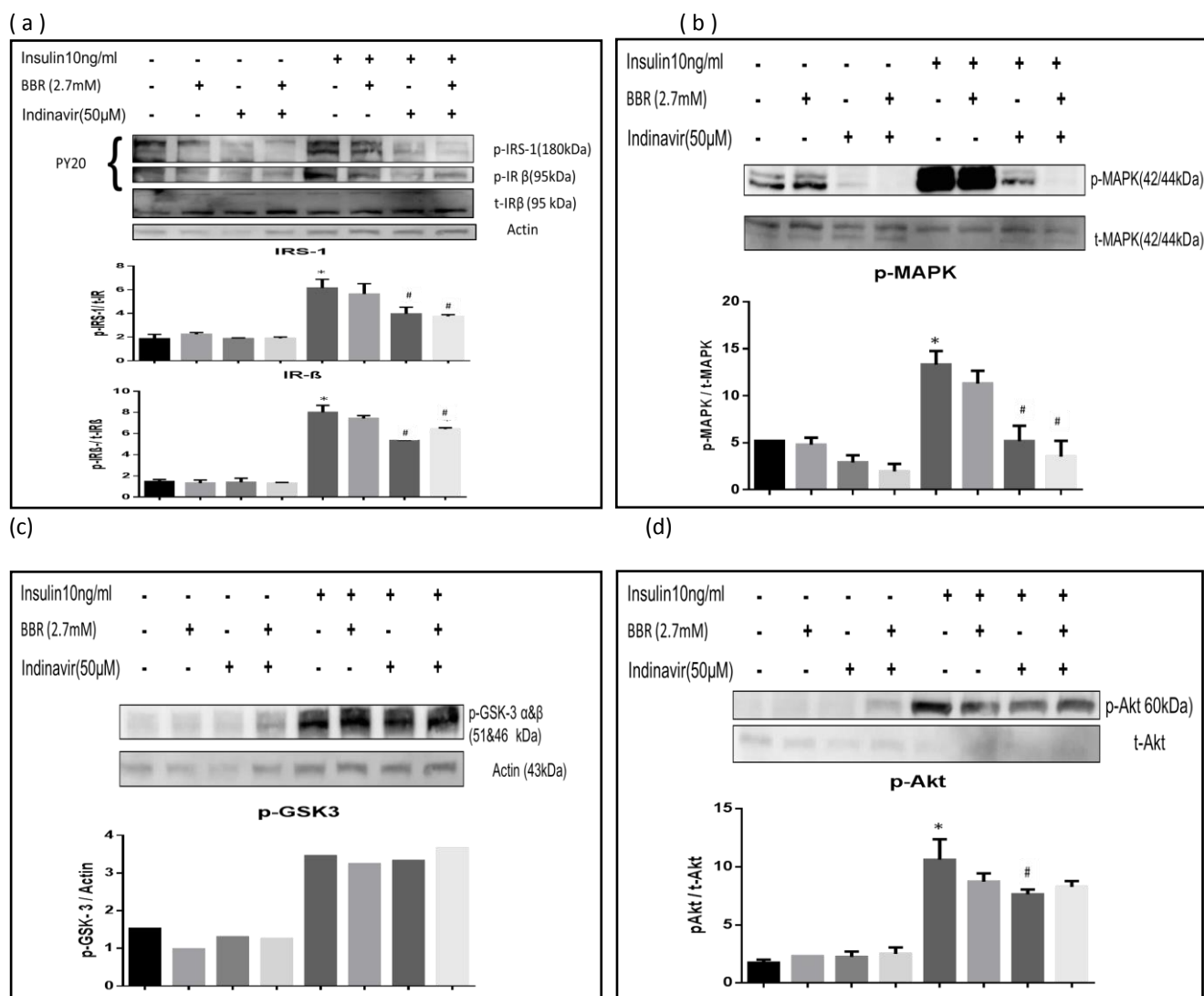
Figure 3.11: Effects of BBR on the insulin signaling pathway of CHO-IR cells treated with nelfinavir. CHO-IR cells were pre-treated with berberine chloride (2.7 mM) for 1 h prior to treatment with nelfinavir (30 µM) for 16 h. The cells were stimulated with insulin for 5min. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: (a) IR-βand IRS-1with anti-phosphotyrosine PY20 antibody (b) Phospho-p44/42 MAPK (Erk1/2) (c) phospho-Akt and (d) Phsopho-GSK-3α/β (upper panels). The blots were reprobed with anti t-IR-β (a) ,p44/42 MAPK (Erk1/2)(b) total Akt (c) and actin (d) respectively (lower panel) . Bound antibodies were visualized by chemiluminescence and quantified by ChemiImager. The band intensity of the proteins from 2 independent experiments was normalized to the band intensity of total IR. Data are shown as the mean ± S.E.M. (n=2). p-IR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B, P-GSK3= phospho-Glycogen synthase kinase-3 alpha/beta. *p< 0.05, using one way ANOVA.“*” statistically significant change in phosphorylation after insulin stimulation as copmared to basel level.“#” statistically significant change in the phosphorylation after drug treatment as compared to the insulin alone.

Sodium salicylate slightly increased phosphorylation of the IR-β and IRS-1 in the presence of nelfinavir (Figure 3.12 (a)) but did not affect the phosphorylation of MAPK and Akt (Figure 3.12 (c) and (d) respectively).

(a)

(b)

(c)

(d)
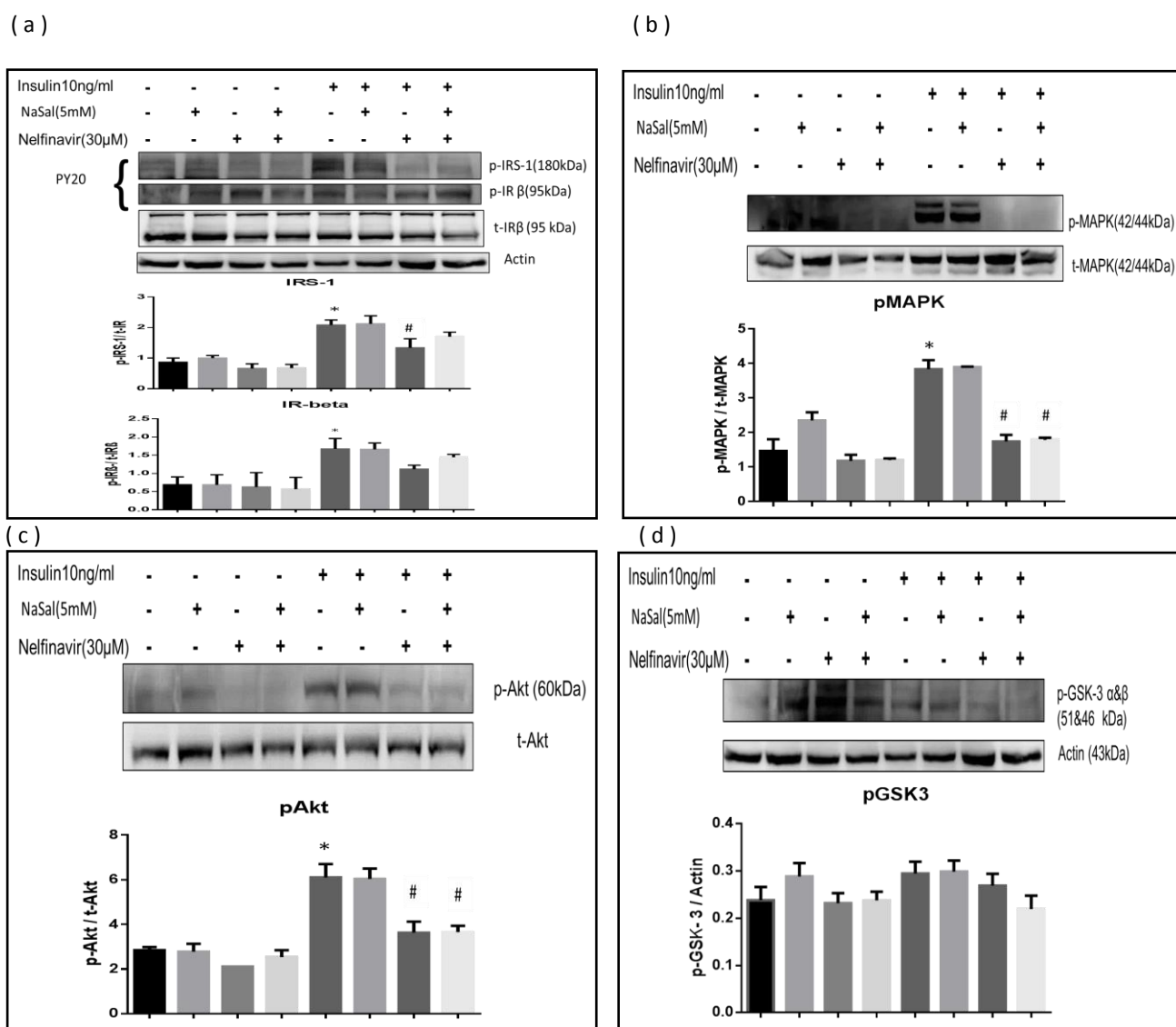
Figure 3.12: Effects of sodium salicylate on the insulin signalling pathway of CHO-IR cells treated with nelfinavir. CHO-IR cells were pre-treated with sodium salicylate (5 mM) for 1 h prior to treatment with nelfinavir (30 μM) for 16 h. The cells were stimulated with insulin for 5min. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: **(a)** IR-βand IRS-1with anti-phosphotyrosine PY20 antibody **(b)** Phospho-p44/42 MAPK (Erk1/2) **(c)** phospho-Akt and **(d)** Phospho-GSK-3α/β (upper panels). The blots were then reprobed with anti t-IR-β (a) ,p44/42 MAPK (Erk1/2)(b) total Akt (c) and actin (d) respectively (lower panel) . Bound antibodies were visualized by chemiluminescence and quantified by ChemiImager. The band intensity of the proteins from 2 independent experiments was normalised to the band intensity of total IR. Data are shown as the mean ± S.E.M. (n=2). p-IR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B, P-GSK3= phospho-Glycogen synthase kinase-3 alpha/beta. *p< 0.05, using one way ANOVA."*" statistically significant change in phosphorylation after insulin stimulation as copmared to basel level."#" statistically significant change in the phosphorylation after drug treatment as compared to the insulin alone.

5μM of IKK-16 was used to pre-treat cells .This may have been toxic to cells and affected the total quantities of t-IRβ, actin, t-MAPK and t-Akt (lower panels) (Figure 3.13 (a), (b), (c) and (d) respectively (lower panels). But this toxic effect was apparently reduced in the presence of the nelfinavir (Figure 3.13). There was no significant change in the dephosphorylation induced in nelfinavir-treated CHO-IR cells at insulin receptor, IRS-1, Akt and MAPK (Figure 3.13(a), (b) and (c)).
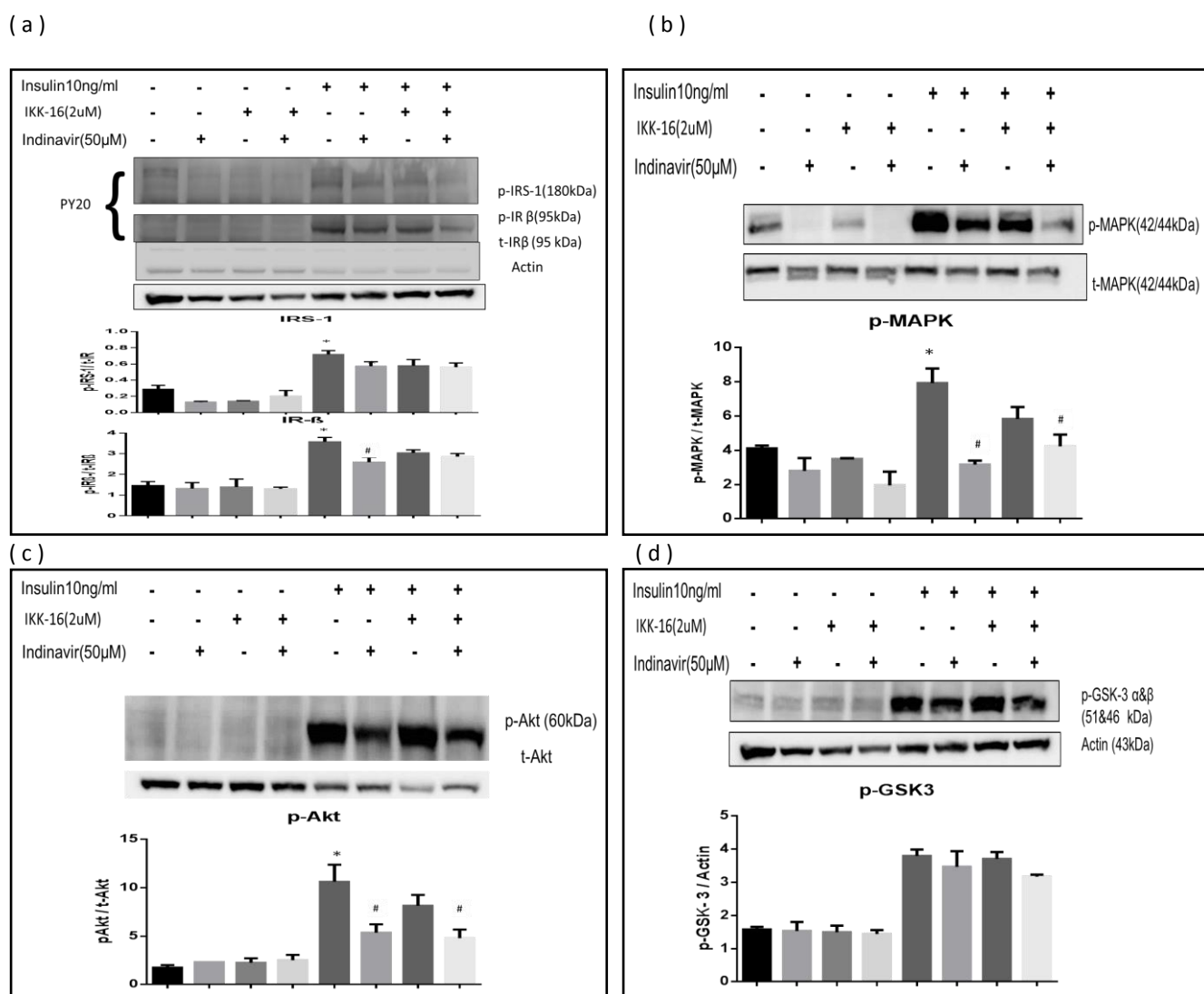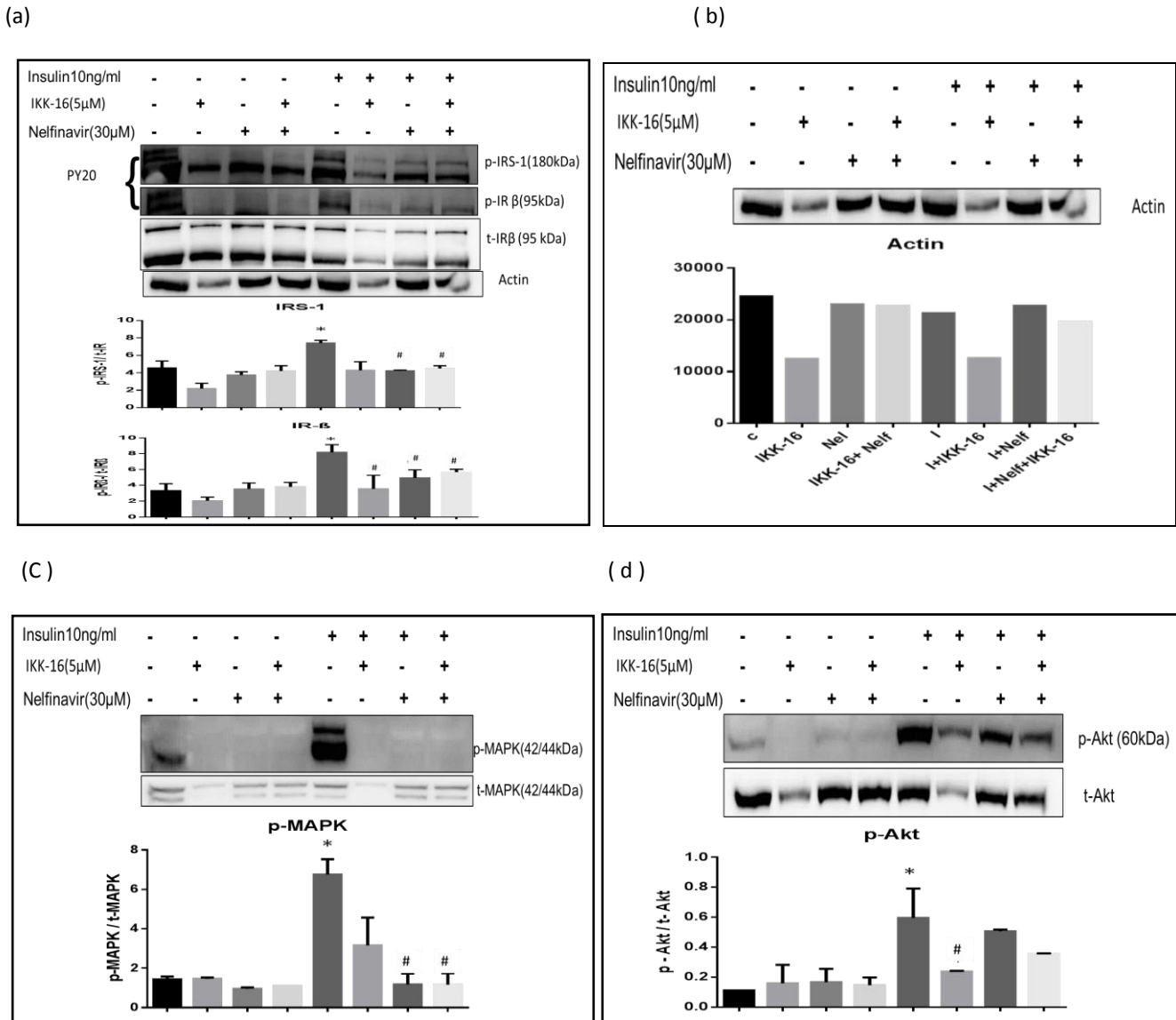
Figure 3.13: Effects of IKK-16on the insulin signaling pathway of CHO-IR cells treated with nelfinavir. CHO-IR cells were pre-treated with IKK-16 (5 µM) for 1 h prior to treatment with nelfinavir (50 µM) for 16 h. The cells were stimulated with insulin for 5min. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: **(a)** IR-βand IRS-1with anti-phosphotyrosine PY20 antibody **(b)** Actin **(c)** Phospho-p44/42 MAPK (Erk1/2) and (**d**) phospho-Akt (upper panels). The blots were then reprobed with anti t-IR-β (a), p44/42 MAPK (Erk1/2) (c) and total Akt (d) respectively (lower panel) . Bound antibodies were visualised by chemiluminescence and quantified by ChemiImager. The band intensity of the proteins from 2 independent experiments was normalised to the band intensity of total IR. Data are shown as the mean ± S.E.M. (n=2). p-IR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B, *p< 0.05, using one way ANOVA."*" statistically significant change in phosphorylation after insulin stimulation as copmared to basel level."#" statistically significant change in the phosphorylation after drug treatment as compared to the insulin alone.

3.3.5 Effect of berberine chloride on cells treated with NRTIs.

3.3.5.1 Effects of tenofovir

For the first time in this study, CHO-IR cells were used to examine the molecular effects of NRTIs (Tenofovir and Stavudine) on the insulin signaling pathway. The cells were treated with NRTIs for 16 h followed by insulin stimulation (10 ng/ml) (1.7 nM) for 5 min. In order to evaluate the effects of the NaSal and BBR on the NRTIs, the cells were pre-treated with (5 mM) NaSal and (2.7 mM) BBR for one hour before the drug treatment for 16 h.

Tenofovir showed no effect on the insulin-stimulated phosphorylation at IR-β and IRS-1 as shown in (Figure 3.14(a)). However tenofovir decreased the phosphorylation at MAPK and Akt (Figure 3.14(b) and (c)).In the same experiment, the effect of NaSal was also evaluated. NaSal caused a small increase in Akt phosphorylation in the tenofovir-treated cells (Figure 3.14(c)) but Nasal did not increase the phosphorylation of MAPK in tenofovir treated CHO-IR cells (Figure 3.14(b)). In further experiments BBR chloride was evaluated (Figure 3.15 (a) and (b)). BBR treatment did not alter phosphorylation of MAPK and Akt (Figure 3.15(a) and (b)).
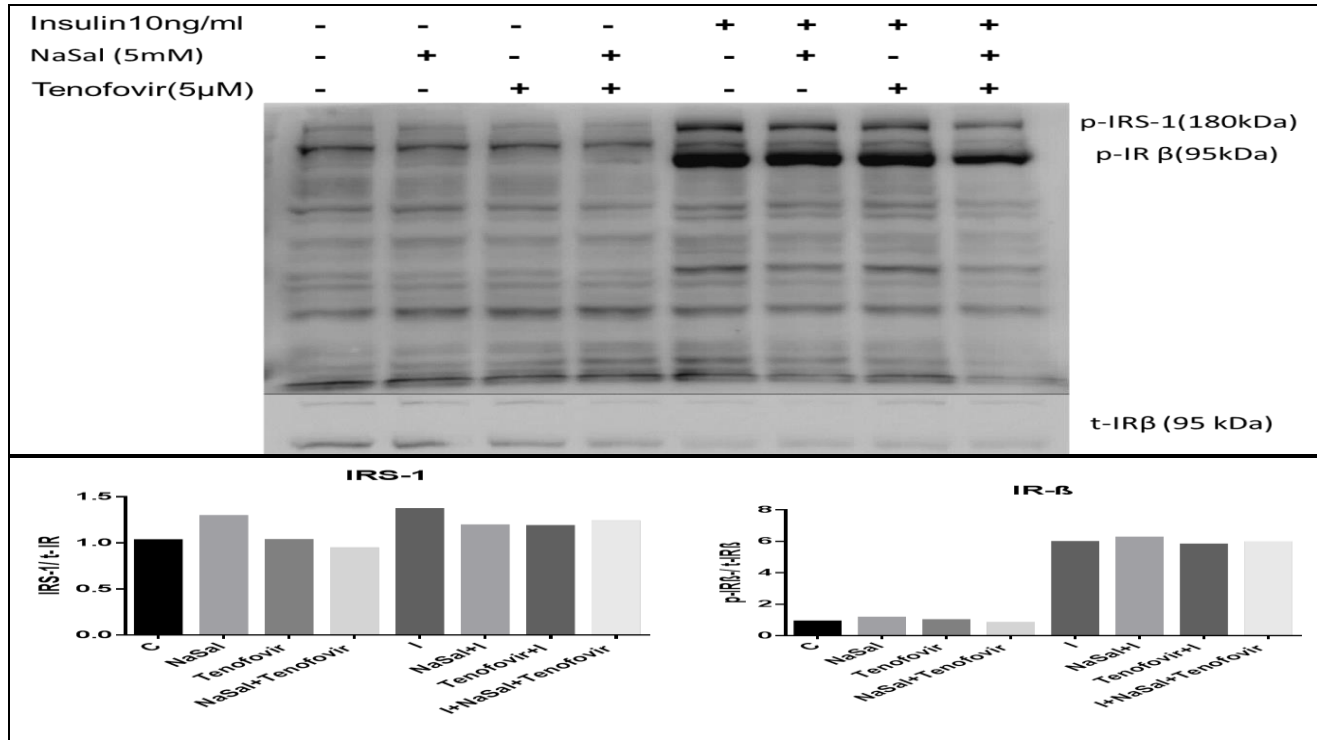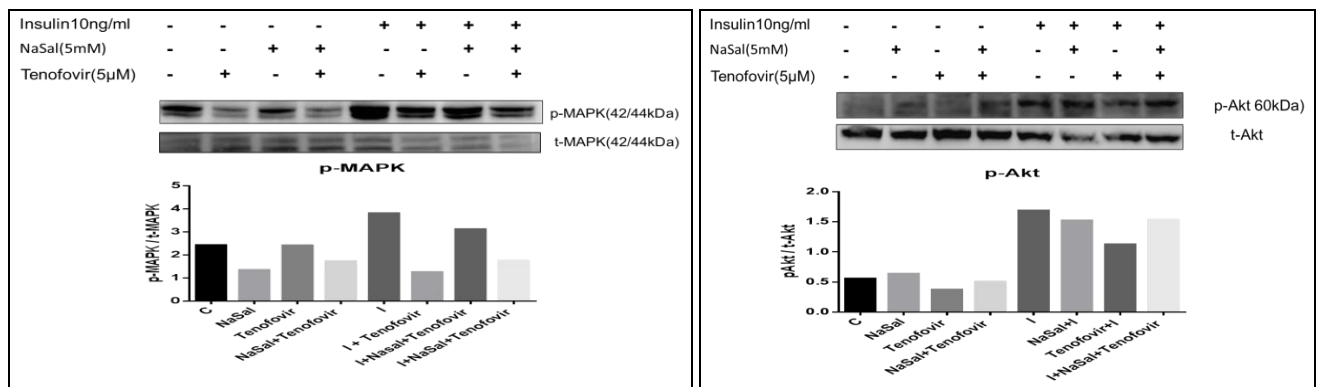
Figure 3.14: Effects of sodium salicylate on the insulin signaling pathway of CHO-IR cells treated with tenofovir. CHO-IR cells were pre-treated with sodium salicylate (5 mM) for 1 h prior to treatment with tenofovir (5 μM) for 16 h. The cells were stimulated with insulin for 5min. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: **(a)** IR-βand IRS-1with anti-phosphotyrosine PY20 antibody **(b)** Phospho-p44/42 MAPK (Erk1/2) and **(c)** phospho-Akt (upper panels). The blots were reprobed with anti t-IR-β (a), p44/42 MAPK (Erk1/2) (b) and total Akt (c) respectively (lower panel). Bound antibodies were visualized by chemiluminescence and quantified by ChemiImager. The band intensity of the phosphorylated proteins was normalized to the band intensity of total proteins. p-IR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B.
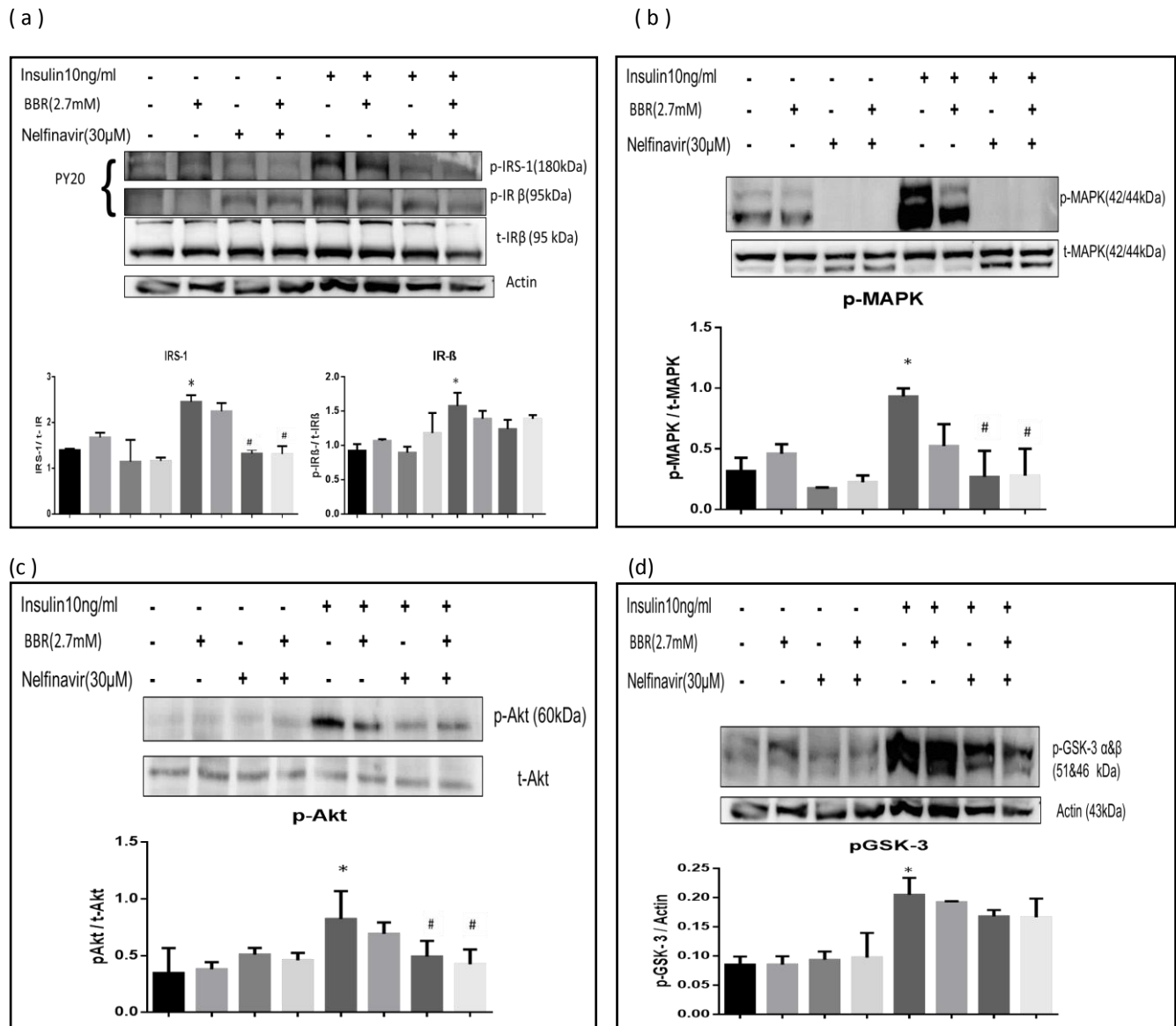
Figure 3.15: Effects of BBR on the insulin signaling pathway of CHO-IR cells treated with tenofovir. CHO-IR cells were pre-treated with berberine chloride (2.7mM) for 1 h prior to treatment with tenofovir (5 μM) for 16 h. The cells were stimulated with insulin for 5min. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: **(a)** Phospho-p44/42 MAPK (Erk1/2) and **(b)** phospho-Akt (upper panels). The blots were reprobed with p44/42 MAPK (Erk1/2) **(a)** and total Akt **(b)** respectively (lower panel) . Bound antibodies were visualized by chemiluminescence and quantified by ChemiImager. The band intensity of the phosphorylated proteins was normalized to the band intensity of total proteins. p-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinase B,t-Akt = Total protein kinase B.

3.3.5.2 Effects of stavudine

In further experiemnts, stavudine treatment had no effect on the proximal end of insulin signaling pathway as observed by the phosphorylation of IR-β and IRS-1of CHO-IR cells (Figure 3.16 (a)) .Also no change was observed in the total quatities of the insulin receptor-β as shown in the same Figure 3.16(a). Stavudine caused a significant decrease in the phosphorylation of MAPK and Akt (Figure 3.16 (b) and (c)) .BBR treatment did not alter protein phosphorylation in cells treated with stavudine in

(Figure 3.16 (b) and (c)) .
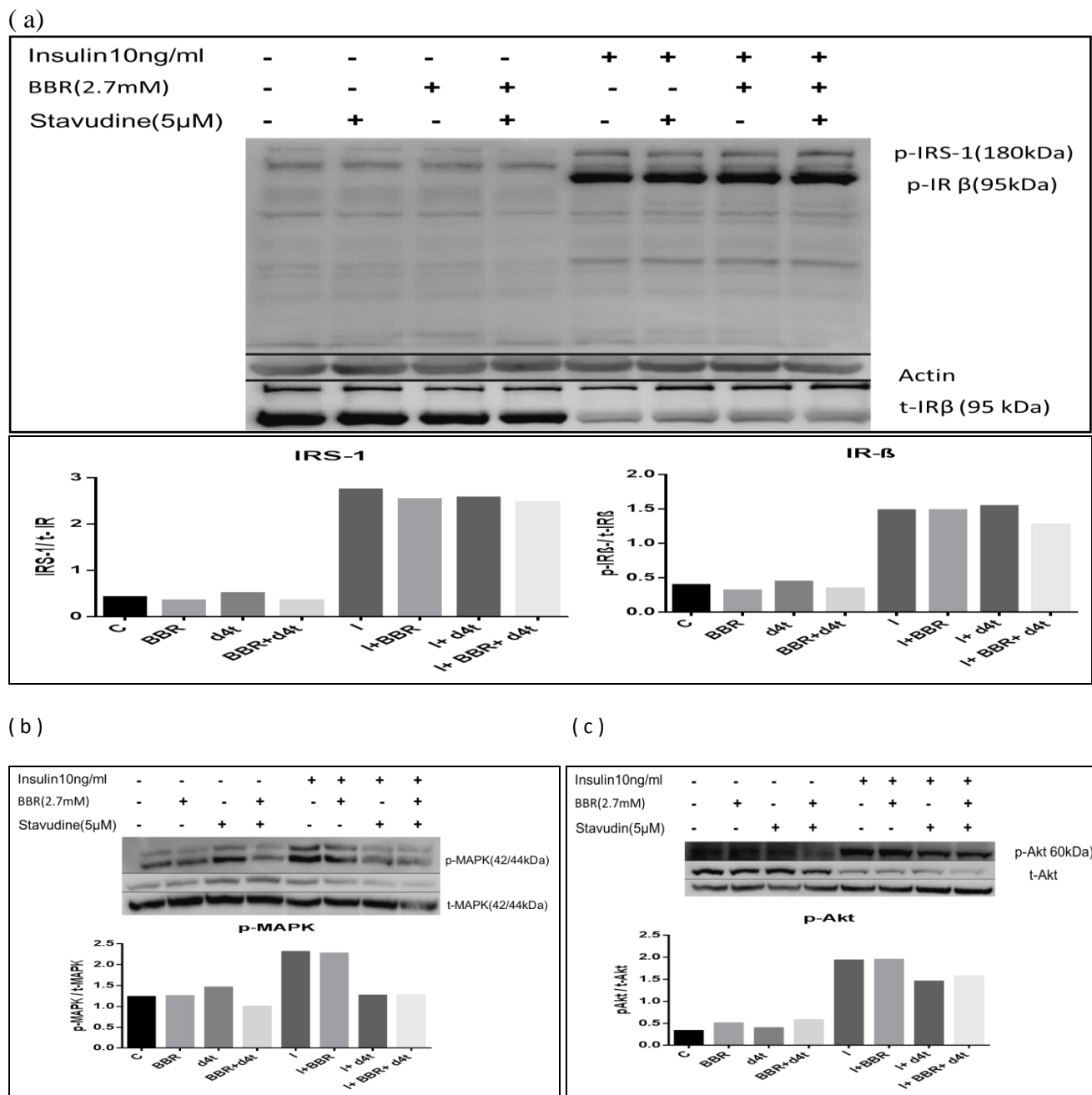
Figure 3.16: Effects of BBR on the insulin signaling pathway of CHO-IR cells treated with stavudine. CHO-IR cells were pre-treated with berberine chloride (2.7 mM) for 1 h prior to treatment with stavudine (5 μM) for 16 h. The cells were stimulated with insulin for 5min. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: **(a)** IR-βand IRS-1with anti-phosphotyrosine PY20 antibody **(b)** Phospho-p44/42 MAPK (Erk1/2) and **(c)** phospho-Akt (upper panels). The blots were reprobed with anti t-IR-β (a), p44/42 MAPK (Erk1/2) (b) and total Akt (c) respectively (lower panel) . Bound antibodies were visualized by chemiluminescence and quantified by ChemiImager. The band intensity of the phosphorylated proteins was normalized to the band intensity of total proteins. p-IR (PY20) = tyrosine phosphorylation of IR, tIRβ = total insulin receptor β-subunit,p-IRS-1 =phosphorylation of Insulin receptor substrate ,P-MAPK = Phospho-p44/42 Mitogen-activated protein kinase, t-MAPK= Total Mitogen-activated protein kinase, p- Akt = phosphorylation of protein kinaseB.

Table 3.2:Summary of the effects of ARVs on phosphorylation of different insulin signalling proteins with and without Berberine chloride and sodium salicylate.

	Protease inhibitors			NRTIs	
	Indinavir	Nelfinavir	Ritonavir	Tenofovir	Stavudine
IR-β	↓	↓	↓	NC	NC
IRS-1	↓	↓	slight↓	NC	NC
p-AKt	*↓	↓	slight↓	Slight ↓	↓
p-MAPK	↓	↓	↓	↓	↓
P-GSK-3α/β	No significant change	Slight decrease	NC	NC	NC
	Effects of BBR			BBR	
IR-β	Slight ↑	NC			
IRS-1	NC	NC			
p-AKt	Slight ↑	NC		NC	slight↑
p-MAPK	NC	NC		NC	NC
P-GSK-3α/β	NC	NC			
	Sodium Salicylate			Sodium Salicylate	
IR-β	Slight↑	Slight ↑			
IRS-1	Slight↑	NC			
p-AKt	NC	NC		slight↑	
p-MAPK	NC	NC		NC	
P-GSK-3α/β					
	IKK-16				
	2µ	5µM			
IR-β	NC	Toxic to cells(NC)			
IRS-1	NC	NC			
p-AKt	NC	NC			
p-MAPK	NC	NC			
P-GSK-3α/β	NC				

NC =No change

↑ = Increased in phosphorylation

↓ = inhibition of phosphorylation

*↓= Decrease in the phosphorylation with new drug vial

Slight↑= slightly increased in phosphorylation but not statistically significantly

3.4 Discussion

The insulin resistance that arises in HIV infected individuals is largely due to the use of PI-based ARV therapy (Ismail et al., 2009), but a few studies have also shown the involvement of NRTIs in insulin resistance without detailing its molecular basis (Fleischman et al., 2007; van Vonderen et al., 2010). Similarly, a number of studies using PIs (indinavir, nelfinavir and saquinavir) have mainly described downstream events in the insulin signalling pathway (Ben-Romano et al., 2003; Murata et al., 2002; Noor et al., 2002; Ranganathan & Kern, 2002; Y. Yang et al., 2006). However, the precise molecular mechanisms leading to insulin resistance have not been completely explored.

In this study, CHO cells transfected with a high number of insulin receptors was used to analyse various effects of protease inhibitors and NRTIs on insulin receptor β-subunit tyrosine kinase activation and subsequent phosphorylation. For the first time, NRTIs were also used to investigate their role in inducing insulin resistance at the cellular level. In this study, not only was the proximal part of the insulin signalling studied but also downstream signalling events were also evaluated including phosphorylation of Akt, MAPK and GSK-3α/β. The high expression of insulin receptors facilitates the detection of downstream insulin-dependent signaling events such that any potential inhibitory effects will be prominent.

The results obtained in this study reveal that CHO-IR cells treated with nelfinavir (30μM) and indinavir (50 μM) for 16 h, followed by insulin stimulation, showed inhibition of tyrosine phosphorylation of the insulin receptor β-subunit, IRS-1 and Akt but there was no significant inhibition observed with ritonavir at the level of IRS-1. This inhibition of phosphorylation by PIs occurs in the proximal and distal parts of insulin signalling after insulin stimulation. Whereas in the case of NRTIs, both tenofovir (5 μM) and stavudine (5 μM) showed no effect on the proximal part of the insulin signaling pathway at the level of phosphorylation of IR-β sub unit and IRS-1. The total expression of these phosphorylated proteins were also determined to rule out any changes in protein expression and

cellular toxicity caused by protease inhibitors and NRTIs on CHO-IR cells and no significant decrease in expression was observed in insulin receptor-β subunit , Akt, MAPK and actin. Initially old vial of indinavir was used which showed only mild decrease in the phosphorylation at Akt but later on new vial received from NIH-USA (national institute of health) which showed significant decrease in the phosphorylation in Akt levels after treatment.

Insulin (10 ng/ml, equivalent to1.7 nM) was used in this study to determine the effects of protease inhibitors and NRTIs on the signalling pathway. In previous studies, in cultured cells, 10 nM or higher concentrations of insulin (supra physiological concentrations) were used to analyse IR signalling pathways (Li, Barrett, Wang, Chai, & Liu, 2005; Murata et al., 2000). In this study we used a submaximal concentration of 10ng/ml (1.7nM) of insulin, because the effects of PIs and NRTIs may only be apparent at low insulin concentrations (1.7 nM). The maximal activation of the insulin receptor tyrosine phosphorylation was observed at 100ng/ml (17 nM). Owing to the overexpression of the insulin receptor and a high level insulin receptor phosphorylation, inhibitory effects of protease inhibitor and NRTIs may be masked if pharmacological concentrations of insulin are used.

Fetal calf serum used to in the growth medium contains small molecules like amino acids, sugars, lipids, and hormones which are essential for the maintenance and growth of cultured cells(Even, Sandusky, & Barnard, 2006; Shah, 1999) . Trypsin treatment is also known for having insulin like effects in rat adipocytes(Tamura, Fujita-Yamaguchi, & Larner, 1983). Both these factors are considered to be responsible in CHO cells to cause basal level phosphorylation insulin receptors in the absence of insulin. Protease inhibitors also showed a small decrease in this basal level phosphorylation in the absence of insulin as shown in fig 3.12 and 3.13.But this impact is not apparent in all blots.

In some cases the decrease in phosphorylation of IR-β and IRS-1 may not be rate limiting steps in insulin signal transduction and it suggested that key functional defects may occur further downstream

(Y. B. Kim, Nikoulina, Ciaraldi, Henry, & Kahn, 1999; Leng, Karlsson, & Zierath, 2004). So in the post-receptor steps of insulin signaling, phosphorylation at Akt, MAPK and GSK-3α/β were also analysed. The phosphorylation results indicated that protease inhibitors and NRTIs significantly affected phosphorylation of MAPK at both high and low concentrations. Similarly phosphorylation of Akt was inhibited by the protease inhibitors. GSK-3α/β phosphorylation step was not significantly affected by either protease inhibitors or NRTIs and was not different from the control. In a prior study using 3T3 L1 adipocyte cells treated with nelfinavir (30 μM) for 18 h, decreased phosphorylation of the Akt and GSK-3α/β protein (Kachko et al., 2009) were observed. The effect of protease inhibitors may differ for different cellular proteins owing to differences in cell lines (Ben-Romano et al., 2003; Noor et al., 2006).

Impairment during the early steps of insulin signalling (phosphorylation at IR-β and IRS-1) is most often the proposed mechanism responsible for insulin resistance however insulin resistance induced without affecting the insulin receptor activation was observed with ceramide (Summers, Garza, Zhou, & Birnbaum, 1998; C. N. Wang, O'Brien, & Brindley, 1998), oxidative stress (Rudich et al., 1998), and GH (Takano et al., 2001) suggested that downstream signalling step(s) of insulin receptor can be functionally affected and rate limiting. Furthermore, decreased insulin-stimulated PKB/Akt phosphorylation was also reported in adipocytes, with or without impairment of the proximal insulin signalling pathway (Danielsson et al., 2005; Tirosh, Potashnik, Bashan, & Rudich, 1999). Bearing in mind that NRTI were used for the first time to evaluate the distal steps of insulin signalling, our results showed that tenofovir significantly decreased the phosphorylation of MAPK and slightly affected the phosphorylation of Akt. Stavudine was also shown for the first time to inhibit the phosphorylation of Akt and MAPK pathway without affecting the initial steps in insulin signalling. Activation of PKB/Akt is a critical step for most of insulin's key functions (Lawlor & Alessi, 2001). Numerous molecular mechanisms have been described as being responsible for the impairment of PKB/Akt activation with

normal activation of PI 3-kinase in vitro. Such mechanisms would include increased activity of protein phosphatase 2A (PP2A; a major PKB/Akt phosphatase) (Cazzolli, Carpenter, Biden, & Schmitz-Peiffer, 2001), impaired sub cellular localization of PI 3-kinase and/or PKB/Akt (Ogihara et al., 2004; Tirosh et al., 1999), defective PIP3 synthesis with normal kinase activity (C. Yang, Watson, Elmendorf, Sacks, & Pessin, 2000), protein kinase C-ξ (PKC) triggered phosphorylation at PH domain of PKB/Akt (Powell, Hajduch, Kular, & Hundal, 2003; Weyrich et al., 2007) and complex formation between PKB/Akt and Drosophila Tribbles homologue protein 3 (TRB3) (Du, Herzig, Kulkarni, & Montminy, 2003).

Mitogen-activated protein kinases are a widely conserved family of serine/ threonine protein kinases involved in many cellular programs such as cell proliferation, differentiation, motility, and death. The p44/42 MAPK (ERK1/2) signaling pathway is activated in response to a wide range of extracellular stimuli including mitogens, growth factors, and cytokines (Baccarini, 2005; Meloche & Pouyssegur, 2007; Roux & Blenis, 2004). MEK1 (MAPK kinase 1) is the primary regulatory MAPK kinase in this pathway (Rubinfeld & Seger, 2005). MEK1 activate p44 and p42 through phosphorylation of activation loop residues Thr202/Tyr204. The protein, p44/42 are negatively regulated by a family of dual-specificity (Thr/Tyr) MAPK phosphatases, known as DUSPs or MKPs (Owens & Keyse, 2007), along with MEK inhibitors such as U0126 and PD98059.

A recently-discovered novel regulatory mechanism affecting insulin sensitivity was described to affect the extracellular signal-regulated kinase/MAP kinase signaling pathway (ERK/MAPK) during an insulin response by controlling the expression of the insulin-like receptor *(inr)* gene in Drosophila (W. Zhang, Thompson, Hietakangas, & Cohen, 2011). This transcriptional regulation is mediated through the Ets-1 (a transcription factor regulated by the MAPK/ERK pathway) (O'Neill, Rebay, Tjian, & Rubin, 1994).

Berberine is used as an anti-hyperglycaemic agent by many physicians in China and has been known to reduce body weight and improve glucose metabolism in animal models of metabolic syndrome (Y. S. Lee et al., 2006). Many studies describe its multiple affects at different levels in the cells to increase glucose uptake by positive impact on the activation of AMP-activated protein kinase-(AMPK) p38 MAPK-GLUT4, JNK PPARα pathways (Yin et al., 2008; Q. Zhang et al., 2011), insulin receptor expression (H. Zhang et al., 2010) and ERK1/2 (Cui et al., 2009) in different cell lines.

For the first time, in this study, berberine was used to minimize the inhibitory effects of ARVs on insulin sensitivity in CHO-IR cells. However, there was no significant change observed with berberine. One reason could be that CHO-IR cells are transfected with a high number of insulin receptors and therefore the augmented effect of berberine was not discernible under these conditions.

Sodium salicylate and aspirin are known to decrease blood glucose levels in diabetic patients via inhibition of NFκB and its upstream activator IKKβ (Yuan et al., 2001). In this study, sodium salicylate was used in the context of cells treated with protease inhibitors. In CHO-IR cells treated with protease inhibitors, our findings revealed that salicylate did not inhibit the effects of protease inhibitor on insulin receptor tyrosine phosphorylation. The inhibition of NFκB pathway and IKKβ in pre-treated with sodium salicylate showed a slight increase in insulin-stimulated tyrosine phosphorylation of insulin receptor β-subunit and tyrosine phosphorylation of IRS-1 in cells. In order to evaluate and confirm the results of sodium salicylate and the role of NFκB pathway in insulin resistance induced by protease inhibitors, IKK-16 a selective inhibitor of IKKβ was used. In the IKK-16 treated cells no significant change was observed in the inhibition of phosphorylation at IRS-1 and IR-β induced by PIs. These results were consistent with the results obtained with sodium salicylate. On the basis of these results further investigations are required to evaluate whether other mechanisms are involved in insulin resistance during the early as well as distal stages of the insulin signalling steps.

3.5 Conclusion

The PIs indinavir, nelfinavir, ritonavir inhibited insulin-stimulated tyrosine phosphorylation of the insulin receptor β-subunit, IRS-1 Akt and MAPK in CHO-IR cells. Similarly NRTIs tenofovir and stavudine also inhibited the downstream parts of insulin signaling such as Akt and MAPK without affecting the phosphorylation of IR-β subunit and IRS-1.The role of NFκB pathway was also evaluated in this study by using the sodium salicylate and IKK-16 but inhibition of NFκB pathways did not alter the impact of protease inhibitors and NRTIs on the insulin stimulated phosphorylation. Therapeutic compound Berberine chloride also failed to reverse the effects of ARV at different levels of its signaling pathway.

We conclude that protease inhibitors inhibit phosphorylation at both proximal (IR-β and IRS-1) and distal ends (Akt and MAPK) of insulin signaling pathways while NRTIs only affect the downstream signaling proteins (Akt and MAPK) of insulin signaling pathway without any effect on proximal steps. In addition it was found that GSK-3 phosphorylation was not altered by both protease inhibitors and NRTIs.

CHAPTER 4: THE EFFECT OF NUCLEOSIDE/NUCLEOTIDE REVERSE TRANSCRIPTASE INHIBITORS AND PROTEASE INHIBITORS ON LIPOPROTEIN LIPASE

4.1 Introduction

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

4.1.1 The synthesis, processing and regulation of lipoprotein lipase

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 (Braun & Severson, 1992; Camps et al., 1991; Camps, Reina, Llobera, Vilaro, & Olivecrona, 1990). The mature LPL enzyme is glycosylated with approximately 12% of carbohydrates and is comprised of two complex oligosaccharide chains, and in few species two complexes and one high mannose chain (Doolittle, Ben-Zeev, Elovson, Martin, & Kirchgessner, 1990; Masuno et al., 1991; Semb & Olivecrona, 1989; Vannier & Ailhaud, 1989). Catalytically active LPL is a homodimer that forms in the endoplasmic reticulum and it has been proposed that these chains are formed by the trimming of glucose residues via glucosidase in the endoplasmic reticulum. It has been shown by several lines of experimental evidence that N-linked glycosylation and dimerization of LPL are vital for its catalytic activity (Braun & Severson, 1992).

The synthesis and release of the LPL is regulated at multiple levels by multiple factors as described in Figure 4.1.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 level. 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 furthermore it also modulates other cellular functions and hormonal effects on the local tissues (H. Wang & Eckel, 2009).
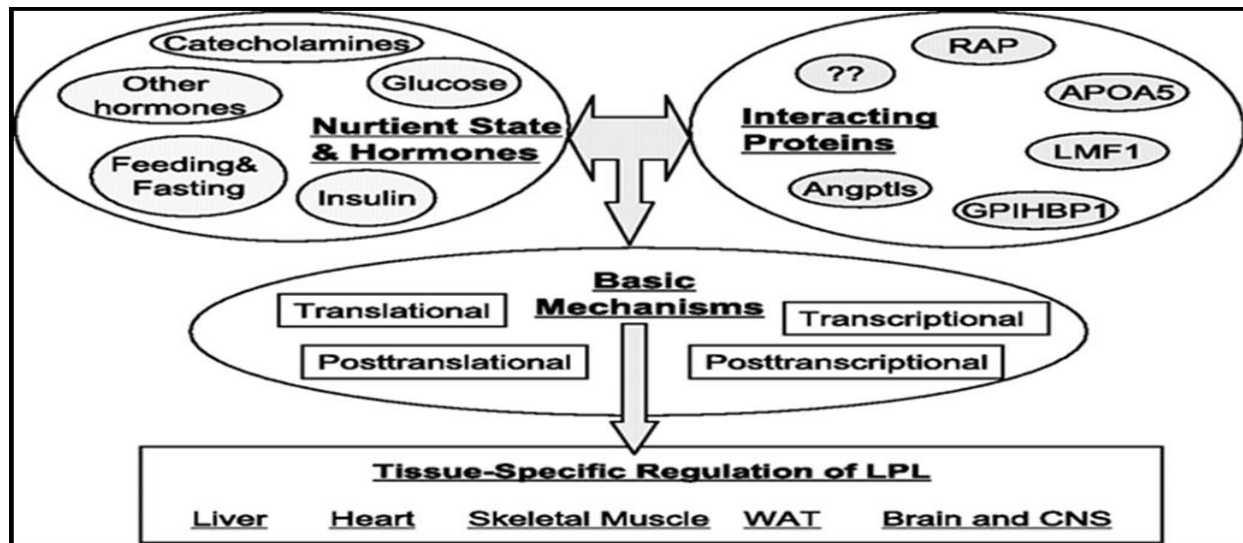
Figure 4.1: Levels of LPL regulation in different tissues and factors (hormonal nutritional and regulatory proteins) which affect its synthesis, translocation, glycosylation and secretion. RAP, receptor-associated protein; APOA5, Apo lipoprotein A5; LMF1, lipase maturation factor 1; Angptls, angiopoietin like; GPIHBP1, glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1; WAT, white adipose tissue; CNS, central nervous system(H. Wang & Eckel, 2009).

4.1.2 Role of lipoprotein lipase in lipid and lipoprotein metabolism

Triacylglycerol (TAG) is transported and secreted from the intestine and liver in the form of chylomicrons and VLDL respectively. Both classes of lipoproteins enter the circulation in the form of large multi-molecular lipoprotein particles in order to distribute TAG to tissues and other organs.

TAG-rich lipoprotein particles are too large to cross the capillary endothelium in most tissues. LPL is found attached via highly charged, membrane bound chains of heparin sulphaate-proteoglycans (HSPG) to the luminal surface of the capillary endothelial cells and this is the location of the LPL activity where TAG on lipoproteins are easily accessible (Braun & Severson, 1992; Cryer, 1981;

Enerback & Gimble, 1993; Mead, Cryer, & Ramji, 1999; Mead & Ramji, 2002; C. S. Wang, Hartsuck, & McConathy, 1992).

LPL plays an important role in lipid metabolism and transport. It catalyses the hydrolysis of TAG attached to circulating chylomicrons and VLDL. The resulting reaction produces non-esterified fatty acids (NEFA) and 2-monoacylglycerol for tissue utilization (Braun & Severson, 1992; Cryer, 1981; Enerback & Gimble, 1993; C. S. Wang et al., 1992). NEFAs in white adipose tissue are re-esterified for energy storage as TAG (Cryer, 1981). Additionally, fatty acids are oxidized to provide an energy source in the heart and to regulate thermogenesis in brown adipose tissue (Cryer, 1981).

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

As discussed above the regulation of LPL synthesis is very important 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 (Olivecrona, Chernick, Bengtsson-Olivecrona, Garrison, & Scow, 1987; Raynolds et al., 1990; Vannier, Amri, Etienne, Negrel, & Ailhaud, 1985). Insulin affects different steps of LPL synthesis in adipocytes such as during the process of differentiation by enhancing gene transcription (Semenkovich, Wims, Noe, Etienne, & Chan, 1989) 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 (Albalat et al., 2007; Albalat, Sanchez-Gurmaches, Gutierrez, & Navarro, 2006; Semenkovich et al., 1989).

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 exactly 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 (Bengtsson, Olivecrona, Hook, Riesenfeld, & Lindahl, 1980; Cisar, Hoogewerf, Cupp, Rapport, & Bensadoun, 1989; Rojas, Enerback, & Bengtsson-Olivecrona, 1990).

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 normal 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 (Kern, Mandic, & Eckel, 1987; Ong & Kern, 1989). Different studies associate insulin resistance with decreased LPL activity, hypertriglyceridaemia, elevated cholesterol and chylomicronaemia (Bijvoet et al., 1996; Brunzell, 1995)

4.1.4 Pathophysiology of lipoprotein lipase

4.1.4.1 Role of lipoprotein lipase in atherosclerosis

Increased LPL activity can result in either pro or anti-atherogenic effects, depending on the cellular location of LPL synthesis. LPL secreted by the parenchymal cells of adipose tissue in the vascular endothelium and muscle tissue (major sources) provides a protective role, whilst LPL secreted by monocyte-derived macrophages has pathophysiological effects that promote foam cell formation and, ultimately, atherosclerosis(Braun & Severson, 1992; Glass & Witztum, 2001).

4.1.4.2 Role of lipoprotein lipase in insulin resistance

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 (Morino et al., 2012). Normally 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 hydrolyse the serum triglycerides and deliver free fatty acids to the muscle cells as described above.

Moreover, this study also confirmed this association when LPL was knocked down in muscle cells resulting in decreased fatty acid influx and reduced biogenesis of mitochondria by decreased activation of PPAR-δ (Morino et al., 2012).

4.1.4.3 Role of lipoprotein lipase in adipose tissue distribution

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, lipaemia retinalis and pancreatitis. There are three naturally occurring mutations in LPL that affect lipid transport and metabolism and result in hypertriglyceridaemia and obesity in mice (Peterfy et al., 2007; Ullrich, Purnell, & Brunzell, 2001).

Disturbances in body fat distribution, dyslipidaemia 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 (G. Behrens et al., 1999; Grunfeld et al.,

1989; Vigouroux et al., 1999). It has already been reported that HIV infection itself has an effect on triglyceride metabolism and lipoprotein lipase activity (Grunfeld et al., 1992).

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 dyslipidaemia and insulin resistance (Dube et al., 2001; Purnell et al., 2000; Vigouroux et al., 1999).

With the advent of HAART, it has become well known that the two drug families; the NRTI and the PIs are often associated with lipid abnormalities and body fat distribution, yet the cellular and biochemical mechanisms underlying these effects are not well understood (Noor et al., 2002; Vigouroux et al., 1999). The NNRTI component of HAART also contributes to the dyslipidaemia although the relationship to lipodystrophy is unclear (Clotet, van der Valk, Negredo, & Reiss, 2003; Mallal et al., 2000; Petit et al., 2003).

A study in transgenic mice overexpressing LPL in liver and skeletal muscle shows that this led to a 3-fold increase in muscle triglyceride and 2-fold increase in liver triglyceride. This increase in triglyceride resulted in insulin resistance involving defects in IRS-I and IRS-2-associated phosphatidylinositol-3- kinase activities in muscle and liver respectively. Contrary to these findings, in liver-specific LPL knockout mice expressed no change in IRS-2 associated PI-3 kinase activity in liver cells as compared to the control (J. K. Kim et al., 2001).

4.1.5 Methods for measuring lipoprotein lipase

A number of methods have been used to measure LPL activity in adipocytes and in serum or plasma. These are mostly based on the use of radioactive or fluorescently-labelled substrates, such as the hydrolysis of radioactive emulsion ([^{3}H]triolein (glycerol tri-[9,10(N)-^{3}H] oleate) and fluorescently-labelled triacylglycerol (Dousset, 1988; Nilsson-Ehle & Schotz, 1976). Recently, a fluorescent LPL

activity assay kit (Roar LPL activity assay kit, Roar Biomedical, Inc.) has been used to measure the activity of the enzyme (Yokota, Nagashima, Ghazizadeh, & Kawanami, 2009). This method uses a ready-made non-fluorescent substrate emulsion (proprietary substrate) which becomes intensely fluorescent when it is hydrolysed by LPL.

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 (Shirai & Jackson, 1982).

4.1.6 Rationale for measuring lipoprotein lipse in CHO cells

Many studies carried out with different cell lines and with primary cultures of cells, indicated that the expression of lipoprotein lipase is usually dependent on the growth conditions (Cupp, Bensadoun, & Melford, 1987; Semb & Olivecrona, 1987, 1989; Severson, Lee, & Carroll, 1988). In Chinese-hamster ovary (CHO) cells LPL is expressed both in growing and in confluent cells (Cisar et al., 1989). In CHO cells, LPL is consistently produced independent of cell differentiation stage (Rojas et al., 1990). The increased level of expression of insulin receptors also makes it easy to modulate the insulin response. Moreover, since the model system for much of the work described in this thesis to study insulin signalling makes use of a CHO cell line, it appeared prudent to use this cell line to quantify the regulation of LPL activity by insulin and ARVs. We therefore analysed 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.

4.2 Method

4.2.1 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 1 h at 37 °C in an incubator, to stimulate LPL activity. After 1 h, heparin sodium-fresenius (final concentration 100 U/ml) was added for a further 2 h to release the enzyme from the cell surface (Knutson, 2000; Ong, Kirchgessner, Schotz, & Kern, 1988; Ranganathan & Kern, 2002; Stewart & Schotz, 1974). 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 (D. Quinn, Shirai, & Jackson, 1983). PMSF (10 mM) was prepared in ethanol.

4.2.2 Assay of lipoprotein lipase activity

LPL activity in supernatants from CHO-IR cells was determined as follows (Shirai & Jackson, 1982):

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900 μl buffer + 100 μl supernatants from CHO-IR cells + 10 μl pNPB in acetonitrile

Total volume = 1010 μl

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)) (D. M. Quinn, Shirai, Jackson, & Harmony, 1982; Shirai & Jackson, 1982). 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 (Shirai & Jackson, 1982) for specific periods of time. Absorbance was then measured using a visible light spectrophotometer at 400 nm.

Enzyme activity was calculated using the following formula (D. Quinn et al., 1983) :

$$\text{Units/ml enzyme} = \frac{(\Delta A400nm/min\ test - (\Delta A400nm/min\ substrate\ blank))\ (1.01)\ (df)}{(0.0148)(0.1)}$$

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 nmole of p-nitrophenol phosphate per minute at pH 7.2 and 37° C, when using p-nitrophenyl butyrate as the substrate.

For these 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, entricitubine 20µM, atazanavir sulphate 20µM (Minami et al., 2011), stavudine 10µM (Dudley et al., 1992), lamivudine 10µM (Heald et al., 1996), tenofovir 1 µM (Cihlar et al., 2002).

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

4.3.1 Changes in LPL activity measured in CHO-IR cells after insulin and heparin

Fig 4.2(a)

Fig 4.2(b)
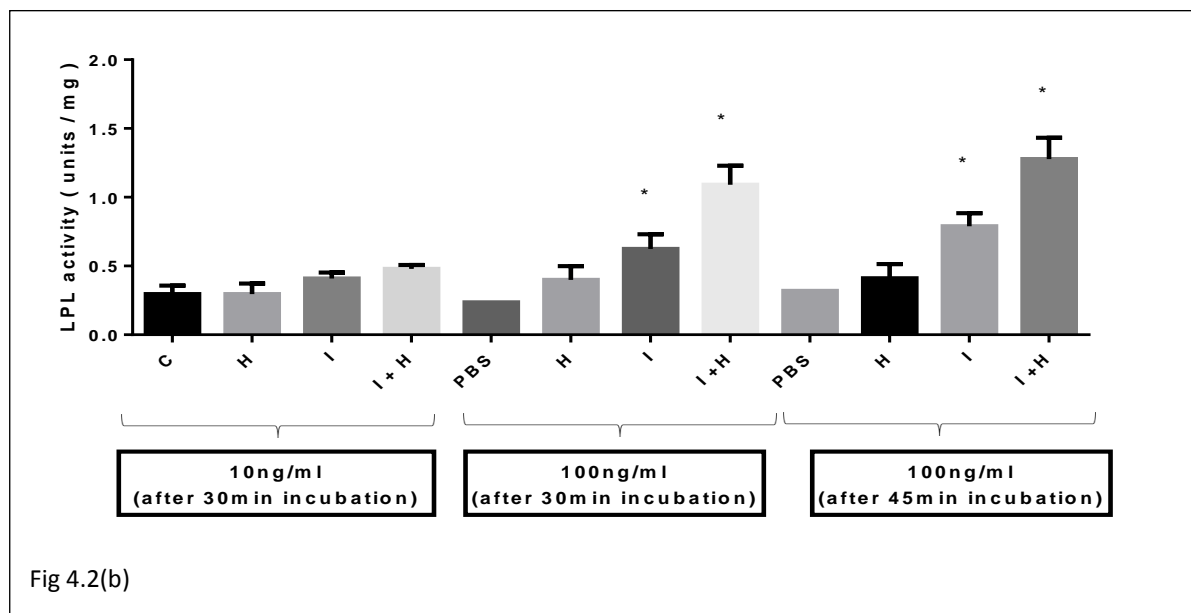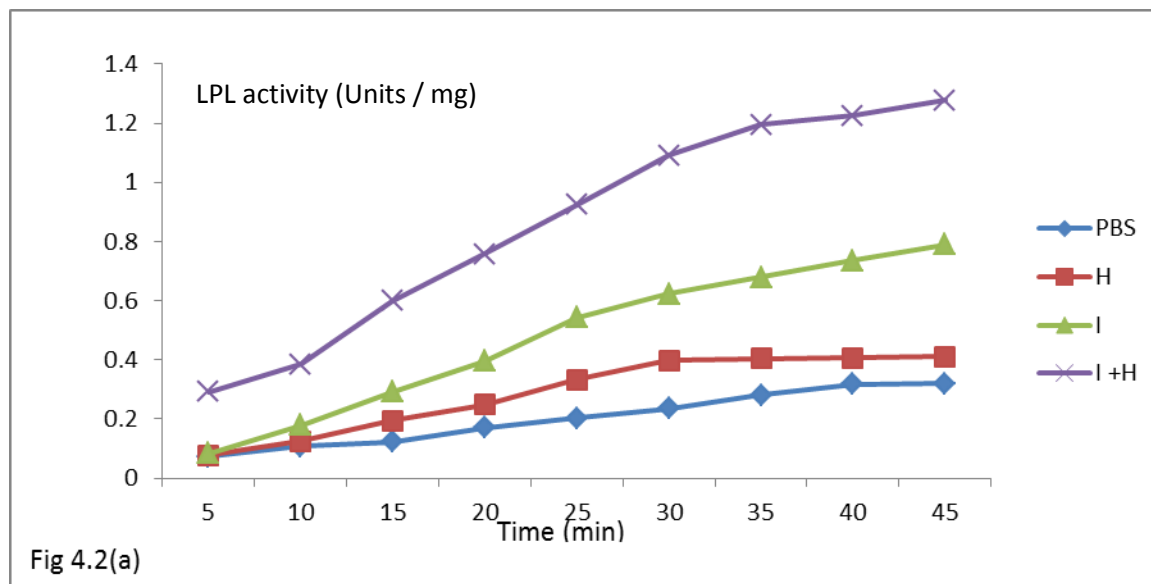
Figure 4.2(a) Time course of LPL activity in supernatants of CHO-IR cells stimulated with insulin and heparin. CHO-IR cells were stimulated with insulin (100ng) for 1 h followed by the addition of heparin (100U/ml). Cell culture supernatants were harvested to measure LPL activity at indicated incubation periods using spectrophotometry (400 nm). **Fig 4.2(b)** show differences in LPL activity after incubation of 30 min with10 ng/ml and with 100ng/ml after 30 and 45 min .LPL activity was measured in three independent experiments. PBS = phosphate buffered saline, H = heparin, I = insulin. $p < 0.05$, using Student's *t*-test.

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 = 0.0129) incubation and then at 45 min (p=0.0127). The cells which were stimulated with insulin only also showed increased LPL after 30 min (p = 0.0361) up to 45 min (p = 0.0197) incubation as shown in figure 4.2(b). In order to observe the effect of insulin concentration on LPL activity a low dose of insulin 10 ng (1.7nM) were used to stimulate the cells .A statistically insignificant (p=0.0625) low activity of LPL was measured in the supernatants as shown in Figure 4.2(b).

4.3.2 Effect of nucleoside/nucleotide reverse transcriptase inhibitors on lipoprotein lipase activity in supernatant from the CHO-IR cells

LPL activity was measured in CHOIR 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 of 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.
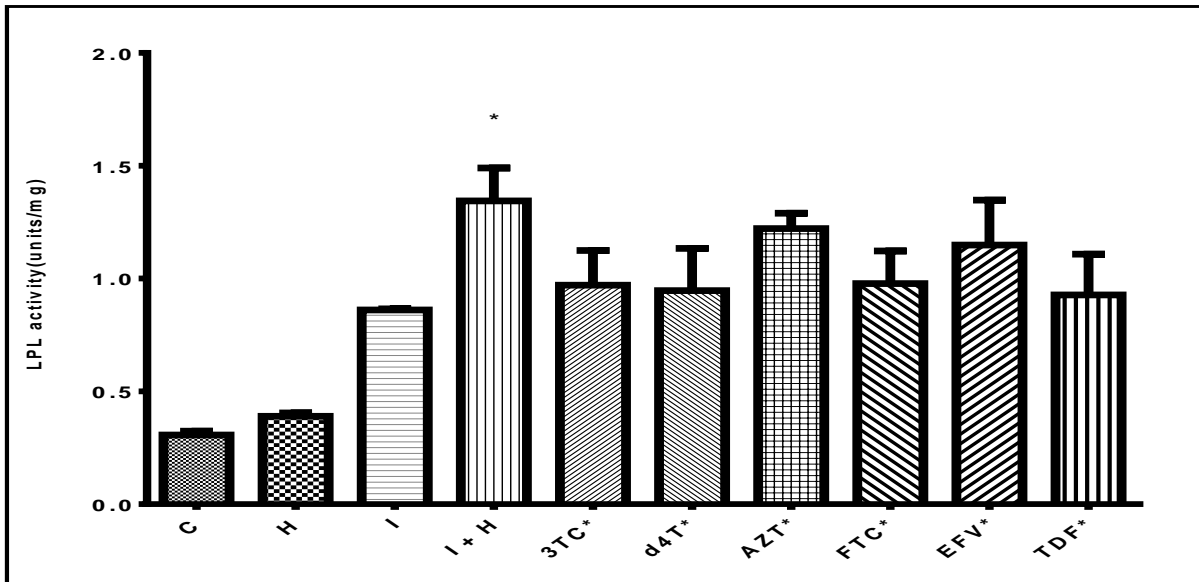
Figure 4.3: Effect of NRTIs on LPL activity after adding directly in supernatants of CHO-IR cells.

CHO-IR cells were grown in 6-well plates and upon 70-80% confluency the cells were serum starved for 16 h, followed by exposure to both insulin (100)ng/ml) and heparin. LPL activity was measured in duplicate in two independent experiments. Data are shown as the mean and S.E.M. (n=2). C= control, H= Heparin, I = insulin, AZT* = zidovudine, EFV*= efavirenz, FTC* = emtricitabine, d4T* = stavudine, 3TC*= lamivudine and, TDF*= tenofovir. $p* < 0.05$ using unpaired *t* test (*) indicate the drugs that were added directly in the reaction mixture of the LPL assay before incubation.

In the first experiment the NRTIs were added directly to 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 in Figure 4.3.
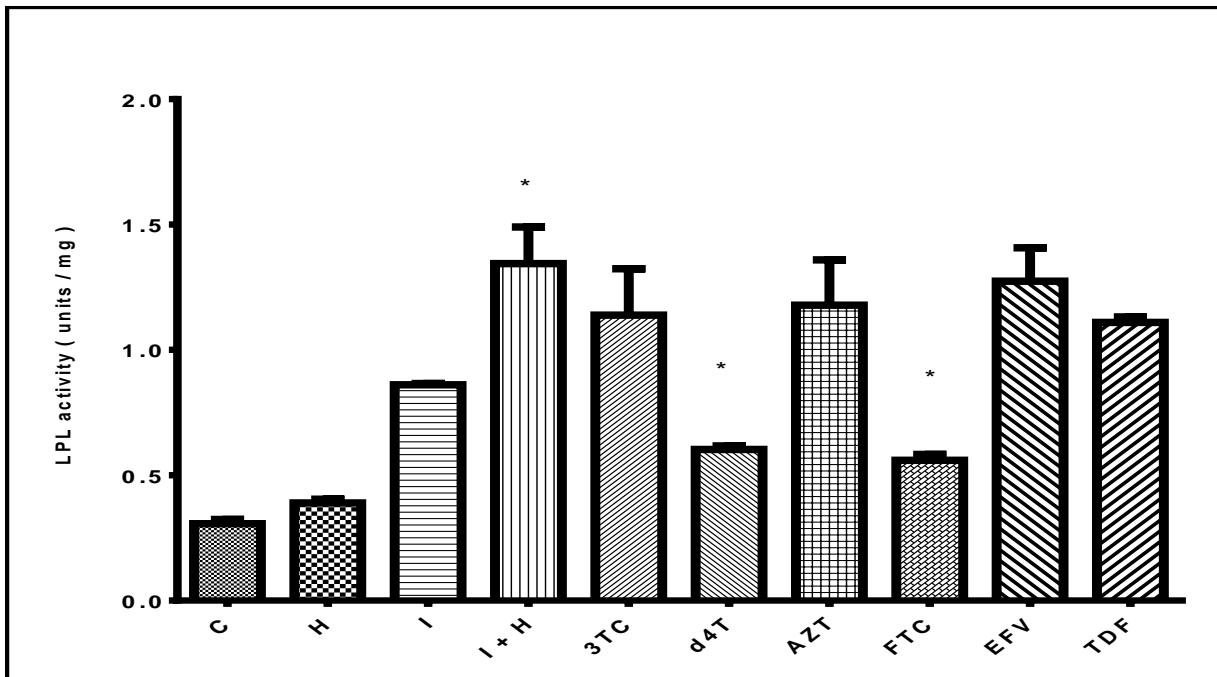
Figure 4.4: LPL activity in supernatants of CHO-IR cells treated for 16 hours with NRTIs.

CHO-IR Cells were grown to 70-80 % confluency, usually taking 24 to 48 hours in 6-well plates .The cells were then serum starved for 16 h in the absence or presence of the nucleoside reverse transcriptase inhibitors, followed by exposure to both 100ng/ml insulin (17nM) and heparin. LPL activity was measured in duplicate as two independent experiments. Data are shown as the mean and S.E.M. (n=2). C= control, H= Heparin, I = insulin, AZT = zidovudine, FTC = emtricitabine, EFV= efavirenz, d4T = stavudine, 3TC= lamivudine and, TDF= tenofovir. p*< 0.05 using unpaired t-test

In order to examine the effect of the NRTIs on 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.

4.3.3 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.
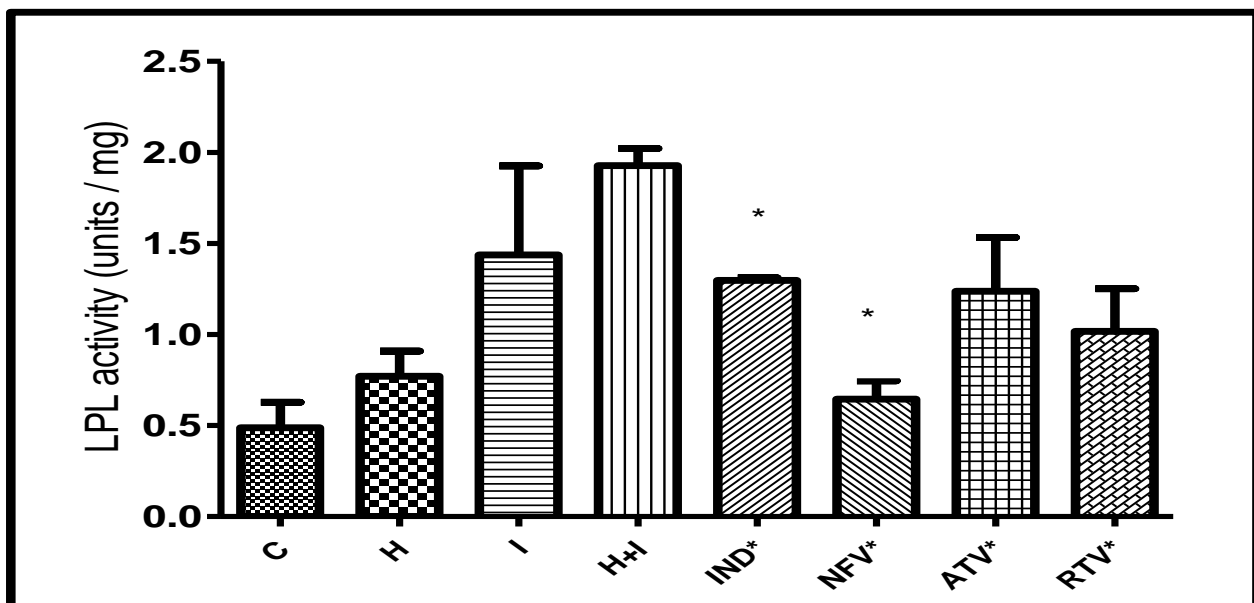
Figure 4.5: Effect of HPIs on LPL activity after adding HPIs directly in supernatants of CHO-IR cells.

CHO-IR cells were grown in 6-well plates and upon 70-80% confluency the cells were serum starved for 16 h, followed by exposure to both100ng/ml (17nM) insulin and heparin. LPL activity was measured in duplicate in two independent experiments. Data are shown as the mean and S.E.M. (n=2). C= control, H= Heparin, I = insulin, IND* = indinavir, NFV*= nelfinavir ATV* = atazanavir sulfate, RTV* = ritonavir. p*< 0.05 using unpaired t-test .(*) indicate that drugs were added directly in the reaction assay before incubation.
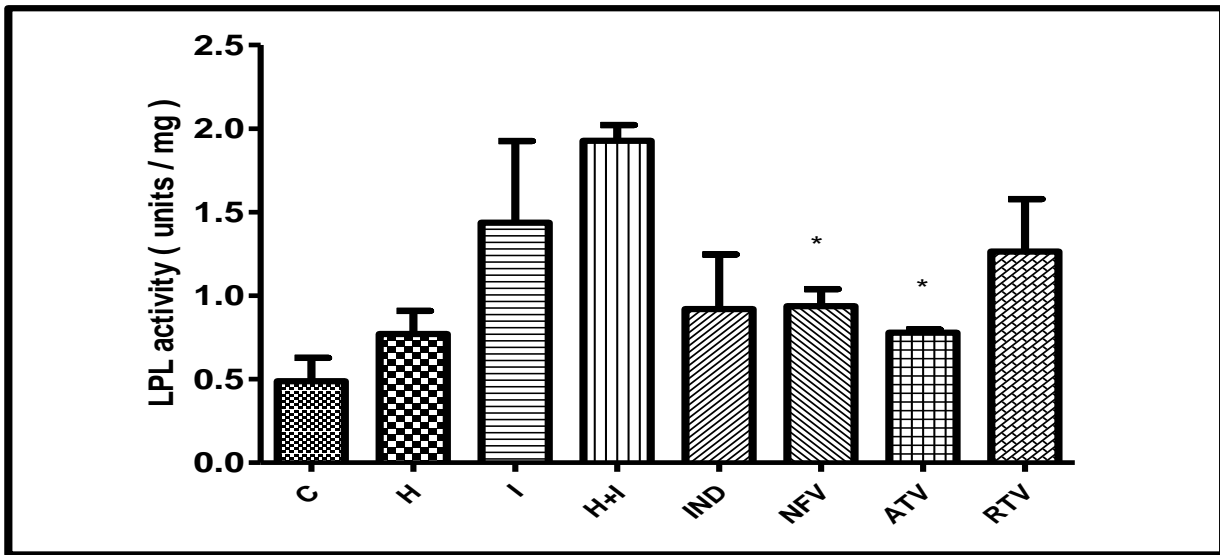
Figure 4.6: LPL activity in supernatants of CHO-IR cells treated for 16 hours with HPIs.

CHO-IR cells were grown in 6-well plates and upon 70-80 % confluency the cells were treated with HPIs indicated in serum starvation medium for 16 h, followed by exposure to both insulin (100ng/ml) and heparin. LPL activity was measured in duplicate in two independent experiments. Data are shown as the mean and S.E.M. (n=2). C= control, H= Heparin, I = insulin, IND = indinavir, NFV= nelfinavir ATV = atazanavir sulfate, RTV = ritonavir. $p* < 0.05$ using unpaired t-test.

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 supernatant were 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 secret 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 sulphate (p=0069) was observed while smaller (statistically insignificant) decreases in LPL were observed with indinavir and ritonavir treated cell supernatants (Figure4.6).

4.4 Discussion

In this study, for the first time CHO-IR cells were used to measure LPL activity using a colorimetric method employing pNPB as substrate. LPL has been shown to catalyse the hydrolysis of short-chain fatty acyl esters such as tributyrin, p-nitrophenylacetate and pNPB *in vivo (Shirai & Jackson, 1982).* 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.(D. M. Quinn et al., 1982).

CHO cells provide a convenient model to study intracellular synthesis and translocation of lipoprotein lipase. A major 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. (Rojas et al., 1990). 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 measurable 3-fold increase in LPL activity. Insulin plays an important dose-dependent role in LPL synthesis and significantly affects the posttranscriptional and posttranslational levels with minimal effects at mRNA level during differentiation of adipocytes (Pradines-Figueres, Vannier, & Ailhaud, 1988; Semenkovich et al., 1989; Spooner, Chernick, Garrison, & Scow, 1979). These results indicate that the insulin-induced synthesis of LPL is dose-dependent and there 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 (Palacios et al., 2007).

Major PIs such as indinavir, nelfinavir, atazanavir sulphate and ritonavir are still being used in South Africa and these were used in this study as well (Meintjes G et al., 2012). 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 sulphate 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. These 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. In addition ritonavir also decreased the total LPL activity in the plasma (den Boer et al., 2006).

Similarly few other studies describe a relationship between the intracellular lipid content and insulin resistance in muscle cells (Krssak et al., 1999; Perseghin et al., 1999). 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 (Morino et al., 2012). Similarly Tissue-specific over expression 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 PKC‚s (PKCθ, PKCδ, and PKCβ) responsible of insulin resistance by effecting the insulin signalling pathway in to the cells (Morino et al., 2005; Morino, Petersen, & Shulman, 2006; Savage, Petersen, & Shulman, 2007; Shulman, 2000; Yu et al., 2002).

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 (Bijvoet et al., 1996; Brunzell, 1995; Mead & Ramji, 2002).

4.5 Conclusions

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 analyse the effects of PIs and NRTIs on LPL activity. This is the first study to report that stavudine, emtricitabine, indinavir atazanavir sulphate and nelfinavir significantly decrease LPL activity.

CHAPTER 5: METABOLOMIC ANALYSIS OF CELL CULTURE SUPERNATANTS TREATED WITH PROTEASE INHIBITORS AND NUCLEOSIDE/NUCLEOTIDE REVERSE TRANSCRIPTASE INHIBITORS

5.1 Introduction

5.1.1 Highly active antiretroviral therapy and HIV associated metabolic complication

The introduction of HAART (a combination of the different drug groups of ARV) has completely changed the status of HIV/AIDS from a fatal disease to a chronic infection by decreasing the mortality and morbidity in the infected patients and improving the quality of the life (Gurunathan et al., 2009; Pendyala, Want, Webb, Siuzdak, & Fox, 2007). The use of HAART for long periods of time results in a number of different metabolic complications such as insulin resistance lipodystrophy, atherosclerosis and cardiovascular diseases.

Currently there are only two clinical markers, CD4 counts and viral loads that are available for the prognostication of HIV infection (Friis-Moller, Weber, et al., 2003; Jain, Furfine, Pedneault, White, & Lenhard, 2001; Schuster et al., 2008; Williams. A 2012). There are no useful biochemical markers available. Routine biochemical assays are not useful in understanding the detailed metabolic complications owing to their limited ability to analyse single metabolites at a time, along with low sensitivity and specificity make their application restricted in this area.

The requirement of an advanced sophisticated, sensitive multitask analytical instrumentation to analyse the metabolic changes induced by HIV/HAART was fulfilled by the mass spectrometry (MS) and nuclear magnetic resonance (NMR). These advanced technology-based instruments are capable of analysing very low molecular weight metabolites in very low concentrations in a broad range of samples and body fluids (Lungile J. Sitole, 2012). The failure of genomic and proteomic methods to provide phenotypic knowledge makes metabolomics more applicable for the study of cellular

interactions with the environment (Pendyala & Fox, 2010). Two interchangeable terms metabolomics and metabonomics are used which differ in application rather than definition. Metabolomics is the quantitative and qualitative analysis of low molecular weight metabolites from different cells, tissues or any biological sample under certain circumstances while metabonomics measures the overall variations in the metabolic profiles of living system under the effect of any external stimuli such as drug or disease (Pendyala et al., 2007; Wishart et al., 2007).

In HIV-infected patients, metabolic complications are the result of the combined contribution of the virus, immune response against the virus and side effects induced by the ARVs used against the virus and these are often quite obvious (Alberti, Zimmet, & Shaw, 2006; Powderly, 2004; Slama et al., 2009).

 A number of metabolic alterations are detected in the early asymptomatic stage of   HIV infection before the initiation of HAART in HIV-infected subjects with normal phenotypes (Gurunathan et al., 2009; Hommes et al., 1990; Martin & Emery, 2009; Slama et al., 2009). A group of studies in the normal asymptomatic HIV-infected patients showed increased energy utilization at the resting stage which is proposed to be linked to the high energy requirement of the infected cells, having increased beta oxidation rate, higher viral load and resulting in increased catabolism in the body (Hommes et al., 1990; Lane & Provost-Craig, 2000; Slama et al., 2009). Another study, looking at clinically stable HIV-infected patients using magnetic resonance imaging (MRI) and positron emission tomography, indicated high glucose utilization rates by the brain and other metabolic changes before any structural changes were observed (Pascal et al., 1991; Salas-Salvado & Garcia-Lorda, 2001).

Metabolic profiling could be very useful in prognostication of the progress of the infection and for the development of corrective therapy to prevent further development of clinical complications. The metabolic defects induced by HIV involve almost all the main metabolic pathways of lipid, carbohydrate and proteins. HIV infection is known to cause mitochondrial dysfunction and

lipodystrophy (Garrabou et al., 2011; Lane & Provost-Craig, 2000; Martin & Emery, 2009; Polo, Martinez, Madrigal, & Gonzalez-Munoz, 2003). Other metabolic complications caused by HIV infection include effects on calcium metabolism, hypercatabolism, bone disease and liver disease (Pascal et al., 1991; Safrin & Grunfeld, 1999; Slama et al., 2009). Lipodystrophy induced by the NRTIs is the combination of lipoatrophy and lipohypertrophy associated with hyperlipidaemia, hypertriglyceridaemia and hypercholesterolaemia, all of which suggest dysregulation of lipid metabolism. With the involvement of two main NRTIs, stavudine and azidothymidine in apoptosis and mitochondrial toxicity at DNA level and resultant lipodystrophy, led to the removal of stavudine from the first line of therapy in developed countries (Caron-Debarle, Lagathu, Boccara, Vigouroux, & Capeau, 2010; Falutz, 2011; Haubrich et al., 2009). Insulin resistance is among the first metabolic complications reported in HIV/HAART patients and is associated with inflammation and the development of type-2 diabetes mellitus (Feeney ER, 2011; Hofstede, Burger, & Koopmans, 2003). A number of studies indicate the involvement of many PIs in the development of insulin resistance, lipoatrophy and fat deposition which eventually after long term use results in the development of the metabolic syndrome (Silva et al., 1998; Wu et al., 2012). Metabolic syndrome is the term used to describe a cluster of conditions origination from insulin resistance, dyslipidaemia, central obesity and hypertension (Alberti et al., 2006). The relationship between exact drug regimens and specific metabolic problems has been shown using conventional biochemical assays and has yet to be investigated and confirmed using metabolomic approaches.

5.1.2 The role of metabolomics in the investigation of HIV infection and highly active antiretroviral therapy

In the last ten years, great improvements in the field of metabolomics have provided new understanding about the mechanism of diseases as well as methods for diagnosing the onset of disease.

Metabolomics, in common with other "omics" techniques, such as genomics, transcriptomics or proteomics, is a systems approach and metabolomics was first introduced by Nicholson and colleagues (Nicholson, Lindon, & Holmes, 1999).

Metabolomics measures the metabolome, which constitutes the total collection of all small-molecule metabolites present in any biological organism (Oliver, Winson, Kell, & Baganz, 1998; Tweeddale, Notley-McRobb, & Ferenci, 1998). The advantages of metabolomics over other "omics" technologies include its high level of sensitivity and its ability to enable the analysis of relatively few metabolites compared with the complex and large number of corresponding genes or mRNA molecules. Metabolites represent the final products of the complex interaction between genes and other factors such as environmental, health condition or pharmaceutical interventions, which gives metabolomics yet another advantage over the other "omic" fields. Similarly, levels of metabolites represent the activity of metabolic pathways therefore, allowing the detection of temporary as well permanent physiological or pathological changes in cells, tissues or body fluids and offer a convenient tool for biomarker detection (Friedrich, 2012).

5.1.3 Insulin resistance and amino acid metabolism.

The levels of branched-chain amino acids (BCAA) leucine, isoleucine and valine in the circulation are regulated by insulin. In many studies, the levels of BCAA were found to be at abnormal levels related to conditions such as insulin deficiency or insulin resistance. Abnormal BCAA metabolism has also been observed in obesity, type 2 diabetes as well as conditions associated with kidney and liver failure (Adeva, Calvino, Souto, & Donapetry, 2012).

Normally high protein diets or high leucine oral administration act as a positive modulator for insulin release and result in lower glucose and amino acid levels in circulation by enhancing their entry into the tissues and muscles (Stanley et al., 1998). These levels of BCAAs, tyrosine, phenylalanine and

methionine decrease significantly in plasma after insulin release (Aoki, Brennan, Fitzpatrick, & Knight, 1981; Aoki et al., 1976; Elia & Livesey, 1983; Felig, 1975).

Insulin release also negatively affects protein degradation and the rate of proteolysis. BCAA are highly responsive to these inhibitory actions of insulin on cells (Louard, Fryburg, Gelfand, & Barrett, 1992; Pozefsky, Felig, Tobin, Soeldner, & Cahill, 1969) resulting in a decrease of BCAA released from muscle tissue (Felig, 1975). Similar effects have also been seen after insulin infusion particularly on BCAAs, tyrosine, phenylalanine, threonine, and glycine, while alanine release is not significantly affected (Aoki et al., 1976; Elia & Livesey, 1983; Felig, 1975; Pozefsky et al., 1969) . Insulin infusion also decreases branched chain keto acids (BCKAs) in plasma (Schauder, Schroder, Matthaei, Henning, & Langenbeck, 1983).

5.1.4 Metabolomics technologies

There are two main techniques for metabolomics; these are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Both methods enable the comprehensive exploration of metabolic profiles (Dunn, Bailey, & Johnson, 2005; Hollywood, Brison, & Goodacre, 2006; Lenz & Wilson, 2007) and provide useful information of the metabolome of body fluids such as plasma, urine or cerebrospinal fluid (Bictash et al., 2010).

5.1.4.1 Nuclear magnetic resonance spectroscopy

 NMR is a generally used spectroscopic technique for metabolomics. It is based on the magnetic properties of the atomic nucleus (e.g., ^{1}H, ^{13}C, or ^{31}P). This method was introduced in 1980's for the analysis of body fluids (Bell, Brown, & Sadler, 1989; Iles, Hind, & Chalmers, 1985; Nicholson et al., 1984). The characteristic behaviour of NMR active nuclei in a strong magnetic field provides information about the structural and chemical properties of a molecule. ^{1}H nucleus is present in high

numbers in biological fluids thus makings ^{1}H-NMR spectroscopy the first choice for the body fluid analysis. Each separate signal in a ^{1}H-NMR spectrum corresponds to a certain compound. Based on measurements the identification and quantification of single metabolite is possible. Advantages of NMR spectroscopy over MS include the non-destructive nature of the analysis, robust and reproducible measurements with minimal sample preparation requirements, as no separation or ionization steps are necessary. However, in comparison to MS, the analytical sensitivity of NMR is relatively low, even if stronger magnetic fields are used to increase the analytical sensitivity.

5.2 Methodology

For NMR analyses of cell supernatants, the lyophilised supernatant was resuspended in 540 µL of sodium phosphate buffer in D_2O (0.1M, pH 7.4) containing 0.1mM of deuterated 3-trimethylsilylpropionic acid (TSP) as the internal chemical shift standard. Samples were vortexed, centrifuged at13 000xg, 8^oC for 10 min, after which, 520 µL of the sample was added to a 5 mm NMR tube and analysed on the same day. ^{1}H- NMR measurements were performed on a Bruker Avance III (Bruker Biospin, Karlsruhe, Germany) spectrometer operating at 600.10 MHz, equipped with a TXI-probe and a BCU Xtreme for maintaining the probehead temperature at 300K. 1H NMR data were acquired using a pulse sequence to the first increment of a nuclear overhauser effect spectroscopy (NOESY) pulse sequence , using a 90°-3µs-90°-100ms-90° pulse sequence with irradiation during a 2s relaxation delay and also during the 100ms mixing time. A pulse width was automatically determined for each sample. A low frequency irradiation applied at the frequency of the water resonance during the relaxation delay and mixing time. Sixty four free induction decays (FIDS) were collected at a spectral resolution of 64K data points. The total acquisition time was ~7min per sample. An exponential line-broadening factor of 0.3Hz was applied to each FID, after which the resulting spectra were fourier transformed, phase and baseline corrected. Spectra were calibrated by assigning the lactate CH_3 doublet to 1.33 ppm. Additional 2D J-resolved (JRES) 1H NMR

spectra were acquired for each sample, using 4 transients per increment for 1 increments that were collected into 8K data points, using spectral widths of 10KHz in F2 (chemical shift axis) and 0.1 Hz in F1 (spin-spin coupling constant axis). A 1s relaxation delay was employed. Data sets were zero filled to 128 points in F1, and both dimensions multiplied by sine-bell window functions prior to double complex FT. JRES spectra were tilted and symmetrized. In addition, 2D ^{1}H-^{1}H COSY and ^{1}H,^{13}C HSQC (Heteronuclear single quantum coherence spectroscopy) NMR experiments were performed on a single sample to aid in the assignment of metabolites.

5.2.1 Data processing

An in-house database of reference metabolites was created using the AMIX program, version 3.9 (Bruker Biospin, Rheinstetten, Germany). This was done by obtaining 1D, 2D NMR spectra of reference compounds from the human metabolome database (http://www.hmdb.ca/scripts/Biofluid_browse.cgi), the Madison metabolomics consortium database (http://mmcd.nmrfam.wisc.edu/) and the NMR metabolomics database (http://www.liu.se/hu/mdl/main/). Prior to importing the spectra into the in-house database, in AMIX, the spectra were cleaned; by removing background noise and artefacts, and spectral peaks were annotated.

For analyses of the CHO cell supernatant metabolites, integral regions were defined around selected peaks of interest in an interactive manner while observing all spectra superimposed or stacked vertically to ensure proper inclusion of the peaks of interest and exclusion of extraneous peaks across the entire dataset. The assigned regions contain to the most part combinations of metabolites, and this taken together formed a well-defined pattern for the CHO-IR cell supernatant metabolites.

Prior to integration, all spectra were treated with a background removal tool in AMIX, using a 40 MHz frequency filter. A total of 53 spectral regions were assigned for integration using the statistical binning option for integration in AMIX. The integral regions were normalised to total integral region and results exported for further analyses in MatLAB (2011b).

5.2.2 Statistical analyses

Paired t-tests were performed in MatLab for each integral region. Each experiment was considered paired, having no treatment, treatment of the drug, treatment with insulin and finally treatment with insulin and drug. For each drug treatment 3 separate (6) repeats were done. The first t-test was performed to determine the effects of drug treatment, thus the control sample were paired with drug treated samples. A second test was done to compare the effects of insulin treatment in the presence of drug, thus drug treated cells were paired with dug and insulin treated cells. The final test was to observe changes due to insulin treatment alone and was used to also validate the degree of distribution of metabolites here control samples were paired with treatment of insulin.

5.3 Results

Initially, the concentrations of amino acids and organic acids in untreated and ARV-treated cell culture supernatants (followed by insulin stimulation) were compared to those in the basal culture medium (control). Changes in the amino acid and organic acid levels were analysed using the Student's t-test.

A total of 28 amino acids and organic acids derivatives were detected in untreated and ARVs (PI and NNRTI) treated cell culture supernatants before and after insulin stimulation, as mentioned in table 5.1. In two different sets of experiments the cells were treated with two PIs (indinavir and nelfinavir) and two NRTIs (stavudine and tenofovir).

Table 5.1: Amino acids and organic acids identified in the drug-treated or untreated cell culture supernatants using ¹H-NMR. (CHO-IR cells were left untreated or treated with PI and NRTIs for 16 h, followed by insulin stimulation for 5 mins in serum starved media).

Table5.1 : ^{1}H chemical shifts of metabolites		
Metabolite	**Abbr**	**^{1}H (ppm)**
Acetate	Ace	1.91
Acetoacetate	AcAc	2.27, 3.42
Alanine	Ala	1.48, 3.78
Arginine	Arg	1.65, 1.71, 1.90, 3.25, 3.76
Asparagine	Asp	2.85, 2.94, 3.99
Butyrate-n	Byt	0.88
Formate	For	8.45
Glucose	Glcs	3.24, 3.41, 3.47, 3.53, 3.74, 3.84, 3.89, 4.65, 5.23
Glutamate	Glu	2.06, 2.13, 2.35, 3.76
Glutamine	Gln	2.13, 2.45, 3.77
Histidine	His	3.14, 3.25, 3.98, 7.05, 7.80
Isoleucine	Ile	0.95, 1.01, 1.26, 1.45, 1.98, 2.06, 3.67
Lactate	Lac	1.33, 4.11
Leucine	Leu	0.96,1.71, 3.74
Lysine	Lys	1.46, 1.71, 1.89, 3.02, 3.74
Methionine	Met	2.14, 2.18, 2.64, 3.86
N-Acetyl-aspartate	NAc-asp	2.01
N-Acetyl-amino acid	NAc-aa	2.07
2-oxoisovalerate	Oxv	1.09, 3.00
Phenylalanine	Phe	3.13, 3.29, 4.03, 7.33, 7.37, 7.43
Pyruvate	Pyr	2.37
Threonine	Thr	1.32, 3.57, 4.24
Tryptophan	Trp	3.03, 3.49, 4.06, 7.19, 7.28, 7.31, 7.54, 7.74
Tyrosine	Tyr	3.05, 3.20, 3.94, 6.89, 7.19
Valine	Val	0.99, 1.04, 2.27, 3.60
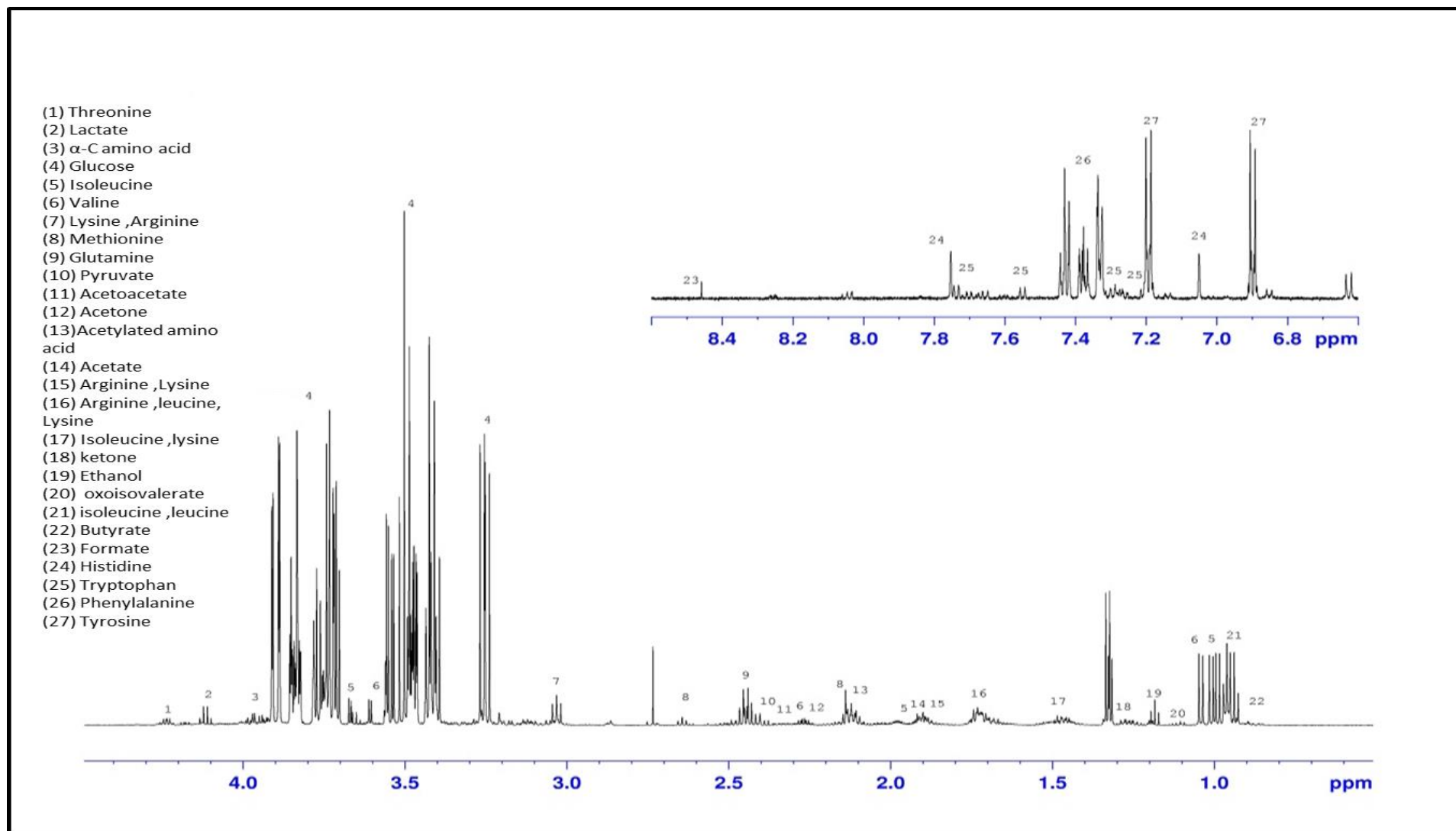
Figure 5.1: A typical representation of spectra generated from 600-MHz 1H NMR spectra of cell supernatants obtained from the CHO-IR cells control, serum starved for 16 hours. Metabolites identified and quantified on the spectrum are listed in the figure with their position on the spectrum.

5.3.1 Amino acid and organic acid analysis after protease inhibitors treatment in CHO-IR cells supernatant

The quantities of different metabolites (amino acids and organic acids) were determined in CHO-IR cells supernatants after treatment for 16 hours with PIs (nelfinavir and indinavir) (table 5.2). In the first analysis, statistical differences in amino acids in untreated and indinavir-treated supernatants of CHO-IR cells with and without insulin were analysed. A significant difference in two metabolites formate ($p = 0.034$) and glutamate ($p = 0.017$) were found. Formate was significantly reduced in indinavir treated cells as compared to the untreated cell supernatants. While glutamine was found in increase concentration in indinavir treated cells followed by insulin as compared to insulin-stimulated cell supernatants alone (see Figure 5.2).

In the other experiment, when nelfinavir-treated cell supernatants were compared with basal level cell supernatant (control), two metabolites were significantly reduced histidine ($p=0.038$) and 2-oxo-isovalerate ($p=0.020$) similarly when nelfinavir treated cell supernatants (with insulin) were compared with insulin stimulated (alone) cell supernatants, metabolites glutamate ($p=0.044$), glutamine ($p=0.0205$) and butyrate ($p=0.008$) were significantly decreased in the nelfinavir treated cell supernatants with insulin as compared to the inulin alone. Only two metabolites lactate ($p=0.039$) and tyrosine ($p=0.009$) increased significantly in supernatants with nelfinavir treated cell supernatants with inulin as compared to insulin alone. Similar increasing trend in lactate was also observed in the nelfinavir treated cell supernatants without insulin as compared to control (basal level) was (see in figure 5.3). A few other metabolites also showed some trends in either increasing or decreasing in levels which are shown in Table 5.2. The levels of amino acids and organic acids are reported as fold change after comparing drug treated with untreated supernatants (without insulin) and then followed by insulin stimulation in the supernatants (with and without drug) (Figures 5.2 and5.3) .

Table 5.2: List of the metabolites showing significant changes and trends in their concentration after protease inhibitor treatment in CHO-IR cell supernatant with and without insulin stimulation.

Metabolite	Nelfinavir Fold change		Indinavir Fold change	
	Control/Nelfianvir	Insulin / Nelfinavir + Insulin	Control / Indinavir	Insulin /Indinavir + Insulin
Acetate	1.3	1.00		
Acetoacetate		0.95		
Alanine	0.74	0.62		
Butyrate		0.34*		
Formate	0.72	0.33	0.89*	
Glucose	0.93	0.93	0.91	
Glutamate-l	0.68	0.33*	0.62	1.10*
Glutamine-l		1.08*		
Histidine	0.83*	0.91		
Isoleucine				0.83
Ketones	0.79	1.18		
Lactate	2.02	2.11*		
Lysine + Arginine			0.79	0.87
Oxoisovalerate	0.28	0.24	0.76	0.97
2-Oxo-isovalerate	0.47*	0.47	0.84	
Phenylalanine			0.79	
Threonine		0.75	0.67	
Tryptophan	1.14			
Tyrosine		1.01*		
158ppm(ukn)			1.21	1.21

Figure 5.2.Fold change in amino acid and organic acid levels of untreated and drug-treated cell culture supernatants, before and after insulin stimulation. Blue: Indinavir treated and untreated without insulin .Red: insulin stimulated (alone) compared with insulin stimulated + indinavir treated

 CHO-IR cells were treated with indinavir (50 μM) for 16 h followed by insulin stimulation. Cell culture supernatants were harvested for amino acid and organic acid analysis using ^{1}H NMR. Peak areas of organic acids and derivatives in the untreated and treated cell culture supernatants were subtracted from those in the basal culture medium. Amino acid and organic acid analysis is from three independent experiments. Data are shown as the mean fold change (n=3). *p < 0.05, using paired *t*-test, untreated or treated cell culture supernatants versus basal culture medium and insulin treated culture medium. For details of amino acids organic acids (1-28), please refer to Table 5.2.
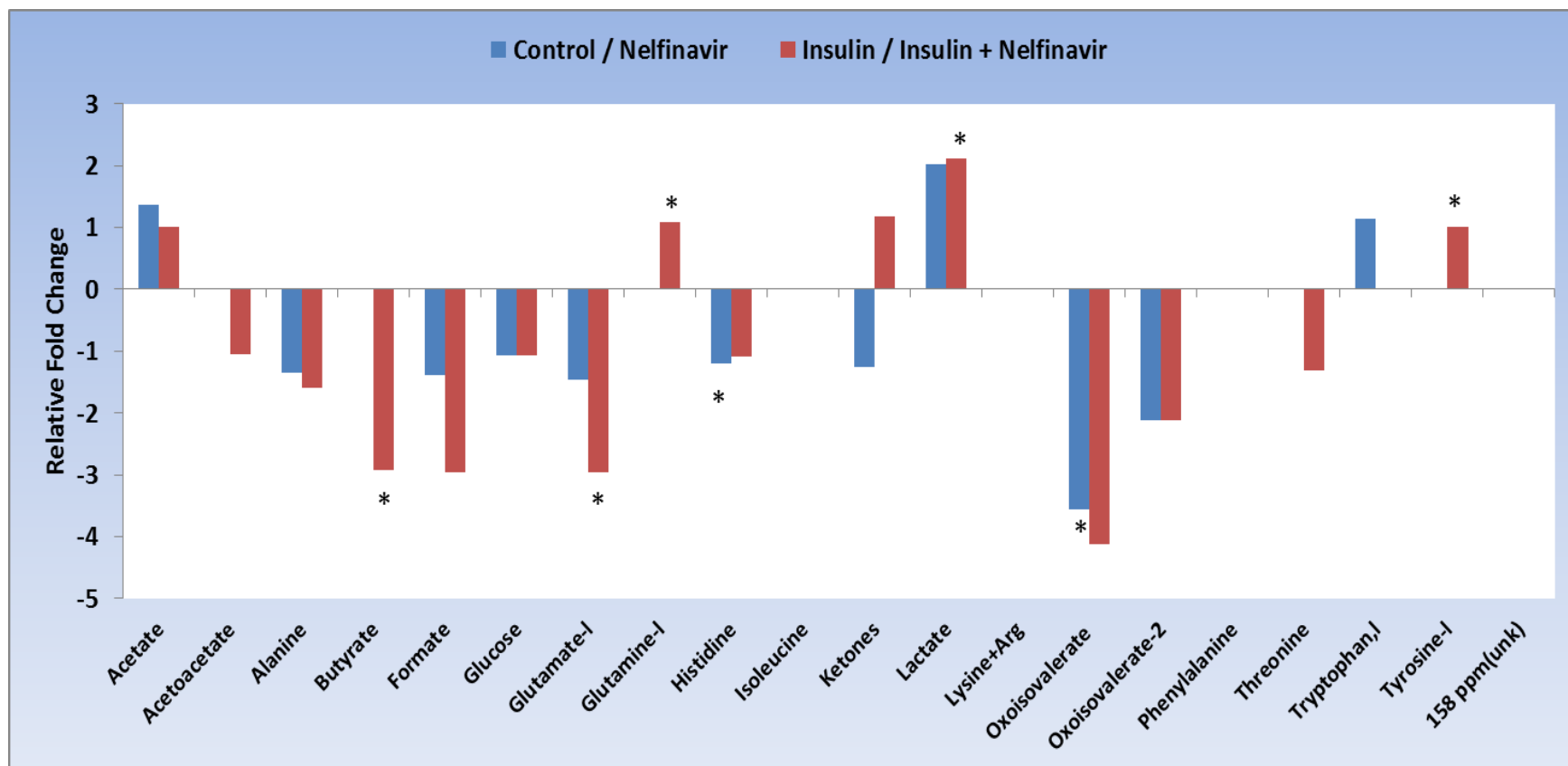
Figure 5.3.Fold change in amino acid and organic acid levels of untreated and drug-treated cell culture supernatants with and without insulin stimulation cell culture supernatants .Blue: Nelfinavir treated and untreated without insulin .Red: Insulin stimulated (alone) compared with insulin stimulated + nelfinavir treated. CHO-IR cells were treated with nelfinavir (30µM) for 16 h followed by insulin stimulation. Cell culture supernatants were harvested for amino acid and organic acid analysis using ^{1}H NMR. Peak areas of organic acids and derivatives in the untreated and treated cell culture supernatants were subtracted from those in the basal culture medium. Amino acid and organic acid analysis is from three independent experiments. Data are shown as the mean fold change (n=3). *$p < 0.05$, using paired *t*-test, untreated or treated cell culture supernatants versus basal culture medium and insulin treated culture medium. For details of amino acids organic acids (1-28), please see Table 5.2.

5.3.2 Amino acid and organic acid analysis in CHO-IR cells supernatant after treatment with NRTIs with and without insulin

The quantities of different metabolites (amino acids and organic acids) were determined in CHO-IR cells supernatants after treatment for 16 h with NRTIs (stavudine and tenofovir). Following treatment with stavudine (without insulin), the levels of methionine (p=0.006) phenylalanine (p=0.058) and tryptophan (p=0.0357) were significantly reduced in the supernatants of the treated cells, whilst only one metabolite acetone (p=0.014 was significantly increased in the supernatant. Similarly when metabolite levels were determined in stavudine treated and untreated supernatants followed by insulin stimulation, the levels of acetate (p=0.019), glucose (p=0.021) and histidine (p=0.017) were significantly reduced in the stavudine treated supernatants as shown in table 5.3. Few other metabolites also showed some trend in the levels (non-significant) also mentioned in the Table 5.3 and presented in Figure 5.4. The significant decrease in acetoacetate (p=0.0349) was found in tenofovir treated cell supernatants followed by insulin stimulation (see in Figure 5.5) .The levels of amino acids and organic acid are presented in fold change and parts per million (ppm) in the drug treated and untreated supernatants (without insulin) and then followed by insulin stimulation in the supernatants shown in Table 5.3.

Table 5.3: List of the metabolites altered significantly or expressed trends in CHO-IR cells supernatants after NRTIs treatment with and without insulin (Fold change in chemical shift of in major metabolites).

Metabolite	Fold change in Stavudine		Fold change in Tenofovir	
	Control/Stavudine	Insulin / Stavudine + Insulin	Control / Tenofovir	Insulin / Tenofovir + Insulin
Acetate	0.89	0.61*		
Acetoacetate			1.0	0.91*
Acetone	2.06*			
Alanine				1.0
Butyrate		0.90		
Formate		1.04		
Glucose		0.93*		
Glutamate-l		1.2		1.11
Glutamine-l	0.81	1.0		
Histidine		0.80*		
Isoleucine + Leucine		0.52		
Isoleucine		0.98		
Lactate		1.0		
Leucine-d	0.97			
Lysine + Arginine	0.77	1.20		
Methionine	0.18*			
2-oxoisovalerate	0.88		1.0	1.4
Phenylalanine	0.73*	0.87		
Tryptophan	0.86*	0.81	0.762	0.79
Tyrosine-l		1.03		
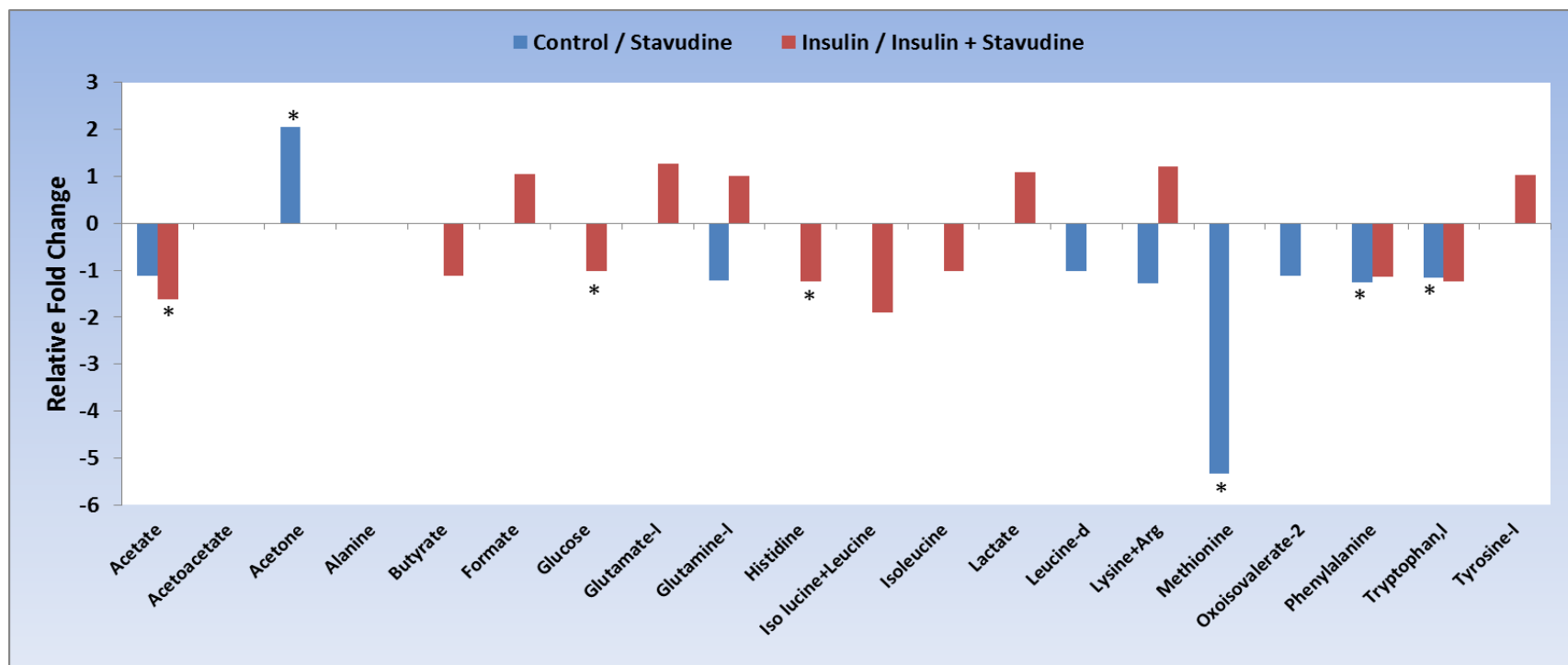
Figure 5.4.Fold change in amino acid and organic acid levels of untreated and drug-treated cell culture supernatants with and without insulin stimulation. Blue: Stavudine treated and untreated without insulin .Red: Insulin stimulated compared with insulin stimulated + Stavudine treated. CHO-IR cells were treated with nelfinavir (5μM) for 16 h followed by insulin stimulation. Cell culture supernatants were harvested for amino acid and organic acid analysis using ^{1}H NMR. Peak areas of organic acids and derivatives in the untreated and treated cell culture supernatants were subtracted from those in the basal culture medium. Amino acid and organic acid analysis is from three independent experiments. Data are shown as the mean fold change (n=3). *p < 0.05, using paired *t*-test, untreated or treated cell culture supernatants versus basal culture medium and insulin treated culture medium. For details of amino acids organic acids (1-28), see Table 5.3.
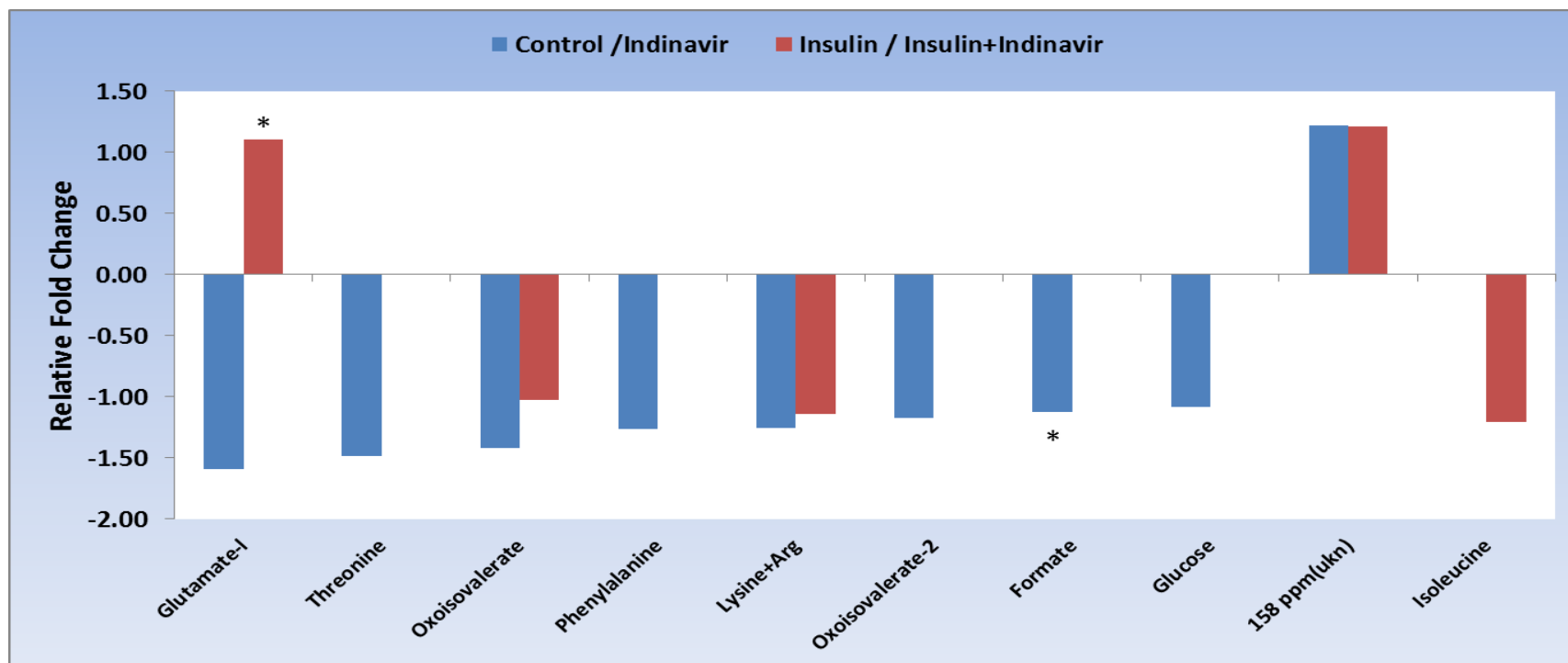
Figure 5.5.Fold change in amino acid and organic acid levels of untreated and drug-treated cell culture supernatants with and without insulin stimulation. Blue: Tenofovir treated and untreated without insulin .Red insulin stimulated compared with insulin stimulated + Tenofovir treated. CHO-IR cells were treated with nelfinavir (5μM) for 16 h followed by insulin stimulation. Cell culture supernatants were harvested for amino acid and organic acid analysis using ^{1}H NMR. Peak areas of organic acids and derivatives in the untreated and treated cell culture supernatants were subtracted from those in the basal culture medium. Amino acid and organic acid analysis is from three independent experiments. Data are shown as the mean fold change (n=3). *p < 0.05, using paired *t*-test, untreated or treated cell culture supernatants versus basal culture medium and insulin treated culture medium. For details of amino acids organic acids (1-28), see Table 5.3.

5.4 Discussion

Cellular metabolism is crucial for the biological functions of higher animals. Any change in the cellular metabolism induced by protease inhibitors is likely to affect insulin action. For the first time CHO-IR cells were used in this study to evaluate the effects of protease inhibitors and NRTIs on amino acid and organic acid metabolism, using metabolomic analysis by ^{1}H-NMR. It is convenient to analyse metabolites in the supernatants secreted by the cells, as they require minimal sample preparation and require less cells compared to that of cell extracts. Any alteration in various cellular metabolisms under the influence of ARVs which result in change of released metabolites can be detected by analysing cell supernatant. Importantly, the number of comprehensive studies are limited that investigate cellular metabolites after treating with HIV protease inhibitors and NRTIs using sensitive techniques such as ^{1}H-NMR and GC-MS.

The reason for using CHO cells for this metabolic analysis was based on a recent study on metabolic flux estimation using NMR where metabolite balancing was used for flux estimation. Two methods (one dimensional proton NMR and 2-Dimensional (^{1}H,^{13}C) NMR correlation spectroscopy (COSY)) were used to determine the metabolic flux and showed good agreement. This study validated CHO cells as a simplified model and suggested CHO cells can routinely be used in bioprocess development experiments to estimate metabolic fluxes with much reduced analytical investment(Goudar et al., 2010).

Normally the synthesis of amino acids and organic acids with and without insulin stimulation was found to be higher in cell culture supernatants as compared to the drug treated supernatants. However, significant decrease and trends were observed in the amino acids and organic acids synthesis after the drug-treated cell supernatants.

In amino acid analysis, glutamate increased the most significantly among the amino acids, when comparing insulin stimulated alone with indinavir-treated cell culture supernatants followed by insulin

stimulation. An opposite trend was observed between untreated and indinavir-treated cells without insulin. Glutamate is the central amino acid in nitrogen metabolism and is involved in many cellular pathways.

Glutamate metabolism is also linked with other biochemical pathways such as aminotransferase reactions, ureagenesis, the tricarboxylic acid (TCA) cycle, γ-Amino butyric acid (GABA) synthesis, and glutathione synthesis. Two enzymes are very important in glutamate metabolism: one is glutamate dehydrogenase (GDH) (mitochondrial matrix enzyme responsible for conversion of glutmate to the α-ketoglutarate); the other one is glutaminase (catalyzes the hydrolysis of glutamine to glutamate and ammonia). A regulation link also exists between GDH and insulin secretion from the pancreatic β cells (Stanley, 2009). Glutaminase is the major enzyme of glutamine catabolism in liver cells and is also located within the mitochondria (Kalra & Brosnan, 1973) .

Oxidative deamination of glutamate by GDH supplies α-ketoglutarate (α-KG) to the TCA cycle and generates ATP. This increase in the ATP/ADP ratio triggers insulin release through the sulfonylurea receptor/potassium channel complex (Matschinsky FM, 1996). This step in cellular metabolism connects amino acid metabolism with carbohydrate metabolism (TCA) for energy production. (Karaca, Frigerio, & Maechler, 2011).

 Hyperinsulinism/hyperammonemia syndrome (HI/HA) is a rare congenital disorder associated with hyperinsulinism (HI) due to mutations of GDH (Stanley et al., 2000; Weinzimer et al., 1997; Zammarchi, Filippi, Novembre, & Donati, 1996). The children suffering from HI/HA express recurrent hypoglycemia due to inappropriate secretion of insulin. It is possible that the inhibition of these enzymes affecting glutamate levels induced by indinavir suggests possible links between protein and carbohydrate metabolism, which is often the case among those receiving ART.

Synthesis of lactate and tyrosine was significantly increased in cell culture supernatants treated with nelfinavir versus untreated supernatants, followed by insulin stimulation. Similar trends in lactate were

also noticed in the nelfinavir treated cell supernatants as compared to the untreated without insulin stimulation.

Lactate is continuously produced by the cells converting pyruvate to lactate through reaction catalysed by the enzyme lactate dehydrogenase (LDH) during normal metabolism and exercise. In cellular metabolism, lactate is known to decrease insulin-stimulated glycolysis and this effect precedes the effect of lactate to decrease insulin-stimulated glucose uptake in the skeletal muscle (Choi et al., 2002). The other enzyme pyruvate dehydrogenase complex (PDC) in several mammalian tissues is responsible for the conversion of pyruvate to acetyl Co-A. This enzyme complex exists both in an inactive phosphorylated form (pyruvate dehydrogenase phosphate) and an active unphosphorylated form (pyruvate dehydrogenase) (Harris et al., 1995; Patel & Korotchkina, 2006) However the regulation of this critical enzyme is controlled by insulin (S. I. Taylor & Jungas, 1974) . Overproduction of lactate and ketones in the nelfinavir treated cell supernatants may be as a result of the alteration in the activity of these enzymes.

The amino acid analysis of cells treated with nelfinavir with and without insulin showed high levels of glutamine and significantly reduced levels of glutamate in the cell supernatants. Glutamine is the most abundant non-essential amino acid in human plasma. It makes up almost 6% of all bound amino acids (Newsholme, Procopio, Lima, Pithon-Curi, & Curi, 2003). It functions as a precursor in the synthesis of purines, pyrimidines, glucosamine, and NAD. Glutamine is synthesized in the cell cytoplasm predominantly from glutamate and branched-chain amino acids. Glutamine increases glucose-stimulated insulin secretion via the metabolism of the gamma-glutamyl cycle, glutathione synthesis and mitochondrial function (Brennan et al., 2003). Moreover a unique metabolic pathway also exists in adipocytes responsible for insulin desensitization of the insulin-responsive GTS (glucose transport system). It involves an early step of pathway , the conversion of fructose 6-phosphate to glucosamine

6-phosphate via glutamine:fructose-6-phosphate amidotransferase (first rate-limiting enzyme of the hexosamine pathway) (Marshall, Bacote, & Traxinger, 1991).

A trend in decreasing concentrations of 2-oxo-isovalerate was also identified in cell culture supernatants treated with nelfinavir, indinavir and stavudine. However, this was not seen with tenofovir. This organic acid is derived from leucine catabolism,2-oxoisovalerate is a branched chain organic acid which is a precursor to leucine and valine synthesis. It is also a degradation product of valine. The enzyme dihydroxy-acid dehydratase catalyzes the fourth step in the biosynthesis of isoleucine and valine, through the dehydration of 2, 3-dihydroxy-isovaleic acid into oxo-isovalerate (http://www.hmdb.ca/). An underutilization of branched chain amino acids suggests a decrease in synthesis of 2-oxoisovalerate in the treated cell culture supernatants. However, the function of 2-oxoisovalerate is unclear but increased 2-oxoisovalerate has been found to be associated with certain diseases such as Reye's syndrome (A potential fatal disease mainly caused by liver dysfunction and which can cause hypoglycaemia and which is associated with aspirin use in children) (Trauner, Nyhan, & Sweetman, 1975).

Stavudine resulted in a significant decrease in the levels of acetate. Similar affect were also seen with the neutral amino acids levels such as methionine tryptophan and phenylalanine and basic amino acid histidine. Acetone was the only organic acid that significantly increased in the supernatants. Overproduction of acetone and ketone (in case on nelfinavir) may produce ketone bodies and is likely an indication of induced ketosis and ketoacidosis. Overproduction of ketone bodies is associated with insulin deficiency in type 1 diabetes mellitus (Laffel, 1999).

A trend of increasing acetate levels was observed in supernatants of nelfinavir-treated cells, whereas a decrease in acetate was observed with stavudine treatment. Plasma concentrations of acetate depends on endogenous sources, amino acids, fatty acids and glucose metabolism in non- ruminants (human and rats).

The high plasma concentration of acetate is associated with type 2 diabetes (Akanji, Ng, & Humphreys, 1988; Smith, Humphreys, & Hockaday, 1986; Todesco et al., 1993).Acetate and glucose concentration in the blood, are associated by a key enzyme [acetyl-coenzyme A (CoA) synthetase (ACAS)] of acetate metabolism that is regulated by insulin, which has been shown with *in vitro* models (Sone et al., 2002). Increase of acetate in the nelfinavir treated cells supernatants may be as a result of the alteration in the activity of this enzyme.

Both insulin and amino acids play a role in the activation of the mTOR/p70S6k pathway (Hara et al., 1998; Patti, Brambilla, Luzi, Landaker, & Kahn, 1998) but long term activation of the mTOR and 70S6k pathway by amino acid exposure decrease the IRS-1 dependent PI3 kinase activity. Furthermore, the same study also revealed that high concentration of tyrosine amino acid decreased in insulin-dependent glucose uptake in L6 skeletal muscle cells (Tremblay & Marette, 2001). In our study, a significant increase was observed in tyrosine levels in nelfinavir- treated cell supernatants followed by insulin stimulation. This increase in tyrosine may mediate the inhibition of insulin receptor signalling pathways in cells treated with antiretroviral protease inhibitors.

Specifically no significant changes were observed in the metabolite concentration after treatment with insulin as compared to the basal level(data not here). It may be due to fact that change in metabolite concentration require time. So it is suggested for the future work to use high concentration insulin (100ng) for long time exposure (possibly one hour) .These alterations in the metabolism as a result of protease inhibitors and NRTIs provide very basic information about the metabolite synthesis and utilization at the cellular level. As CHO cell are very rapidly growing cells compared to the differentiated adipocytes and it is difficult to treat these cells for longer periods of time. Moreover these cells are transfected with high levels of insulin receptors, which may result in the cells having abnormally high glucose related metabolic pathways that could mask the effects of possible more slow active protease inhibitors and NRTIs on different metabolitic pathways .Future experiments are

required that will include prolonged treatment of cells with drugs, with more than three replicates, and multiple drug combinations.

5.5 Conclusion

The application of metabolite analysis (Metabolite profiling with GC-MS and LC-MS) is expanding and it is developing into a very powerful analytical tool for analysing different health conditions. The results from this study suggest that HIV protease inhibitors and NRTIs affect different cellular metabolic pathways and induce alterations. Protease inhibitors are more potent and diverse in affecting metabolitic pathways as compared to NRTIs. Changes in metabolite such as lactate, glutamate, glutamine, acetone tyrosine and acetate strongly correlate with insulin resistance. However to confirm and develop a more detailed understanding, analyses of intracellular metabolites is needed. A comprehensive analysis of these affects of protease inhibitors and NRTIs on cellular metabolism may open new doors for drug improvement.

One drawback of NMR during quantitative analyses is that in some instances, an integration-of-defined region has to be applied where spectral peaks of more than one metabolite are being reported. Here even for well pronounced peaks, the information regarding the defined region is cannot be used to report metabolites in isolations because of underlying overlapping peaks of sometimes other less-well pronounced metabolites. However, in these cases this approach still allows for non-targeted analyses of the metabolic changes occurring.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

6.1 Major finding about the effects of protease inhibitors and nucleoside/nucleotide reverse transcriptase inhibitors on insulin signaling pathway

In the treatment of HIV/AIDS, protease inhibitor and nucleoside/ nucleotide analogue reverse transcriptase inhibitor drugs, are the major components of HAART, and cause various metabolic disorders in HIV patients. The primary side-effects induced by the drugs in HAART are insulin resistance and lipodystrophy. The precise mechanistic basis of insulin resistance and lipodystrophy remain largely unknown although there has been some progress in recent years.

A wide range of published data from the last decade suggests an increase in the development of metabolic side effects associated with HIV infection and anti-retroviral therapy (ART) (Hruz, 2011). Insulin resistance is the primary metabolic disorder in type 2 diabetes, obesity, metabolic syndrome and atherosclerotic cardiovascular disease (Meyer et al., 2002; Nolan et al., 1994; Savage et al., 2005).Previous studies have shown the involvement of PIs in inhibition of insulin-stimulated glucose uptake in the insulin signaling pathway (Murata et al., 2002; Rudich, Ben-Romano, Etzion, & Bashan, 2005). In these studies, the major focal points were PIs and their effect on the selected steps (proximal or distal) in the insulin signaling pathway. The inhibition of glucose uptake may occur at a proximal step, which has been observed in cases of insulin resistance in type 2 diabetes and obesity (Arner, Pollare, Lithell, & Livingston, 1987; Caro et al., 1987; Meyer et al., 2002; Nolan et al., 1994).

Furthermore, it has been also observed that many inducers of insulin resistance (ceramide oxidative stress and GH) also affect the downstream steps without altering insulin receptor activation (Rudich et al., 1998; Summers et al., 1998; Takano et al., 2001; C. N. Wang et al., 1998). These findings proposed

that rate limiting steps in insulin signaling pathway exist more distal to the insulin receptor and its immediate substrates.

The effect of PIs and NRTIs on the insulin dependent tyrosine phosphorylation of proximal end (IR-β and IRS-1) and distal end (Akt, MAPK and GSK-3α\β) were investigated using CHO-IR cells (transfected with high numbers of insulin receptor). This is the first time CHO-IR cells were used to study the effects of NRTIs on the insulin signaling pathways. CHO-IR cells have a high level of insulin receptor expression, which expedites sensitive detection of any alteration in the expression of signaling proteins as compared to 3T3-L1 adipocytes. This may have facilitated easier analysis of changes in the insulin receptor β-subunit than has been possible previously.

Three PIs, indinavir, nelfinavir and ritonavir were used in this study. Indinavir and nelfinavir treatment significantly reduced the insulin-stimulated phosphorylation of the IRβ, IRS-1, Akt and MAPK in CHO-IR cells. However phosphorylation of GSK-3α\β was not affected by the PIs. Ritonavir also decreased (not statistically significant) the phosphorylation of IR-β and IRS-1 but its effect to decrease MAPK was the same as by the other PIs.

Similarly two NRTIs, tenofovir and stavudine, were studied for the first time to investigate their role in inducing insulin resistance. No effect was observed on the insulin stimulated tyrosine phosphorylation of IRβ and IRS-1 but phosphorylation at MAPK and Akt was decreased by the both NRTIs. In order to understand the role of NFκ-B pathway in blocking phosphorylation of insulin receptor-β, IKK-16, selective inhibitor of IkB kinase (IKK) was used. Similarly two therapeutic compounds sodium salicylate and berberine chloride were used to reverse the side effects induced by protease inhibitors and NRTIs in CHO-IR cells.

The inhibition of NFκB activated IKK did not alter the impact of PIs on the inhibition of tyrosine phosphorylation of IR-β and IRS-1. It had a very limited effect on NFκB pathway induction of insulin resistance via IKK.

Similarly sodium salicylate and berberine reduced (but not significantly) the effects of PIs (indinavir and nelfinavir) on IR-β and IRS-1. In order to block the affects of PIs and NRTIs on the latter steps of insulin signaling, cells pre-treated with sodium salicylate and berberine chloride were used but no significant change was observed. These findings suggest that PIs induce insulin resistance by affecting multiple steps in the signaling pathway. At the proximal end of the insulin signalling pathway, protease inhibitors affect IR-β and IRS-1 while at the distal end they affect phosphorylation of Akt and MAPK while NRTIs only affect distal end, Akt and MAPK of insulin signaling.

6.2 Metabolomic changes induced by antiretroviral drugs

For the first time CHO-IR cells were used to analyse changes in metabolic profiles after treatment with protease inhibitors and NRTIs. ^{1}H-NMR was used to quantify the changes in amino acids and organic acids in the cell culture supernatants after treatment with PIs (indinavir and nelfinavir) and NRTIs (tenofovir and stavudine) in the presence and absence of insulin stimulation. In the amino acid analysis, it was found that indinavir significantly increased glutamate levels. Glutamate is the central amino acid in nitrogen metabolism and is involved in many cellular pathways. Similarly, indinavir also decreased amino acid such as threonine, 2-oxo-isovalerate, phenylalanine lysine arginine and isoleucine in cell supernatants. It is well established that branched chain amino acids (BCAA) particularly leucine are associated with insulin resistance via activating mTOR and S6K1 and serine phosphorylation of IRS-1(Krebs et al., 2007; Tremblay, Brule, et al., 2007; Tremblay, Lavigne, Jacques, & Marette, 2007; Um, D'Alessio, & Thomas, 2006).However in this study increase synthesis of glutamate maybe associated with increased BCAA catabolism. Furthermore glutamate accumulation may increase transamination of pyruvate to alanine (Newgard et al., 2009). The increasing trend in synthesis of alanine with glutamate was also observed in the tenofovir-treated cell supernatants after insulin stimulation. This increase in

alanine (gluconeogenic amino acid) may contribute in glucose intolerance in insulin resistance (Newgard et al., 2009).

Nelfinavir also caused many significant changes and trend in the metabolite concentrations. It appeared that nelfinavir caused more extensively effects on cellular metabolism when compared to indinavir. Lactate and tyrosine synthesis was significantly increased in cell culture supernatants treated with nelfinavir versus untreated supernatants, followed by insulin stimulation. Similar trend was also noticed in the lactate level in the nelfinavir treated without insulin. Also significant decrease in the synthesis of butyrate, glutamate, histidine and 2-oxo-isovalerate were observed in the nelfinavir treated supernatants. In the cell metabolism lactate is known to decrease insulin-stimulated glycolysis and this effect precedes the effect of lactate to decrease insulin-stimulated glucose uptake in the skeletal muscle with affecting the GLUT4 content of the cell (Choi et al., 2002). The other enzyme pyruvate dehydrogenase Complex (PDC) in several mammalian tissues exists responsible for conversion of pyruvate to acetyl Co-A. This enzyme complex exist both in an inactive phosphorylated form (pyruvate dehydrogenase phosphate) and an active un-phosphorylated form (pyruvate dehydrogenase) (Harris et al., 1995; Patel & Korotchkina, 2006) however the regulation of this critical enzyme is controlled by insulin (S. I. Taylor & Jungas, 1974) . Overproduction of in lactate levels and ketone in the nelfinavir treated cells supernatants may associate with alteration in the activity of these enzymes.

The activation of the mTOR/p70S6K pathway by insulin and amino acids is very important (Hara et al., 1998; Patti et al., 1998) but long term activation of the mTOR and 70S6k pathway by amino acid exposure decrease the IRS-1 dependent PI3 kinase activity. Furthermore, the same study also revealed that high concentration of tyrosine amino acid decreased in insulin-dependent glucose uptake in L6 skeletal muscle cells (Tremblay & Marette, 2001) .In our study, a significant increase was observed in tyrosine levels in nelfinavir-treated cell supernatants followed by insulin stimulation. This increase in

tyrosine may mediate the inhibition of insulin receptor signalling pathways in cells treated with antiretroviral protease inhibitors.

Stavudine treatment produced pronounced effects on many amino acids and organic acid .It caused significant decrease the synthesis of acetate, histidine, methionine, phenylalanine and tryptophan while significant increase in the acetone was observed in stavudine treated cell supernatants without insulin. The other NRTI, tenofovir induced a significant decrease in the acetoacetate followed by the insulin treatment but opposite trend were analysed without insulin. Similar patterns in gluconeogenic amino acid (aspartate, glutamine glycine histidine) were observed in the serum of zucker diabetic fatty (ZDF) rats (Wijekoon, Skinner, Brosnan, & Brosnan, 2004) which may correlate these alterations with insulin resistance.

6.3 Findings about lipoprotein lipase activity in CHO-IR cells supernatants.

CHO-IR cells were also used to measure LPL activity using a colorimetric method employing pNPB as substrate. LPL has been shown to catalyse the hydrolysis of short-chain fatty acyl esters such as tributyrin, p-nitrophenylacetate and pNPB *in vivo* (Shirai & Jackson, 1982). 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. In addition ritonavir also decreased the total LPL activity in the plasma (den Boer et al., 2006).

The results indicate that NRTIs did induce changes in LPL activity in the supernatant. Stavudine and emtricitabine significantly inhibited the LPL activity from the CHO-IR cells. 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 sulphate 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 inhibits LPL activity intracellularly as well as extracellularly. These 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.

Any alteration in the LPL activity is critical in the regulation lipid metabolism and may induce metabolic disorders such as hypertriglyceridaemia, chylomicronaemia, pancreatitis, atherosclerosis, coronary artery disease and lipodystrophy associated with insulin resistance and diabetes mellitus.

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. In addition ritonavir also decreased the total LPL activity in the plasma as described above (den Boer et al., 2006).The hydrolysis and release of lipids for storage from triglyceride-rich lipoproteins such as chylomicrons and very low density lipoproteins, and their subsequent storage in adipose tissue, cannot take place without lipoprotein lipase. Therefore regulation of LPL expression is crucial for normal lipid-lipoprotein homeostasis (Bijvoet et al., 1996; Brunzell, 1995; Mead & Ramji, 2002).

6.4 Conclusion

It is first basic study in which role of two groups of ARVs (PIs and NRTIs) were evaluated at molecular level to understand the cellular basis of different side effects in HIV infected patients as compared to previous studies which were only focusing the PIs only. Furthermore CHO-IR cells were used previously only for one drug (described in section 1.5.1 page 34) side effects evaluation but in this study cells were used for multiple drugs .Similarly in this study not only signalling pathway of insulin was evaluated but also the effects on cellular secreted enzyme (lipoprotein lipase) and metabolites were

also analysed. Both methods (NMR and p-NPB) which were used in this study also never been used before for such kind of studies.

Taken together, the data from this study suggests that PIs and NRTIs treatment inhibit insulin signaling pathways at several steps in CHO-IR cells. The NFkB pathway has a very limited involvement in the induction of insulin resistance. The effects of PIs and NRTIs in CHO-IR cells report that stavudine, emtricitabine, indinavir atazanavir sulphate and nelfinavir significantly decrease LPL activity. High level of glutamate, lactate, acetone and ketone and low levels glutamine, 2-oxo-isovalerate and histidine may be secondary effects of insulin resistance by PIs and NRTIs.

6.5 Future work

This study give a new cellular mode CHO-IR cells to evaluate different therapeutic compounds which can be used as possible reversing agents for the side effects induced by ARVs , insulin resistance and lipodystrophy. It's fast, consistent and specific in nature as compared to the other available cellular models such as adipocyte. Due to competent role in evaluating the side effects of ARVs, in future CHO-IR cells can also be used to evaluate the side effects of newly designed or developed ARV .

The present study suggests very limited involvement of NFκB pathway in induction of insulin resistance by protease inhibitors. Activation of mTOR, S6K1 and JNK need to be evaluated in the possible involvement in causing insulin resistance by protease inhibitor (Dann, Selvaraj, & Thomas, 2007; Hirosumi et al., 2002). In order to confirm this finding, the effects of protease inhibitors should be investigated in NFκB-knockout cells.

In addition, as the present study showed, it would be useful to perform metabolomic analysis with protease inhibitors and NRTIs for longer exposure and multiple repeat (more than 3) to confirm and identify maximum alterations in the metabolites. To get more insight about the effects of ARVs on

metabolism cellular metabolites need to be analysed and compared with the current supernatant metabolite data.

Similarly in HIV patients protease inhibitors and NRTIs are used in fixed dose combinations (Meintjes G et al., 2012)(according to the guidelines for the antiviral therapy by southern African society of clinicians) so for the future work it would be excellent approach to use different protease inhibitors and NRTIs in combinations to get more detailed picture of the side effects induced by the ARVs . The results may shed new light on drug development in insulin resistance induced by protease inhibitors and NRTIs.

REFERENCES

(SAHCS), S. A. H. c. s. (2008). post-expoure prophylaxis. *Southern African journal of HIV medicine, 9*, 36-45.

Adeva, M. M., Calvino, J., Souto, G., & Donapetry, C. (2012). Insulin resistance and the metabolism of branched-chain amino acids in humans. *Amino Acids, 43*(1), 171-181. doi: 10.1007/s00726-011-1088-7

Africa, S. S. (2011). Mid-year population estimates. (27 July 2011).

Agarwal, A. K., Fryns, J. P., Auchus, R. J., & Garg, A. (2003). Zinc metalloproteinase, ZMPSTE24, is mutated in mandibuloacral dysplasia. *Hum Mol Genet, 12*(16), 1995-2001.

Ahmed, Z., & Pillay, T. S. (2001). Functional effects of APS and SH2-B on insulin receptor signalling. *Biochem Soc Trans, 29*(Pt 4), 529-534.

AIDSinfo. (2011). Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents.

Akanji, A. O., Ng, L., & Humphreys, S. (1988). Plasma acetate levels in response to intravenous fat or glucose/insulin infusions in diabetic and non-diabetic subjects. *Clin Chim Acta, 178*(1), 85-94.

Albalat, A., Saera-Vila, A., Capilla, E., Gutierrez, J., Perez-Sanchez, J., & Navarro, I. (2007). Insulin regulation of lipoprotein lipase (LPL) activity and expression in gilthead sea bream (Sparus aurata). *Comp Biochem Physiol B Biochem Mol Biol, 148*(2), 151-159. doi: 10.1016/j.cbpb.2007.05.004

Albalat, A., Sanchez-Gurmaches, J., Gutierrez, J., & Navarro, I. (2006). Regulation of lipoprotein lipase activity in rainbow trout (Oncorhynchus mykiss) tissues. *Gen Comp Endocrinol, 146*(3), 226-235. doi: 10.1016/j.ygcen.2005.11.011

Alberti, K. G., Zimmet, P., & Shaw, J. (2006). Metabolic syndrome--a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet Med, 23*(5), 469-480. doi: 10.1111/j.1464-5491.2006.01858.x

Aldrovandi, G. M., Lindsey, J. C., Jacobson, D. L., Zadzilka, A., Sheeran, E., Moye, J., . . . Mulligan, K. (2009). Morphologic and metabolic abnormalities in vertically HIV-infected children and youth. *AIDS, 23*(6), 661-672. doi: 10.1097/QAD.0b013e3283269dfb

Algenstaedt, P., Daneshi, S., Schwarzloh, B., Hennigs, N., Hamann, A., Hansen-Algenstaedt, N., & Beisiegel, U. (2003). Therapeutic dose of HIV-1 protease inhibitor saquinavir does not permanently influence early insulin signaling. *Exp Clin Endocrinol Diabetes, 111*(8), 491-498. doi: 10.1055/s-2003-44709

Anuurad, E., Bremer, A., & Berglund, L. (2010). HIV protease inhibitors and obesity. *Curr Opin Endocrinol Diabetes Obes, 17*(5), 478-485. doi: 10.1097/MED.0b013e32833dde87

Aoki, T. T., Brennan, M. F., Fitzpatrick, G. F., & Knight, D. C. (1981). Leucine meal increases glutamine and total nitrogen release from forearm muscle. *J Clin Invest, 68*(6), 1522-1528.

Aoki, T. T., Brennan, M. F., Muller, W. A., Soeldner, J. S., Alpert, J. S., Saltz, S. B., . . . Cahill, G. F., Jr. (1976). Amino acid levels across normal forearm muscle and splanchnic bed after a protein meal. *Am J Clin Nutr, 29*(4), 340-350.

Arner, P., Pollare, T., Lithell, H., & Livingston, J. N. (1987). Defective insulin receptor tyrosine kinase in human skeletal muscle in obesity and type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia, 30*(6), 437-440.

Arvind, H., George, R., Raju, P., Ve, R. S., Mani, B., Kannan, P., & Vijaya, L. (2008). Neural rim characteristics of healthy South Indians: the Chennai Glaucoma Study. *Invest Ophthalmol Vis Sci, 49*(8), 3457-3464. doi: 10.1167/iovs.07-1210

Baccarini, M. (2005). Second nature: biological functions of the Raf-1 "kinase". *FEBS Lett, 579*(15), 3271-3277. doi: 10.1016/j.febslet.2005.03.024

Bacchetti, P., Gripshover, B., Grunfeld, C., Heymsfield, S., McCreath, H., Osmond, D., . . . Tien, P. (2005). Fat distribution in men with HIV infection. *J Acquir Immune Defic Syndr, 40*(2), 121-131.

Bastard, J. P., Caron, M., Vidal, H., Jan, V., Auclair, M., Vigouroux, C., . . . Capeau, J. (2002). Association between altered expression of adipogenic factor SREBP1 in lipoatrophic adipose tissue from HIV-1-infected patients and abnormal adipocyte differentiation and insulin resistance. *Lancet, 359*(9311), 1026-1031. doi: 10.1016/S0140-6736(02)08094-7

Behrens, G., Dejam, A., Schmidt, H., Balks, H. J., Brabant, G., Korner, T., . . . Schmidt, R. E. (1999). Impaired glucose tolerance, beta cell function and lipid metabolism in HIV patients under treatment with protease inhibitors. *AIDS, 13*(10), F63-70.

Behrens, G. M., Boerner, A. R., Weber, K., van den Hoff, J., Ockenga, J., Brabant, G., & Schmidt, R. E. (2002). Impaired glucose phosphorylation and transport in skeletal muscle cause insulin resistance in HIV-1-infected patients with lipodystrophy. *J Clin Invest, 110*(9), 1319-1327. doi: 10.1172/JCI15626

Bell, J. D., Brown, J. C., & Sadler, P. J. (1989). NMR studies of body fluids. *NMR Biomed, 2*(5-6), 246-256.

Ben-Romano, R., Rudich, A., Etzion, S., Potashnik, R., Kagan, E., Greenbaum, U., & Bashan, N. (2006). Nelfinavir induces adipocyte insulin resistance through the induction of oxidative stress: differential protective effect of antioxidant agents. *Antivir Ther, 11*(8), 1051-1060.

Ben-Romano, R., Rudich, A., Tirosh, A., Potashnik, R., Sasaoka, T., Riesenberg, K., . . . Bashan, N. (2004). Nelfinavir-induced insulin resistance is associated with impaired plasma membrane recruitment of the PI 3-kinase effectors Akt/PKB and PKC-zeta. *Diabetologia, 47*(6), 1107-1117. doi: 10.1007/s00125-004-1408-5

Ben-Romano, R., Rudich, A., Torok, D., Vanounou, S., Riesenberg, K., Schlaeffer, F., . . . Bashan, N. (2003). Agent and cell-type specificity in the induction of insulin resistance by HIV protease inhibitors. *AIDS, 17*(1), 23-32. doi: 10.1097/01.aids.0000042954.95433.b7

Bengtsson, G., Olivecrona, T., Hook, M., Riesenfeld, J., & Lindahl, U. (1980). Interaction of lipoprotein lipase with native and modified heparin-like polysaccharides. *Biochem J, 189*(3), 625-633.

Berg, A. H., Combs, T. P., Du, X., Brownlee, M., & Scherer, P. E. (2001). The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat Med, 7*(8), 947-953. doi: 10.1038/90992

Berger, E. A., Murphy, P. M., & Farber, J. M. (1999). Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol, 17*, 657-700. doi: 10.1146/annurev.immunol.17.1.657

Bhattacharya, S., Dey, D., & Roy, S. S. (2007). Molecular mechanism of insulin resistance. *J Biosci, 32*(2), 405-413.

Bictash, M., Ebbels, T. M., Chan, Q., Loo, R. L., Yap, I. K., Brown, I. J., . . . Elliott, P. (2010). Opening up the "Black Box": metabolic phenotyping and metabolome-wide association studies in epidemiology. *J Clin Epidemiol, 63*(9), 970-979. doi: 10.1016/j.jclinepi.2009.10.001

Bijvoet, S., Gagne, S. E., Moorjani, S., Gagne, C., Henderson, H. E., Fruchart, J. C., . . . Hayden, M. R. (1996). Alterations in plasma lipoproteins and apolipoproteins before the age of 40 in heterozygotes for lipoprotein lipase deficiency. *J Lipid Res, 37*(3), 640-650.

Blumer, R. M., van Vonderen, M. G., Sutinen, J., Hassink, E., Ackermans, M., van Agtmael, M. A., . . . Sauerwein, H. P. (2008). Zidovudine/lamivudine contributes to insulin resistance within 3 months of starting combination antiretroviral therapy. *AIDS, 22*(2), 227-236. doi: 10.1097/QAD.0b013e3282f33557

Braun, J. E., & Severson, D. L. (1992). Regulation of the synthesis, processing and translocation of lipoprotein lipase. *Biochem J, 287 (Pt 2)*, 337-347.

Brennan, L., Corless, M., Hewage, C., Malthouse, J. P., McClenaghan, N. H., Flatt, P. R., & Newsholme, P. (2003). 13C NMR analysis reveals a link between L-glutamine metabolism, D-glucose metabolism and gamma-glutamyl cycle activity in a clonal pancreatic beta-cell line. *Diabetologia, 46*(11), 1512-1521. doi: 10.1007/s00125-003-1184-7

Brinkman, K., Smeitink, J. A., Romijn, J. A., & Reiss, P. (1999). Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. *Lancet, 354*(9184), 1112-1115. doi: 10.1016/S0140-6736(99)06102-4

Brown, T. T., Tassiopoulos, K., Bosch, R. J., Shikuma, C., & McComsey, G. A. (2010). Association between systemic inflammation and incident diabetes in HIV-infected patients after initiation of antiretroviral therapy. *Diabetes Care, 33*(10), 2244-2249. doi: 10.2337/dc10-0633

Bruce, K. D., & Byrne, C. D. (2009). The metabolic syndrome: common origins of a multifactorial disorder. *Postgrad Med J, 85*(1009), 614-621. doi: 10.1136/pgmj.2008.078014

Brunetti, A., Manfioletti, G., Chiefari, E., Goldfine, I. D., & Foti, D. (2001). Transcriptional regulation of human insulin receptor gene by the high-mobility group protein HMGI(Y). *FASEB J, 15*(2), 492-500. doi: 10.1096/fj.00-0190com

Brunzell, J. (1995). Familial lipoprotein lipase deficiency and other causes of the chylomicronemia syndrome. *The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York,*, 1913–1932.

Butt, A. A., McGinnis, K., Rodriguez-Barradas, M. C., Crystal, S., Simberkoff, M., Goetz, M. B., . . . Justice, A. C. (2009). HIV infection and the risk of diabetes mellitus. *AIDS, 23*(10), 1227-1234. doi: 10.1097/QAD.0b013e32832bd7af

Calmy, A., Carey, D., Mallon, P. W., Wand, H., Law, M., Cooper, D. A., & Carr, A. (2008). Early changes in adipokine levels and baseline limb fat may predict HIV lipoatrophy over 2 years following initiation of antiretroviral therapy. *HIV Med, 9*(2), 101-110. doi: 10.1111/j.1468-1293.2007.00527.x

Cammalleri, C., & Germinario, R. J. (2003). The effects of protease inhibitors on basal and insulin-stimulated lipid metabolism, insulin binding, and signaling. *J Lipid Res, 44*(1), 103-108.

Camps, L., Reina, M., Llobera, M., Bengtsson-Olivecrona, G., Olivecrona, T., & Vilaro, S. (1991). Lipoprotein lipase in lungs, spleen, and liver: synthesis and distribution. *J Lipid Res, 32*(12), 1877-1888.

Camps, L., Reina, M., Llobera, M., Vilaro, S., & Olivecrona, T. (1990). Lipoprotein lipase: cellular origin and functional distribution. *Am J Physiol, 258*(4 Pt 1), C673-681.

Caro, J. F., Sinha, M. K., Raju, S. M., Ittoop, O., Pories, W. J., Flickinger, E. G., . . . Dohm, G. L. (1987). Insulin receptor kinase in human skeletal muscle from obese subjects with and without noninsulin dependent diabetes. *J Clin Invest, 79*(5), 1330-1337. doi: 10.1172/JCI112958

Caron-Debarle, M., Lagathu, C., Boccara, F., Vigouroux, C., & Capeau, J. (2010). HIV-associated lipodystrophy: from fat injury to premature aging. *Trends Mol Med, 16*(5), 218-229. doi: 10.1016/j.molmed.2010.03.002

Caron, M., Auclair, M., Sterlingot, H., Kornprobst, M., & Capeau, J. (2003). Some HIV protease inhibitors alter lamin A/C maturation and stability, SREBP-1 nuclear localization and adipocyte differentiation. *AIDS, 17*(17), 2437-2444. doi: 10.1097/01.aids.0000096898.73209.ff

Caron, M., Auclair, M., Vigouroux, C., Glorian, M., Forest, C., & Capeau, J. (2001). The HIV protease inhibitor indinavir impairs sterol regulatory element-binding protein-1 intranuclear localization, inhibits preadipocyte differentiation, and induces insulin resistance. *Diabetes, 50*(6), 1378-1388.

Carper, M. J., Cade, W. T., Cam, M., Zhang, S., Shalev, A., Yarasheski, K. E., & Ramanadham, S. (2008). HIV-protease inhibitors induce expression of suppressor of cytokine signaling-1 in insulin-sensitive tissues and promote insulin resistance and type 2 diabetes mellitus. *Am J Physiol Endocrinol Metab, 294*(3), E558-567. doi: 10.1152/ajpendo.00167.2007

Carr, A., Samaras, K., Burton, S., Law, M., Freund, J., Chisholm, D. J., & Cooper, D. A. (1998). A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. *AIDS, 12*(7), F51-58.

Carr, A., Samaras, K., Chisholm, D. J., & Cooper, D. A. (1998). Pathogenesis of HIV-1-protease inhibitor-associated peripheral lipodystrophy, hyperlipidaemia, and insulin resistance. *Lancet, 351*(9119), 1881-1883. doi: 10.1016/S0140-6736(98)03391-1

Carriere, A., Romeo, Y., Acosta-Jaquez, H. A., Moreau, J., Bonneil, E., Thibault, P., . . . Roux, P. P. (2011). ERK1/2 phosphorylate Raptor to promote Ras-dependent activation of mTOR complex 1 (mTORC1). *J Biol Chem, 286*(1), 567-577. doi: 10.1074/jbc.M110.159046

Carrington, M., Dean, M., Martin, M. P., & O'Brien, S. J. (1999). Genetics of HIV-1 infection: chemokine receptor CCR5 polymorphism and its consequences. *Hum Mol Genet, 8*(10), 1939-1945.

Carter, R. J., Wiener, J., Abrams, E. J., Farley, J., Nesheim, S., Palumbo, P., & Bulterys, M. (2006). Dyslipidemia among perinatally HIV-infected children enrolled in the PACTS-HOPE cohort, 1999-2004: a longitudinal analysis. *J Acquir Immune Defic Syndr, 41*(4), 453-460. doi: 10.1097/01.qai.0000218344.88304.db

Cawthorn, W. P., & Sethi, J. K. (2008). TNF-alpha and adipocyte biology. *FEBS Lett, 582*(1), 117-131. doi: 10.1016/j.febslet.2007.11.051

Cazzolli, R., Carpenter, L., Biden, T. J., & Schmitz-Peiffer, C. (2001). A role for protein phosphatase 2A-like activity, but not atypical protein kinase Czeta, in the inhibition of protein kinase B/Akt and glycogen synthesis by palmitate. *Diabetes, 50*(10), 2210-2218.

Chen, B. K., Gandhi, R. T., & Baltimore, D. (1996). CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1 involves independent activities of vpu, env, and nef. *J Virol, 70*(9), 6044-6053.

Chen, D., Misra, A., & Garg, A. (2002). Clinical review 153: Lipodystrophy in human immunodeficiency virus-infected patients. *J Clin Endocrinol Metab, 87*(11), 4845-4856.

Chene, G., Sterne, J. A., May, M., Costagliola, D., Ledergerber, B., Phillips, A. N., . . . Egger, M. E. (2003). Prognostic importance of initial response in HIV-1 infected patients starting potent antiretroviral therapy: analysis of prospective studies. *Lancet, 362*(9385), 679-686.

Cheng, Z., Tseng, Y., & White, M. F. (2010). Insulin signaling meets mitochondria in metabolism. *Trends Endocrinol Metab, 21*(10), 589-598. doi: 10.1016/j.tem.2010.06.005

Choi, C. S., Kim, Y. B., Lee, F. N., Zabolotny, J. M., Kahn, B. B., & Youn, J. H. (2002). Lactate induces insulin resistance in skeletal muscle by suppressing glycolysis and impairing insulin signaling. *Am J Physiol Endocrinol Metab, 283*(2), E233-240. doi: 10.1152/ajpendo.00557.2001

Cihlar, T., Birkus, G., Greenwalt, D. E., & Hitchcock, M. J. (2002). Tenofovir exhibits low cytotoxicity in various human cell types: comparison with other nucleoside reverse transcriptase inhibitors. *Antiviral Res, 54*(1), 37-45.

Cisar, L. A., Hoogewerf, A. J., Cupp, M., Rapport, C. A., & Bensadoun, A. (1989). Secretion and degradation of lipoprotein lipase in cultured adipocytes. Binding of lipoprotein lipase to membrane heparan sulfate proteoglycans is necessary for degradation. *J Biol Chem, 264*(3), 1767-1774.

Clotet, B., van der Valk, M., Negredo, E., & Reiss, P. (2003). Impact of nevirapine on lipid metabolism. *J Acquir Immune Defic Syndr, 34 Suppl 1*, S79-84.

Coffinier, C., Hudon, S. E., Farber, E. A., Chang, S. Y., Hrycyna, C. A., Young, S. G., & Fong, L. G. (2007). HIV protease inhibitors block the zinc metalloproteinase ZMPSTE24 and lead to an accumulation of prelamin A in cells. *Proc Natl Acad Sci U S A, 104*(33), 13432-13437. doi: 10.1073/pnas.0704212104

Cox, S. W., Aperia, K., Albert, J., & Wahren, B. (1994). Comparison of the sensitivities of primary isolates of HIV type 2 and HIV type 1 to antiviral drugs and drug combinations. *AIDS Res Hum Retroviruses, 10*(12), 1725-1729.

Craigie, R., Fujiwara, T., & Bushman, F. (1990). The IN protein of Moloney murine leukemia virus processes the viral DNA ends and accomplishes their integration in vitro. *Cell, 62*(4), 829-837.

Cryer, A. (1981). Tissue lipoprotein lipase activity and its action in lipoprotein metabolism. *Int J Biochem, 13*(5), 525-541.

Cui, G., Qin, X., Zhang, Y., Gong, Z., Ge, B., & Zang, Y. Q. (2009). Berberine differentially modulates the activities of ERK, p38 MAPK, and JNK to suppress Th17 and Th1 T cell differentiation in type 1 diabetic mice. *J Biol Chem, 284*(41), 28420-28429. doi: 10.1074/jbc.M109.012674

Cupp, M., Bensadoun, A., & Melford, K. (1987). Heparin decreases the degradation rate of lipoprotein lipase in adipocytes. *J Biol Chem, 262*(13), 6383-6388.

Danielsson, A., Ost, A., Lystedt, E., Kjolhede, P., Gustavsson, J., Nystrom, F. H., & Stralfors, P. (2005). Insulin resistance in human adipocytes occurs downstream of IRS1 after surgical cell isolation but at the level of phosphorylation of IRS1 in type 2 diabetes. *FEBS J, 272*(1), 141-151. doi: 10.1111/j.1432-1033.2004.04396.x

Dann, S. G., Selvaraj, A., & Thomas, G. (2007). mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. *Trends Mol Med, 13*(6), 252-259. doi: 10.1016/j.molmed.2007.04.002

De Clercq, E. (2009). Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. *Int J Antimicrob Agents, 33*(4), 307-320. doi: 10.1016/j.ijantimicag.2008.10.010

Debouck, C., T. A. Tomaszek Jr, L. A. Ivanoff and J.Culp. (1995). HIV protease. In: HIV. Biochemistry, molecular biology, and drug discovery.

DeFronzo, R. A., Tobin, J. D., & Andres, R. (1979). Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol, 237*(3), E214-223.

den Boer, M. A., Berbee, J. F., Reiss, P., van der Valk, M., Voshol, P. J., Kuipers, F., . . . Romijn, J. A. (2006). Ritonavir impairs lipoprotein lipase-mediated lipolysis and decreases uptake of fatty acids in adipose tissue. *Arterioscler Thromb Vasc Biol, 26*(1), 124-129. doi: 10.1161/01.ATV.0000194073.87647.10

DiGirolamo, M., Newby, F. D., & Lovejoy, J. (1992). Lactate production in adipose tissue: a regulated function with extra-adipose implications. *FASEB J, 6*(7), 2405-2412.

Doolittle, M. H., Ben-Zeev, O., Elovson, J., Martin, D., & Kirchgessner, T. G. (1990). The response of lipoprotein lipase to feeding and fasting. Evidence for posttranslational regulation. *J Biol Chem, 265*(8), 4570-4577.

Dousset, N., Negre, A., Salvayre, R., Rogalle, P., Dang, Q. Q., and Douste-Blazy, L. (1988). Use of a fluorescent radiolabeled triacylglycerol as a substrate for lipoprotein lipase and hepatic triglyceride lipase. *Lipids, 23,* , 605-608.

Dowell, P., Flexner, C., Kwiterovich, P. O., & Lane, M. D. (2000). Suppression of preadipocyte differentiation and promotion of adipocyte death by HIV protease inhibitors. *J Biol Chem, 275*(52), 41325-41332. doi: 10.1074/jbc.M006474200

Dresner, A., Laurent, D., Marcucci, M., Griffin, M. E., Dufour, S., Cline, G. W., . . . Shulman, G. I. (1999). Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest, 103*(2), 253-259. doi: 10.1172/JCI5001

Du, K., Herzig, S., Kulkarni, R. N., & Montminy, M. (2003). TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. *Science, 300*(5625), 1574-1577. doi: 10.1126/science.1079817

Dube, M. P., Edmondson-Melancon, H., Qian, D., Aqeel, R., Johnson, D., & Buchanan, T. A. (2001). Prospective evaluation of the effect of initiating indinavir-based therapy on insulin sensitivity and B-cell function in HIV-infected patients. *J Acquir Immune Defic Syndr, 27*(2), 130-134.

Dube, M. P., Johnson, D. L., Currier, J. S., & Leedom, J. M. (1997). Protease inhibitor-associated hyperglycaemia. *Lancet, 350*(9079), 713-714. doi: 10.1016/S0140-6736(05)63513-1

Dube, M. P., Komarow, L., Mulligan, K., Grinspoon, S. K., Parker, R. A., Robbins, G. K., . . . Tebas, P. (2007). Long-term body fat outcomes in antiretroviral-naive participants randomized to nelfinavir or efavirenz or both plus dual nucleosides. Dual X-ray absorptiometry results from A5005s, a substudy of Adult Clinical Trials Group 384. *J Acquir Immune Defic Syndr, 45*(5), 508-514. doi: 10.1097/QAI.0b013e3181142d26

Dube, M. P., Parker, R. A., Tebas, P., Grinspoon, S. K., Zackin, R. A., Robbins, G. K., . . . Mulligan, K. (2005). Glucose metabolism, lipid, and body fat changes in antiretroviral-naive subjects randomized to nelfinavir or efavirenz plus dual nucleosides. *AIDS, 19*(16), 1807-1818.

Dube, M. P., Stein, J. H., Aberg, J. A., Fichtenbaum, C. J., Gerber, J. G., Tashima, K. T., . . . Glesby, M. J. (2003). Guidelines for the evaluation and management of dyslipidemia in human immunodeficiency virus (HIV)-infected adults receiving antiretroviral therapy: recommendations of the HIV Medical Association of the Infectious Disease Society of America

and the Adult AIDS Clinical Trials Group. *Clin Infect Dis, 37*(5), 613-627. doi: 10.1086/378131

Dudley, M. N., Graham, K. K., Kaul, S., Geletko, S., Dunkle, L., Browne, M., & Mayer, K. (1992). Pharmacokinetics of stavudine in patients with AIDS or AIDS-related complex. *J Infect Dis, 166*(3), 480-485.

Dunn, W. B., Bailey, N. J., & Johnson, H. E. (2005). Measuring the metabolome: current analytical technologies. *Analyst, 130*(5), 606-625. doi: 10.1039/b418288j

Egger, M., May, M., Chene, G., Phillips, A. N., Ledergerber, B., Dabis, F., . . . Sterne, J. A. (2002). Prognosis of HIV-1-infected patients starting highly active antiretroviral therapy: a collaborative analysis of prospective studies. *Lancet, 360*(9327), 119-129.

Elia, M., & Livesey, G. (1983). Effects of ingested steak and infused leucine on forelimb metabolism in man and the fate of the carbon skeletons and amino groups of branched-chain amino acids. *Clin Sci (Lond), 64*(5), 517-526.

Enerback, S., & Gimble, J. M. (1993). Lipoprotein lipase gene expression: physiological regulators at the transcriptional and post-transcriptional level. *Biochim Biophys Acta, 1169*(2), 107-125.

Even, M. S., Sandusky, C. B., & Barnard, N. D. (2006). Serum-free hybridoma culture: ethical, scientific and safety considerations. *Trends Biotechnol, 24*(3), 105-108. doi: 10.1016/j.tibtech.2006.01.001

Falutz, J. (2011). HIV infection, body composition changes and related metabolic complications: contributing factors and evolving management strategies. *Curr Opin Clin Nutr Metab Care, 14*(3), 255-260. doi: 10.1097/MCO.0b013e3283457a8f

Feeney, E. R., & Mallon, P. W. (2010). Impact of mitochondrial toxicity of HIV-1 antiretroviral drugs on lipodystrophy and metabolic dysregulation. *Curr Pharm Des, 16*(30), 3339-3351.

Feeney, E. R., & Mallon, P. W. (2011). Insulin resistance in treated HIV infection. *Best Pract Res Clin Endocrinol Metab, 25*(3), 443-458. doi: 10.1016/j.beem.2010.11.002

Feeney ER, M. P. (2011). Insulin resistance in treated HIV infection. *Best Pract Res Clin Endocrinol Metab.*, 443-458.

Feingold, K. R., Krauss, R. M., Pang, M., Doerrler, W., Jensen, P., & Grunfeld, C. (1993). The hypertriglyceridemia of acquired immunodeficiency syndrome is associated with an increased prevalence of low density lipoprotein subclass pattern B. *J Clin Endocrinol Metab, 76*(6), 1423-1427.

Felig, P. (1975). Amino acid metabolism in man. *Annu Rev Biochem, 44*, 933-955. doi: 10.1146/annurev.bi.44.070175.004441

Fleischman, A., Johnsen, S., Systrom, D. M., Hrovat, M., Farrar, C. T., Frontera, W., . . . Grinspoon, S. K. (2007). Effects of a nucleoside reverse transcriptase inhibitor, stavudine, on glucose disposal and mitochondrial function in muscle of healthy adults. *Am J Physiol Endocrinol Metab, 292*(6), E1666-1673. doi: 10.1152/ajpendo.00550.2006

Fleischman, A., Shoelson, S. E., Bernier, R., & Goldfine, A. B. (2008). Salsalate improves glycemia and inflammatory parameters in obese young adults. *Diabetes Care, 31*(2), 289-294. doi: 10.2337/dc07-1338

Flint, S. J., L.W. Enquist, V.R. Racaniello, and A.M. Skalka. (2004). Prnciples of Virology. Molecular Biology, Pathogenesis, and Control of Animal Viruses.

Freed, E. O., & Martin, M. A. (1995). The role of human immunodeficiency virus type 1 envelope glycoproteins in virus infection. *J Biol Chem, 270*(41), 23883-23886.

Friedrich, N. (2012). Metabolomics in diabetes research. *J Endocrinol, 215*(1), 29-42. doi: 10.1530/JOE-12-0120

Friis-Moller, N., Sabin, C. A., Weber, R., d'Arminio Monforte, A., El-Sadr, W. M., Reiss, P., . . . Lundgren, J. D. (2003). Combination antiretroviral therapy and the risk of myocardial infarction. *N Engl J Med, 349*(21), 1993-2003. doi: 10.1056/NEJMoa030218

Friis-Moller, N., Weber, R., Reiss, P., Thiebaut, R., Kirk, O., d'Arminio Monforte, A., . . . Lundgren, J. D. (2003). Cardiovascular disease risk factors in HIV patients--association with antiretroviral therapy. Results from the DAD study. *AIDS, 17*(8), 1179-1193. doi: 10.1097/01.aids.0000060358.78202.c1

GA Lee, M. R., JM Schwarz, FT Aweeka and C Grunfeld (2005). A single dose of amprenavir does not induce insulin resistance in healthy normal volunteers. *Antivir Ther, Supplement 3:L11 (abstract no. 15)*

Galic, S., Oakhill, J. S., & Steinberg, G. R. (2010). Adipose tissue as an endocrine organ. *Mol Cell Endocrinol, 316*(2), 129-139. doi: 10.1016/j.mce.2009.08.018

Gan, S. K., Samaras, K., Thompson, C. H., Kraegen, E. W., Carr, A., Cooper, D. A., & Chisholm, D. J. (2002). Altered myocellular and abdominal fat partitioning predict disturbance in insulin action in HIV protease inhibitor-related lipodystrophy. *Diabetes, 51*(11), 3163-3169.

Gao, W. Y., Agbaria, R., Driscoll, J. S., & Mitsuya, H. (1994). Divergent anti-human immunodeficiency virus activity and anabolic phosphorylation of 2',3'-dideoxynucleoside analogs in resting and activated human cells. *J Biol Chem, 269*(17), 12633-12638.

Gao, Z., Zhang, X., Zuberi, A., Hwang, D., Quon, M. J., Lefevre, M., & Ye, J. (2004). Inhibition of insulin sensitivity by free fatty acids requires activation of multiple serine kinases in 3T3-L1 adipocytes. *Mol Endocrinol, 18*(8), 2024-2034. doi: 10.1210/me.2003-0383

Garg, A., & Misra, A. (2004). Lipodystrophies: rare disorders causing metabolic syndrome. *Endocrinol Metab Clin North Am, 33*(2), 305-331. doi: 10.1016/j.ecl.2004.03.003

Garrabou, G., Lopez, S., Moren, C., Martinez, E., Fontdevila, J., Cardellach, F., . . . Miro, O. (2011). Mitochondrial damage in adipose tissue of untreated HIV-infected patients. *AIDS, 25*(2), 165-170. doi: 10.1097/QAD.0b013e3283423219

Gerschenson, M., & Brinkman, K. (2004). Mitochondrial dysfunction in AIDS and its treatment. *Mitochondrion, 4*(5-6), 763-777. doi: 10.1016/j.mito.2004.07.025

Glass, C. K., & Witztum, J. L. (2001). Atherosclerosis. the road ahead. *Cell, 104*(4), 503-516.

Gotte, M., Li, X., & Wainberg, M. A. (1999). HIV-1 reverse transcription: a brief overview focused on structure-function relationships among molecules involved in initiation of the reaction. *Arch Biochem Biophys, 365*(2), 199-210. doi: 10.1006/abbi.1999.1209

Goudar, C., Biener, R., Boisart, C., Heidemann, R., Piret, J., de Graaf, A., & Konstantinov, K. (2010). Metabolic flux analysis of CHO cells in perfusion culture by metabolite balancing and 2D [13C, 1H] COSY NMR spectroscopy. *Metab Eng, 12*(2), 138-149. doi: 10.1016/j.ymben.2009.10.007

Gougeon, M. L., Penicaud, L., Fromenty, B., Leclercq, P., Viard, J. P., & Capeau, J. (2004). Adipocytes targets and actors in the pathogenesis of HIV-associated lipodystrophy and metabolic alterations. *Antivir Ther, 9*(2), 161-177.

Goulbourne, C. N., & Vaux, D. J. (2010). HIV protease inhibitors inhibit FACE1/ZMPSTE24: a mechanism for acquired lipodystrophy in patients on highly active antiretroviral therapy? *Biochem Soc Trans, 38*(Pt 1), 292-296. doi: 10.1042/BST0380292

Griffin, M. E., Marcucci, M. J., Cline, G. W., Bell, K., Barucci, N., Lee, D., . . . Shulman, G. I. (1999). Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes, 48*(6), 1270-1274.

Grinspoon, S., & Carr, A. (2005). Cardiovascular risk and body-fat abnormalities in HIV-infected adults. *N Engl J Med, 352*(1), 48-62. doi: 10.1056/NEJMra041811

Grob, P. M., Wu, J. C., Cohen, K. A., Ingraham, R. H., Shih, C. K., Hargrave, K. D., . . . Merluzzi, V. J. (1992). Nonnucleoside inhibitors of HIV-1 reverse transcriptase: nevirapine as a prototype drug. *AIDS Res Hum Retroviruses, 8*(2), 145-152.

Grunfeld, C., Kotler, D. P., Hamadeh, R., Tierney, A., Wang, J., & Pierson, R. N. (1989). Hypertriglyceridemia in the acquired immunodeficiency syndrome. *Am J Med, 86*(1), 27-31.

Grunfeld, C., Kotler, D. P., Shigenaga, J. K., Doerrler, W., Tierney, A., Wang, J., . . . Feingold, K. R. (1991). Circulating interferon-alpha levels and hypertriglyceridemia in the acquired immunodeficiency syndrome. *Am J Med, 90*(2), 154-162.

Grunfeld, C., Pang, M., Doerrler, W., Shigenaga, J. K., Jensen, P., & Feingold, K. R. (1992). Lipids, lipoproteins, triglyceride clearance, and cytokines in human immunodeficiency virus infection and the acquired immunodeficiency syndrome. *J Clin Endocrinol Metab, 74*(5), 1045-1052.

Gurunathan, S., Habib, R. E., Baglyos, L., Meric, C., Plotkin, S., Dodet, B., . . . Tartaglia, J. (2009). Use of predictive markers of HIV disease progression in vaccine trials. *Vaccine, 27*(14), 1997-2015. doi: 10.1016/j.vaccine.2009.01.039

Hadigan, C., Kamin, D., Liebau, J., Mazza, S., Barrow, S., Torriani, M., . . . Grinspoon, S. (2006). Depot-specific regulation of glucose uptake and insulin sensitivity in HIV-lipodystrophy. *Am J Physiol Endocrinol Metab, 290*(2), E289-298. doi: 10.1152/ajpendo.00273.2005

Hahn, B. H., Shaw, G. M., De Cock, K. M., & Sharp, P. M. (2000). AIDS as a zoonosis: scientific and public health implications. *Science, 287*(5453), 607-614.

Hammer, S. M., Eron, J. J., Jr., Reiss, P., Schooley, R. T., Thompson, M. A., Walmsley, S., . . . Volberding, P. A. (2008). Antiretroviral treatment of adult HIV infection: 2008 recommendations of the International AIDS Society-USA panel. *JAMA, 300*(5), 555-570. doi: 10.1001/jama.300.5.555

Hammer, S. M., Squires, K. E., Hughes, M. D., Grimes, J. M., Demeter, L. M., Currier, J. S., . . . Fischl, M. A. (1997). A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. *N Engl J Med, 337*(11), 725-733. doi: 10.1056/NEJM199709113371101

Hara, K., Yonezawa, K., Weng, Q. P., Kozlowski, M. T., Belham, C., & Avruch, J. (1998). Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem, 273*(23), 14484-14494.

Harris, R. A., Popov, K. M., Zhao, Y., Kedishvili, N. Y., Shimomura, Y., & Crabb, D. W. (1995). A new family of protein kinases--the mitochondrial protein kinases. *Adv Enzyme Regul, 35*, 147-162.

Haubrich, R. H., Riddler, S. A., DiRienzo, A. G., Komarow, L., Powderly, W. G., Klingman, K., . . . Havlir, D. V. (2009). Metabolic outcomes in a randomized trial of nucleoside, nonnucleoside and protease inhibitor-sparing regimens for initial HIV treatment. *AIDS, 23*(9), 1109-1118. doi: 10.1097/QAD.0b013e32832b4377

Haugaard, S. B., Andersen, O., Madsbad, S., Frosig, C., Iversen, J., Nielsen, J. O., & Wojtaszewski, J. F. (2005). Skeletal muscle insulin signaling defects downstream of phosphatidylinositol 3-kinase at the level of Akt are associated with impaired nonoxidative glucose disposal in HIV lipodystrophy. *Diabetes, 54*(12), 3474-3483.

He, Q., Engelson, E. S., Albu, J. B., Heymsfield, S. B., & Kotler, D. P. (2003). Preferential loss of omental-mesenteric fat during growth hormone therapy of HIV-associated lipodystrophy. *J Appl Physiol, 94*(5), 2051-2057. doi: 10.1152/japplphysiol.00845.2002

He, Q., Engelson, E. S., & Kotler, D. P. (2005). A comparison of abdominal subcutaneous adipose tissue pattern in obese and lean HIV-infected women. *J Nutr, 135*(1), 53-57.

Heald, A. E., Hsyu, P. H., Yuen, G. J., Robinson, P., Mydlow, P., & Bartlett, J. A. (1996). Pharmacokinetics of lamivudine in human immunodeficiency virus-infected patients with renal dysfunction. *Antimicrob Agents Chemother, 40*(6), 1514-1519.

Heath, K. V., Chan, K. J., Singer, J., O'Shaughnessy, M. V., Montaner, J. S., & Hogg, R. S. (2002). Incidence of morphological and lipid abnormalities: gender and treatment differentials after initiation of first antiretroviral therapy. *Int J Epidemiol, 31*(5), 1016-1020.

Hertel, J., Struthers, H., Horj, C. B., & Hruz, P. W. (2004). A structural basis for the acute effects of HIV protease inhibitors on GLUT4 intrinsic activity. *J Biol Chem, 279*(53), 55147-55152. doi: 10.1074/jbc.M410826200

Hirano, Y., Yoshida, M., Shimizu, M., & Sato, R. (2001). Direct demonstration of rapid degradation of nuclear sterol regulatory element-binding proteins by the ubiquitin-proteasome pathway. *J Biol Chem, 276*(39), 36431-36437. doi: 10.1074/jbc.M105200200

Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., . . . Hotamisligil, G. S. (2002). A central role for JNK in obesity and insulin resistance. *Nature, 420*(6913), 333-336. doi: 10.1038/nature01137

Hofstede, H. J., Burger, D. M., & Koopmans, P. P. (2003). Antiretroviral therapy in HIV patients: aspects of metabolic complications and mitochondrial toxicity. *Neth J Med, 61*(12), 393-403.

Hollywood, K., Brison, D. R., & Goodacre, R. (2006). Metabolomics: current technologies and future trends. *Proteomics, 6*(17), 4716-4723. doi: 10.1002/pmic.200600106

Hommes, M. J., Romijn, J. A., Godfried, M. H., Schattenkerk, J. K., Buurman, W. A., Endert, E., & Sauerwein, H. P. (1990). Increased resting energy expenditure in human immunodeficiency virus-infected men. *Metabolism, 39*(11), 1186-1190.

Horton, J. D., Goldstein, J. L., & Brown, M. S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest, 109*(9), 1125-1131. doi: 10.1172/JCI15593

Hotamisligil, G. S. (2010). Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell, 140*(6), 900-917. doi: 10.1016/j.cell.2010.02.034

Hruz, P. W. (2011). Molecular mechanisms for insulin resistance in treated HIV-infection. *Best Pract Res Clin Endocrinol Metab, 25*(3), 459-468. doi: 10.1016/j.beem.2010.10.017

Hruz, P. W., Murata, H., Qiu, H., & Mueckler, M. (2002). Indinavir induces acute and reversible peripheral insulin resistance in rats. *Diabetes, 51*(4), 937-942.

Hruz, P. W., & Yan, Q. (2006). Tipranavir without ritonavir does not acutely induce peripheral insulin resistance in a rodent model. *J Acquir Immune Defic Syndr, 43*(5), 624-625. doi: 10.1097/01.qai.0000245883.66509.b4

Hughes, C. A., Robinson, L., Tseng, A., & MacArthur, R. D. (2009). New antiretroviral drugs: a review of the efficacy, safety, pharmacokinetics, and resistance profile of tipranavir, darunavir, etravirine, rilpivirine, maraviroc, and raltegravir. *Expert Opin Pharmacother, 10*(15), 2445-2466. doi: 10.1517/14656560903176446

Iles, R. A., Hind, A. J., & Chalmers, R. A. (1985). Use of proton nuclear magnetic resonance spectroscopy in detection and study of organic acidurias. *Clin Chem, 31*(11), 1795-1801.

Ismail, W. I. W., King, J., and Pillay, T. S. (2009). Insulin resistance induced by antiretroviral drugs: Current understanding of molecular mechanisms. JEMDSA 14(3), 129-132. *JEMDSA, 14(3)*, 129-132.

Jain, R. G., Furfine, E. S., Pedneault, L., White, A. J., & Lenhard, J. M. (2001). Metabolic complications associated with antiretroviral therapy. *Antiviral Res, 51*(3), 151-177.

Jemsek, J. G., Arathoon, E., Arlotti, M., Perez, C., Sosa, N., Pokrovskiy, V., . . . Giordano, M. (2006). Body fat and other metabolic effects of atazanavir and efavirenz, each administered in combination with zidovudine plus lamivudine, in antiretroviral-naive HIV-infected patients. *Clin Infect Dis, 42*(2), 273-280. doi: 10.1086/498505

Jones, S. P., Janneh, O., Back, D. J., & Pirmohamed, M. (2005). Altered adipokine response in murine 3T3-F442A adipocytes treated with protease inhibitors and nucleoside reverse transcriptase inhibitors. *Antivir Ther, 10*(2), 207-213.

Jones, S. P., Qazi, N., Morelese, J., Lebrecht, D., Sutinen, J., Yki-Jarvinen, H., . . . Moyle, G. J. (2005). Assessment of adipokine expression and mitochondrial toxicity in HIV patients with lipoatrophy on stavudine- and zidovudine-containing regimens. *J Acquir Immune Defic Syndr, 40*(5), 565-572.

Kachko, I., Maissel, A., Mazor, L., Ben-Romano, R., Watson, R. T., Hou, J. C., . . . Rudich, A. (2009). Postreceptoral adipocyte insulin resistance induced by nelfinavir is caused by insensitivity of PKB/Akt to phosphatidylinositol-3,4,5-trisphosphate. *Endocrinology, 150*(6), 2618-2626. doi: 10.1210/en.2008-1205

Kalra, J., & Brosnan, J. T. (1973). Localization of glutaminase in rat liver. *FEBS Lett, 37*(2), 325-328.

Karaca, M., Frigerio, F., & Maechler, P. (2011). From pancreatic islets to central nervous system, the importance of glutamate dehydrogenase for the control of energy homeostasis. *Neurochem Int, 59*(4), 510-517. doi: 10.1016/j.neuint.2011.03.024

Katz, R. A., Merkel, G., Kulkosky, J., Leis, J., & Skalka, A. M. (1990). The avian retroviral IN protein is both necessary and sufficient for integrative recombination in vitro. *Cell, 63*(1), 87-95.

Katzmarzyk, P. T., Perusse, L., & Bouchard, C. (1999). Genetics of abdominal visceral fat levels. *Am J Hum Biol, 11*(2), 225-235. doi: 10.1002/(SICI)1520-6300(1999)11:2<225::AID-AJHB10>3.0.CO;2-J

Kelley, D. E., He, J., Menshikova, E. V., & Ritov, V. B. (2002). Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes, 51*(10), 2944-2950.

Kern, P. A., Mandic, A., & Eckel, R. H. (1987). Regulation of lipoprotein lipase by glucose in primary cultures of isolated human adipocytes. Relevance to hypertriglyceridemia of diabetes. *Diabetes, 36*(11), 1238-1245.

Kim, J. K., Fillmore, J. J., Chen, Y., Yu, C., Moore, I. K., Pypaert, M., . . . Shulman, G. I. (2001). Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. *Proc Natl Acad Sci U S A, 98*(13), 7522-7527. doi: 10.1073/pnas.121164498

Kim, R. J., Wilson, C. G., Wabitsch, M., Lazar, M. A., & Steppan, C. M. (2006). HIV protease inhibitor-specific alterations in human adipocyte differentiation and metabolism. *Obesity (Silver Spring), 14*(6), 994-1002. doi: 10.1038/oby.2006.114

Kim, Y. B., Nikoulina, S. E., Ciaraldi, T. P., Henry, R. R., & Kahn, B. B. (1999). Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. *J Clin Invest, 104*(6), 733-741. doi: 10.1172/JCI6928

Kino, T., De Martino, M. U., Charmandari, E., Ichijo, T., Outas, T., & Chrousos, G. P. (2005). HIV-1 accessory protein Vpr inhibits the effect of insulin on the Foxo subfamily of forkhead transcription factors by interfering with their binding to 14-3-3 proteins: potential clinical implications regarding the insulin resistance of HIV-1-infected patients. *Diabetes, 54*(1), 23-31.

Kino, T., Gragerov, A., Slobodskaya, O., Tsopanomichalou, M., Chrousos, G. P., & Pavlakis, G. N. (2002). Human immunodeficiency virus type 1 (HIV-1) accessory protein Vpr induces transcription of the HIV-1 and glucocorticoid-responsive promoters by binding directly to p300/CBP coactivators. *J Virol, 76*(19), 9724-9734.

Knutson, V. P. (2000). The release of lipoprotein lipase from 3T3-L1 adipocytes is regulated by microvessel endothelial cells in an insulin-dependent manner. *Endocrinology, 141*(2), 693-701.

Kong, W., Wei, J., Abidi, P., Lin, M., Inaba, S., Li, C., . . . Jiang, J. D. (2004). Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. *Nat Med, 10*(12), 1344-1351. doi: 10.1038/nm1135

Koska, J., Ortega, E., Bunt, J. C., Gasser, A., Impson, J., Hanson, R. L., . . . Krakoff, J. (2009). The effect of salsalate on insulin action and glucose tolerance in obese non-diabetic patients: results of a randomised double-blind placebo-controlled study. *Diabetologia, 52*(3), 385-393. doi: 10.1007/s00125-008-1239-x

Krebs, M., Brunmair, B., Brehm, A., Artwohl, M., Szendroedi, J., Nowotny, P., . . . Roden, M. (2007). The Mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. *Diabetes, 56*(6), 1600-1607. doi: 10.2337/db06-1016

Krssak, M., Falk Petersen, K., Dresner, A., DiPietro, L., Vogel, S. M., Rothman, D. L., . . . Shulman, G. I. (1999). Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia, 42*(1), 113-116. doi: 10.1007/s001250051123

Laffel, L. (1999). Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. *Diabetes Metab Res Rev, 15*(6), 412-426.

Lagathu, C., Eustace, B., Prot, M., Frantz, D., Gu, Y., Bastard, J. P., . . . Capeau, J. (2007). Some HIV antiretrovirals increase oxidative stress and alter chemokine, cytokine or adiponectin production in human adipocytes and macrophages. *Antivir Ther, 12*(4), 489-500.

Lalezari, J. P., Henry, K., O'Hearn, M., Montaner, J. S., Piliero, P. J., Trottier, B., . . . Salgo, M. (2003). Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. *N Engl J Med, 348*(22), 2175-2185. doi: 10.1056/NEJMoa035026

Lane, B. J., & Provost-Craig, M. A. (2000). Resting energy expenditure in asymptomatic HIV-infected females. *J Womens Health Gend Based Med, 9*(3), 321-327. doi: 10.1089/152460900318524

Langlais, P., Yi, Z., & Mandarino, L. J. (2011). The identification of raptor as a substrate for p44/42 MAPK. *Endocrinology, 152*(4), 1264-1273. doi: 10.1210/en.2010-1271

Large, V., Peroni, O., Letexier, D., Ray, H., & Beylot, M. (2004). Metabolism of lipids in human white adipocyte. *Diabetes Metab, 30*(4), 294-309.

Lau, C. W., Yao, X. Q., Chen, Z. Y., Ko, W. H., & Huang, Y. (2001). Cardiovascular actions of berberine. *Cardiovasc Drug Rev, 19*(3), 234-244.

Lawlor, M. A., & Alessi, D. R. (2001). PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci, 114*(Pt 16), 2903-2910.

Lazzarin, A., Clotet, B., Cooper, D., Reynes, J., Arasteh, K., Nelson, M., . . . Salgo, M. (2003). Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. *N Engl J Med, 348*(22), 2186-2195. doi: 10.1056/NEJMoa035211

Lee, G. A., Lo, J. C., Aweeka, F., Schwarz, J. M., Mulligan, K., Schambelan, M., & Grunfeld, C. (2006). Single-dose lopinavir-ritonavir acutely inhibits insulin-mediated glucose disposal in healthy volunteers. *Clin Infect Dis, 43*(5), 658-660. doi: 10.1086/505974

Lee GA, M. D., Lo JC, Schwarz JM, Aweeka FT, Mulligan M, Schambelan M, Grunfeld C. (2004). Ritonavir acutely induces insulin resistance in healthy normal volunteers. *Antivir Ther, 9*(6).

Lee, G. A., Seneviratne, T., Noor, M. A., Lo, J. C., Schwarz, J. M., Aweeka, F. T., . . . Grunfeld, C. (2004). The metabolic effects of lopinavir/ritonavir in HIV-negative men. *AIDS, 18*(4), 641-649.

Lee, S., Lim, H. J., Park, J. H., Lee, K. S., Jang, Y., & Park, H. Y. (2007). Berberine-induced LDLR up-regulation involves JNK pathway. *Biochem Biophys Res Commun, 362*(4), 853-857. doi: 10.1016/j.bbrc.2007.08.060

Lee, Y. S., Kim, W. S., Kim, K. H., Yoon, M. J., Cho, H. J., Shen, Y., . . . Kim, J. B. (2006). Berberine, a natural plant product, activates AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states. *Diabetes, 55*(8), 2256-2264. doi: 10.2337/db06-0006

Leng, Y., Karlsson, H. K., & Zierath, J. R. (2004). Insulin signaling defects in type 2 diabetes. *Rev Endocr Metab Disord, 5*(2), 111-117. doi: 10.1023/B:REMD.0000021432.84588.f6

Lenhard, J. M., Furfine, E. S., Jain, R. G., Ittoop, O., Orband-Miller, L. A., Blanchard, S. G., . . . Weiel, J. E. (2000). HIV protease inhibitors block adipogenesis and increase lipolysis in vitro. *Antiviral Res, 47*(2), 121-129.

Lenz, E. M., & Wilson, I. D. (2007). Analytical strategies in metabonomics. *J Proteome Res, 6*(2), 443-458. doi: 10.1021/pr0605217

Leow, M. K., Addy, C. L., & Mantzoros, C. S. (2003). Clinical review 159: Human immunodeficiency virus/highly active antiretroviral therapy-associated metabolic syndrome: clinical presentation, pathophysiology, and therapeutic strategies. *J Clin Endocrinol Metab, 88*(5), 1961-1976.

Lewis, W., Day, B. J., & Copeland, W. C. (2003). Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. *Nat Rev Drug Discov, 2*(10), 812-822. doi: 10.1038/nrd1201

Li, G., Barrett, E. J., Wang, H., Chai, W., & Liu, Z. (2005). Insulin at physiological concentrations selectively activates insulin but not insulin-like growth factor I (IGF-I) or insulin/IGF-I hybrid receptors in endothelial cells. *Endocrinology, 146*(11), 4690-4696. doi: 10.1210/en.2005-0505

Liang, J. S., Distler, O., Cooper, D. A., Jamil, H., Deckelbaum, R. J., Ginsberg, H. N., & Sturley, S. L. (2001). HIV protease inhibitors protect apolipoprotein B from degradation by the proteasome: a potential mechanism for protease inhibitor-induced hyperlipidemia. *Nat Med, 7*(12), 1327-1331. doi: 10.1038/nm1201-1327

Lohse, N., Hansen, A. B., Pedersen, G., Kronborg, G., Gerstoft, J., Sorensen, H. T., . . . Obel, N. (2007). Survival of persons with and without HIV infection in Denmark, 1995-2005. *Ann Intern Med, 146*(2), 87-95.

Louard, R. J., Fryburg, D. A., Gelfand, R. A., & Barrett, E. J. (1992). Insulin sensitivity of protein and glucose metabolism in human forearm skeletal muscle. *J Clin Invest, 90*(6), 2348-2354. doi: 10.1172/JCI116124

Lugassy, D. M., Farmer, B. M., & Nelson, L. S. (2010). Metabolic and hepatobiliary side effects of antiretroviral therapy (ART). *Emerg Med Clin North Am, 28*(2), 409-419, Table of Contents. doi: 10.1016/j.emc.2010.01.011

Lungile J. Sitole, A. A. W. a. D. M. (2012). Metabonomic analysis of HIV-infected biofluidsw. *Mol Biosyst*.

Luzi, L., Perseghin, G., Tambussi, G., Meneghini, E., Scifo, P., Pagliato, E., . . . Lazzarin, A. (2003). Intramyocellular lipid accumulation and reduced whole body lipid oxidation in HIV lipodystrophy. *Am J Physiol Endocrinol Metab, 284*(2), E274-280. doi: 10.1152/ajpendo.00391.2001

Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., & Pandolfi, P. P. (2005). Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell, 121*(2), 179-193. doi: 10.1016/j.cell.2005.02.031

Maagaard, A., & Kvale, D. (2009). Mitochondrial toxicity in HIV-infected patients both off and on antiretroviral treatment: a continuum or distinct underlying mechanisms? *J Antimicrob Chemother, 64*(5), 901-909. doi: 10.1093/jac/dkp316

Mallal, S. A., John, M., Moore, C. B., James, I. R., & McKinnon, E. J. (2000). Contribution of nucleoside analogue reverse transcriptase inhibitors to subcutaneous fat wasting in patients with HIV infection. *AIDS, 14*(10), 1309-1316.

Mallon, P. W., Miller, J., Cooper, D. A., & Carr, A. (2003). Prospective evaluation of the effects of antiretroviral therapy on body composition in HIV-1-infected men starting therapy. *AIDS, 17*(7), 971-979. doi: 10.1097/01.aids.0000060348.78202.74

Mantzoros, C. S. (2009). Whither recombinant human leptin treatment for HIV-associated lipoatrophy and the metabolic syndrome? *J Clin Endocrinol Metab, 94*(4), 1089-1091. doi: 10.1210/jc.2009-0340

Marshall, S., Bacote, V., & Traxinger, R. R. (1991). Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. *J Biol Chem, 266*(8), 4706-4712.

Martin, A., & Emery, S. (2009). Metabolic disorders and cardiovascular consequences of HIV infection and antiretroviral therapy. *Expert Rev Clin Pharmacol, 2*(4), 381-390. doi: 10.1586/ecp.09.11

Masuno, H., Schultz, C. J., Park, J. W., Blanchette-Mackie, E. J., Mateo, C., & Scow, R. O. (1991). Glycosylation, activity and secretion of lipoprotein lipase in cultured brown adipocytes of newborn mice. Effect of tunicamycin, monensin, 1-deoxymannojirimycin and swainsonine. *Biochem J, 277 (Pt 3)*, 801-809.

Matschinsky FM, S. I. (1996). Annotated questions about glucose metabolism and insulin secretion of beta-cells. . *Diabetes Rev 4*, 130–144.

McComsey, G. A., & Walker, U. A. (2004). Role of mitochondria in HIV lipoatrophy: insight into pathogenesis and potential therapies. *Mitochondrion, 4*(2-3), 111-118. doi: 10.1016/j.mito.2004.05.008

McComsey, G. A., Ward, D. J., Hessenthaler, S. M., Sension, M. G., Shalit, P., Lonergan, J. T., . . . Hernandez, J. E. (2004). Improvement in lipoatrophy associated with highly active antiretroviral therapy in human immunodeficiency virus-infected patients switched from stavudine to abacavir or zidovudine: the results of the TARHEEL study. *Clin Infect Dis, 38*(2), 263-270. doi: 10.1086/380790

Mead, J. R., Cryer, A., & Ramji, D. P. (1999). Lipoprotein lipase, a key role in atherosclerosis? *FEBS Lett, 462*(1-2), 1-6.

Mead, J. R., & Ramji, D. P. (2002). The pivotal role of lipoprotein lipase in atherosclerosis. *Cardiovasc Res, 55*(2), 261-269.

Meintjes G, M. G., Boulle A,, Conradie F, G. F., Hefer E, Johnson D,Mathe M, Moosa Y,, Osih R, R. T., Cutsem G,Variava E, Francois Venter,, & D., a. S. (2012). Guidelines for antiretroviral therapy in adults. *Southern African Journal of HIV Medicine, 13*(3).

Meloche, S., & Pouyssegur, J. (2007). The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. *Oncogene, 26*(22), 3227-3239. doi: 10.1038/sj.onc.1210414

Merluzzi, V. J., Hargrave, K. D., Labadia, M., Grozinger, K., Skoog, M., Wu, J. C., . . . et al. (1990). Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. *Science, 250*(4986), 1411-1413.

Meyer, M. M., Levin, K., Grimmsmann, T., Beck-Nielsen, H., & Klein, H. H. (2002). Insulin signalling in skeletal muscle of subjects with or without Type II-diabetes and first degree relatives of patients with the disease. *Diabetologia, 45*(6), 813-822. doi: 10.1007/s00125-002-0830-9

Minami, R., Yamamoto, M., Takahama, S., Ando, H., Miyamura, T., & Suematsu, E. (2011). Comparison of the influence of four classes of HIV antiretrovirals on adipogenic differentiation: the minimal effect of raltegravir and atazanavir. *J Infect Chemother, 17*(2), 183-188. doi: 10.1007/s10156-010-0101-5

Mitsuya, H., & Broder, S. (1986). Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. *Proc Natl Acad Sci U S A, 83*(6), 1911-1915.

Morino, K., Petersen, K. F., Dufour, S., Befroy, D., Frattini, J., Shatzkes, N., . . . Shulman, G. I. (2005). Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J Clin Invest, 115*(12), 3587-3593. doi: 10.1172/JCI25151

Morino, K., Petersen, K. F., & Shulman, G. I. (2006). Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. *Diabetes, 55 Suppl 2*, S9-S15. doi: 10.2337/diabetes.

Morino, K., Petersen, K. F., Sono, S., Choi, C. S., Samuel, V. T., Lin, A., . . . Shulman, G. I. (2012). Regulation of mitochondrial biogenesis by lipoprotein lipase in muscle of insulin-resistant offspring of parents with type 2 diabetes. *Diabetes, 61*(4), 877-887. doi: 10.2337/db11-1391

Mulligan, K., Khatami, H., Schwarz, J. M., Sakkas, G. K., DePaoli, A. M., Tai, V. W., . . . Schambelan, M. (2009). The effects of recombinant human leptin on visceral fat, dyslipidemia, and insulin resistance in patients with human immunodeficiency virus-associated lipoatrophy and hypoleptinemia. *J Clin Endocrinol Metab, 94*(4), 1137-1144. doi: 10.1210/jc.2008-1588

Mulligan, K., Parker, R. A., Komarow, L., Grinspoon, S. K., Tebas, P., Robbins, G. K., . . . Dube, M. P. (2006). Mixed patterns of changes in central and peripheral fat following initiation of antiretroviral therapy in a randomized trial. *J Acquir Immune Defic Syndr, 41*(5), 590-597. doi: 10.1097/01.qai.0000214811.72916.67

Murata, H., Hruz, P. W., & Mueckler, M. (2000). The mechanism of insulin resistance caused by HIV protease inhibitor therapy. *J Biol Chem, 275*(27), 20251-20254. doi: 10.1074/jbc.C000228200

Murata, H., Hruz, P. W., & Mueckler, M. (2002). Indinavir inhibits the glucose transporter isoform Glut4 at physiologic concentrations. *AIDS, 16*(6), 859-863.

Nagy, G. S., Tsiodras, S., Martin, L. D., Avihingsanon, A., Gavrila, A., Hsu, W. C., . . . Mantzoros, C. S. (2003). Human immunodeficiency virus type 1-related lipoatrophy and lipohypertrophy are associated with serum concentrations of leptin. *Clin Infect Dis, 36*(6), 795-802. doi: 10.1086/367859

Navia, M. A., Fitzgerald, P. M., McKeever, B. M., Leu, C. T., Heimbach, J. C., Herber, W. K., . . . Springer, J. P. (1989). Three-dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1. *Nature, 337*(6208), 615-620. doi: 10.1038/337615a0

Newgard, C. B., An, J., Bain, J. R., Muehlbauer, M. J., Stevens, R. D., Lien, L. F., . . . Svetkey, L. P. (2009). A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab, 9*(4), 311-326. doi: 10.1016/j.cmet.2009.02.002

Newsholme, P., Procopio, J., Lima, M. M., Pithon-Curi, T. C., & Curi, R. (2003). Glutamine and glutamate--their central role in cell metabolism and function. *Cell Biochem Funct, 21*(1), 1-9. doi: 10.1002/cbf.1003

Nicholson, J. K., Lindon, J. C., & Holmes, E. (1999). 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica, 29*(11), 1181-1189. doi: 10.1080/004982599238047

Nicholson, J. K., O'Flynn, M. P., Sadler, P. J., Macleod, A. F., Juul, S. M., & Sonksen, P. H. (1984). Proton-nuclear-magnetic-resonance studies of serum, plasma and urine from fasting normal and diabetic subjects. *Biochem J, 217*(2), 365-375.

Nilsson-Ehle, P., & Schotz, M. C. (1976). A stable, radioactive substrate emulsion for assay of lipoprotein lipase. *J Lipid Res, 17*(5), 536-541.

Nolan, J. J., Freidenberg, G., Henry, R., Reichart, D., & Olefsky, J. M. (1994). Role of human skeletal muscle insulin receptor kinase in the in vivo insulin resistance of noninsulin-dependent diabetes mellitus and obesity. *J Clin Endocrinol Metab, 78*(2), 471-477.

Noor, M. A., Flint, O. P., Maa, J. F., & Parker, R. A. (2006). Effects of atazanavir/ritonavir and lopinavir/ritonavir on glucose uptake and insulin sensitivity: demonstrable differences in vitro and clinically. *AIDS, 20*(14), 1813-1821. doi: 10.1097/01.aids.0000244200.11006.55

Noor, M. A., Lo, J. C., Mulligan, K., Schwarz, J. M., Halvorsen, R. A., Schambelan, M., & Grunfeld, C. (2001). Metabolic effects of indinavir in healthy HIV-seronegative men. *AIDS, 15*(7), F11-18.

Noor, M. A., Parker, R. A., O'Mara, E., Grasela, D. M., Currie, A., Hodder, S. L., . . . Haas, D. W. (2004). The effects of HIV protease inhibitors atazanavir and lopinavir/ritonavir on insulin sensitivity in HIV-seronegative healthy adults. *AIDS, 18*(16), 2137-2144.

Noor, M. A., Seneviratne, T., Aweeka, F. T., Lo, J. C., Schwarz, J. M., Mulligan, K., . . . Grunfeld, C. (2002). Indinavir acutely inhibits insulin-stimulated glucose disposal in humans: a randomized, placebo-controlled study. *AIDS, 16*(5), F1-8.

O'Neill, E. M., Rebay, I., Tjian, R., & Rubin, G. M. (1994). The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. *Cell, 78*(1), 137-147.

Ogihara, T., Asano, T., Katagiri, H., Sakoda, H., Anai, M., Shojima, N., . . . Fujita, T. (2004). Oxidative stress induces insulin resistance by activating the nuclear factor-kappa B pathway and disrupting normal subcellular distribution of phosphatidylinositol 3-kinase. *Diabetologia, 47*(5), 794-805. doi: 10.1007/s00125-004-1391-x

Olivecrona, T., Chernick, S. S., Bengtsson-Olivecrona, G., Garrison, M., & Scow, R. O. (1987). Synthesis and secretion of lipoprotein lipase in 3T3-L1 adipocytes. Demonstration of inactive forms of lipase in cells. *J Biol Chem, 262*(22), 10748-10759.

Oliver P. Flint, P., Mustafa A. Noor, MD, MSc2, Paul W. Hruz, MD, PhD3, Phil B. Hylemon,, PhD4, K. Y., PhD5, Donald P. Kotler, MD6, Rex A. Parker, PhD1, and Aouatef, & Bellamine, P. (2009). The Role of Protease Inhibitors in the Pathogenesis of HIVAssociated

Lipodystrophy: Cellular Mechanisms and Clinical

Implications. *Toxicol Pathol, 37*(1), 65–77.

Oliver, S. G., Winson, M. K., Kell, D. B., & Baganz, F. (1998). Systematic functional analysis of the yeast genome. *Trends Biotechnol, 16*(9), 373-378.

Ong, J. M., & Kern, P. A. (1989). The role of glucose and glycosylation in the regulation of lipoprotein lipase synthesis and secretion in rat adipocytes. *J Biol Chem, 264*(6), 3177-3182.

Ong, J. M., Kirchgessner, T. G., Schotz, M. C., & Kern, P. A. (1988). Insulin increases the synthetic rate and messenger RNA level of lipoprotein lipase in isolated rat adipocytes. *J Biol Chem, 263*(26), 12933-12938.

Opravil, M., Ledergerber, B., Furrer, H., Hirschel, B., Imhof, A., Gallant, S., . . . Weber, R. (2002). Clinical efficacy of early initiation of HAART in patients with asymptomatic HIV infection and CD4 cell count > 350 x 10(6) /l. *AIDS, 16*(10), 1371-1381.

Oral, E. A., & Chan, J. L. (2010). Rationale for leptin-replacement therapy for severe lipodystrophy. *Endocr Pract, 16*(2), 324-333. doi: 10.4158/EP09155.RA

Owens, D. M., & Keyse, S. M. (2007). Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene, 26*(22), 3203-3213. doi: 10.1038/sj.onc.1210412

Paik, I. J., & Kotler, D. P. (2011). The prevalence and pathogenesis of diabetes mellitus in treated HIV-infection. *Best Pract Res Clin Endocrinol Metab, 25*(3), 469-478. doi: 10.1016/j.beem.2011.04.003

Palacios, R., Galindo, M. J., Arranz, J. A., Lozano, F., Estrada, V., Rivero, A., . . . Santos, J. (2007). Cervical lipomatosis in HIV-infected patients: a case-control study. *HIV Med, 8*(1), 17-21. doi: 10.1111/j.1468-1293.2007.00421.x

Parker, R. A., Flint, O. P., Mulvey, R., Elosua, C., Wang, F., Fenderson, W., . . . Noor, M. A. (2005). Endoplasmic reticulum stress links dyslipidemia to inhibition of proteasome activity and glucose transport by HIV protease inhibitors. *Mol Pharmacol, 67*(6), 1909-1919. doi: 10.1124/mol.104.010165

Pascal, S., Resnick, L., Barker, W. W., Loewenstein, D., Yoshii, F., Chang, J. Y., . . . Duara, R. (1991). Metabolic asymmetries in asymptomatic HIV-1 seropositive subjects: relationship to disease onset and MRI findings. *J Nucl Med, 32*(9), 1725-1729.

Patel, M. S., & Korotchkina, L. G. (2006). Regulation of the pyruvate dehydrogenase complex. *Biochem Soc Trans, 34*(Pt 2), 217-222. doi: 10.1042/BST20060217

Patti, M. E., Brambilla, E., Luzi, L., Landaker, E. J., & Kahn, C. R. (1998). Bidirectional modulation of insulin action by amino acids. *J Clin Invest, 101*(7), 1519-1529. doi: 10.1172/JCI1326

Pendyala, G., & Fox, H. S. (2010). Proteomic and metabolomic strategies to investigate HIV-associated neurocognitive disorders. *Genome Med, 2*(3), 22. doi: 10.1186/gm143

Pendyala, G., Want, E. J., Webb, W., Siuzdak, G., & Fox, H. S. (2007). Biomarkers for neuroAIDS: the widening scope of metabolomics. *J Neuroimmune Pharmacol, 2*(1), 72-80. doi: 10.1007/s11481-006-9041-3

Perseghin, G., Scifo, P., De Cobelli, F., Pagliato, E., Battezzati, A., Arcelloni, C., . . . Luzi, L. (1999). Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes, 48*(8), 1600-1606.

Pessin, J. E., & Saltiel, A. R. (2000). Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest, 106*(2), 165-169. doi: 10.1172/JCI10582

Peterfy, M., Ben-Zeev, O., Mao, H. Z., Weissglas-Volkov, D., Aouizerat, B. E., Pullinger, C. R., . . . Doolittle, M. H. (2007). Mutations in LMF1 cause combined lipase deficiency and severe hypertriglyceridemia. *Nat Genet, 39*(12), 1483-1487. doi: 10.1038/ng.2007.24

Petit, J. M., Duong, M., Florentin, E., Duvillard, L., Chavanet, P., Brun, J. M., . . . Verges, B. (2003). Increased VLDL-apoB and IDL-apoB production rates in nonlipodystrophic HIV-infected patients on a protease inhibitor-containing regimen: a stable isotope kinetic study. *J Lipid Res, 44*(9), 1692-1697. doi: 10.1194/jlr.M300041-JLR200

Piacenti, F. J. (2006). An update and review of antiretroviral therapy. *Pharmacotherapy, 26*(8), 1111-1133. doi: 10.1592/phco.26.8.1111

Pillay, D., Bryant, M., Getman, D., & Richman, D. D. (1995). HIV-1 Protease inhibitors: Their development, mechanism of action and clinical potential.

Plotnikov, A., Zehorai, E., Procaccia, S., & Seger, R. (2011). The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochim Biophys Acta, 1813*(9), 1619-1633. doi: 10.1016/j.bbamcr.2010.12.012

Pneumocystis pneumonia--Los Angeles. (1981). *MMWR Morb Mortal Wkly Rep, 30*(21), 250-252.

Polo, R., Martinez, S., Madrigal, P., & Gonzalez-Munoz, M. (2003). Factors associated with mitochondrial dysfunction in circulating peripheral blood lymphocytes from HIV-infected people. *J Acquir Immune Defic Syndr, 34*(1), 32-36.

Powderly, W. (2004). The experts speak. Metabolic complications associated with HIV infection and antiretroviral therapy. Interview by Vicki Glaser. *AIDS Patient Care STDS, 18*(8), 431-435. doi: 10.1089/1087291041703638

Powell, D. J., Hajduch, E., Kular, G., & Hundal, H. S. (2003). Ceramide disables 3-phosphoinositide binding to the pleckstrin homology domain of protein kinase B (PKB)/Akt by a PKCzeta-dependent mechanism. *Mol Cell Biol, 23*(21), 7794-7808.

Pozefsky, T., Felig, P., Tobin, J. D., Soeldner, J. S., & Cahill, G. F., Jr. (1969). Amino acid balance across tissues of the forearm in postabsorptive man. Effects of insulin at two dose levels. *J Clin Invest, 48*(12), 2273-2282. doi: 10.1172/JCI106193

Pradines-Figueres, A., Vannier, C., & Ailhaud, G. (1988). Short-term stimulation by insulin of lipoprotein lipase secretion in adipose cells. *Biochem Biophys Res Commun, 154*(3), 982-990.

Prot, M., Heripret, L., Cardot-Leccia, N., Perrin, C., Aouadi, M., Lavrut, T., . . . Binetruy, B. (2006). Long-term treatment with lopinavir-ritonavir induces a reduction in peripheral adipose depots in mice. *Antimicrob Agents Chemother, 50*(12), 3998-4004. doi: 10.1128/AAC.00625-06

Purnell, J. Q., Zambon, A., Knopp, R. H., Pizzuti, D. J., Achari, R., Leonard, J. M., . . . Brunzell, J. D. (2000). Effect of ritonavir on lipids and post-heparin lipase activities in normal subjects. *AIDS, 14*(1), 51-57.

Qin, B., Anderson, R. A., & Adeli, K. (2008). Tumor necrosis factor-alpha directly stimulates the overproduction of hepatic apolipoprotein B100-containing VLDL via impairment of hepatic insulin signaling. *Am J Physiol Gastrointest Liver Physiol, 294*(5), G1120-1129. doi: 10.1152/ajpgi.00407.2007

Quinn, D., Shirai, K., & Jackson, R. L. (1983). Lipoprotein lipase: mechanism of action and role in lipoprotein metabolism. *Prog Lipid Res, 22*(1), 35-78.

Quinn, D. M., Shirai, K., Jackson, R. L., & Harmony, J. A. (1982). Lipoprotein lipase catalyzed hydrolysis of water-soluble p-nitrophenyl esters. Inhibition by apolipoprotein C-II. *Biochemistry, 21*(26), 6872-6879.

Rabe, K., Lehrke, M., Parhofer, K. G., & Broedl, U. C. (2008). Adipokines and insulin resistance. *Mol Med, 14*(11-12), 741-751. doi: 10.2119/2008-00058.Rabe

Randle, P. J. (1963). Endocrine control of metabolism. *Annu Rev Physiol, 25*, 291-324. doi: 10.1146/annurev.ph.25.030163.001451

Ranganathan, S., & Kern, P. A. (2002). The HIV protease inhibitor saquinavir impairs lipid metabolism and glucose transport in cultured adipocytes. *J Endocrinol, 172*(1), 155-162.

Raynolds, M. V., Awald, P. D., Gordon, D. F., Gutierrez-Hartmann, A., Rule, D. C., Wood, W. M., & Eckel, R. H. (1990). Lipoprotein lipase gene expression in rat adipocytes is regulated by isoproterenol and insulin through different mechanisms. *Mol Endocrinol, 4*(9), 1416-1422.

Reeds, D. N., Mittendorfer, B., Patterson, B. W., Powderly, W. G., Yarasheski, K. E., & Klein, S. (2003). Alterations in lipid kinetics in men with HIV-dyslipidemia. *Am J Physiol Endocrinol Metab, 285*(3), E490-497. doi: 10.1152/ajpendo.00118.2003

Report, M. a. M. W. (1981b). Kaposi's Sarcoma and Pneumocystis Pneumoniaa mong Homosexual Men New York City and California. *30*, 305–308.

Richman, D. D., Johnson, V. A., Mayers, D. M., Shirasaka, T., O'Brien, M. C., & Mitsuya, H. (2001). In vitro evaluation of experimental agents for anti-HIV activity. *Curr Protoc Immunol, Chapter 12*, Unit 12 19. doi: 10.1002/0471142735.im1209s08

Richmond, S. R., Carper, M. J., Lei, X., Zhang, S., Yarasheski, K. E., & Ramanadham, S. (2010). HIV-protease inhibitors suppress skeletal muscle fatty acid oxidation by reducing CD36 and CPT1

fatty acid transporters. *Biochim Biophys Acta, 1801*(5), 559-566. doi: 10.1016/j.bbalip.2010.01.007

Riddle, T. M., Kuhel, D. G., Woollett, L. A., Fichtenbaum, C. J., & Hui, D. Y. (2001). HIV protease inhibitor induces fatty acid and sterol biosynthesis in liver and adipose tissues due to the accumulation of activated sterol regulatory element-binding proteins in the nucleus. *J Biol Chem, 276*(40), 37514-37519. doi: 10.1074/jbc.M104557200

Riddler, S. A., Smit, E., Cole, S. R., Li, R., Chmiel, J. S., Dobs, A., . . . Kingsley, L. A. (2003). Impact of HIV infection and HAART on serum lipids in men. *JAMA, 289*(22), 2978-2982. doi: 10.1001/jama.289.22.2978

Rojas, C., Enerback, S., & Bengtsson-Olivecrona, G. (1990). Synthesis and secretion of active lipoprotein lipase in Chinese-hamster ovary (CHO) cells. *Biochem J, 271*(1), 11-15.

Ron, D., & Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol, 8*(7), 519-529. doi: 10.1038/nrm2199

Roux, P. P., & Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev, 68*(2), 320-344. doi: 10.1128/MMBR.68.2.320-344.2004

Roux, P. P., Shahbazian, D., Vu, H., Holz, M. K., Cohen, M. S., Taunton, J., . . . Blenis, J. (2007). RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *J Biol Chem, 282*(19), 14056-14064. doi: 10.1074/jbc.M700906200

Rubinfeld, H., & Seger, R. (2005). The ERK cascade: a prototype of MAPK signaling. *Mol Biotechnol, 31*(2), 151-174. doi: 10.1385/MB:31:2:151

Rudich, A., Ben-Romano, R., Etzion, S., & Bashan, N. (2005). Cellular mechanisms of insulin resistance, lipodystrophy and atherosclerosis induced by HIV protease inhibitors. *Acta Physiol Scand, 183*(1), 75-88. doi: 10.1111/j.1365-201X.2004.01383.x

Rudich, A., Tirosh, A., Potashnik, R., Hemi, R., Kanety, H., & Bashan, N. (1998). Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Diabetes, 47*(10), 1562-1569.

Safrin, S., & Grunfeld, C. (1999). Fat distribution and metabolic changes in patients with HIV infection. *AIDS, 13*(18), 2493-2505.

Salas-Salvado, J., & Garcia-Lorda, P. (2001). The metabolic puzzle during the evolution of HIV infection. *Clin Nutr, 20*(5), 379-391. doi: 10.1054/clnu.2001.0429

Saltiel, A. R., & Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature, 414*(6865), 799-806. doi: 10.1038/414799a

Samuel, V. T., Petersen, K. F., & Shulman, G. I. (2010). Lipid-induced insulin resistance: unravelling the mechanism. *Lancet, 375*(9733), 2267-2277. doi: 10.1016/S0140-6736(10)60408-4

Savage, D. B., Petersen, K. F., & Shulman, G. I. (2005). Mechanisms of insulin resistance in humans and possible links with inflammation. *Hypertension, 45*(5), 828-833. doi: 10.1161/01.HYP.0000163475.04421.e4

Savage, D. B., Petersen, K. F., & Shulman, G. I. (2007). Disordered lipid metabolism and the pathogenesis of insulin resistance. *Physiol Rev, 87*(2), 507-520. doi: 10.1152/physrev.00024.2006

Schauder, P., Schroder, K., Matthaei, D., Henning, H. V., & Langenbeck, U. (1983). Influence of insulin on blood levels of branched chain keto and amino acids in man. *Metabolism, 32*(4), 323-327.

Schols, D. (2004). HIV co-receptors as targets for antiviral therapy. *Curr Top Med Chem, 4*(9), 883-893.

Schuster, I., Thoni, G. J., Ederhy, S., Walther, G., Nottin, S., Vinet, A., . . . Obert, P. (2008). Subclinical cardiac abnormalities in human immunodeficiency virus-infected men receiving antiretroviral therapy. *Am J Cardiol, 101*(8), 1213-1217. doi: 10.1016/j.amjcard.2007.11.073

Semb, H., & Olivecrona, T. (1987). Mechanisms for turnover of lipoprotein lipase in guinea pig adipocytes. *Biochim Biophys Acta, 921*(1), 104-115.

Semb, H., & Olivecrona, T. (1989). The relation between glycosylation and activity of guinea pig lipoprotein lipase. *J Biol Chem, 264*(7), 4195-4200.

Semenkovich, C. F., Wims, M., Noe, L., Etienne, J., & Chan, L. (1989). Insulin regulation of lipoprotein lipase activity in 3T3-L1 adipocytes is mediated at posttranscriptional and posttranslational levels. *J Biol Chem, 264*(15), 9030-9038.

Sennepin, A. D., Charpentier, S., Normand, T., Sarre, C., Legrand, A., & Mollet, L. M. (2009). Multiple reprobing of Western blots after inactivation of peroxidase activity by its substrate, hydrogen peroxide. *Anal Biochem, 393*(1), 129-131. doi: 10.1016/j.ab.2009.06.004

Severson, D. L., Lee, M., & Carroll, R. (1988). Secretion of lipoprotein lipase from myocardial cells isolated from adult rat hearts. *Mol Cell Biochem, 79*(1), 17-24.

Shah, G. (1999). Why do we still use serum in the production of biopharmaceuticals? *Dev Biol Stand, 99*, 17-22.

Shen, L., Peterson, S., Sedaghat, A. R., McMahon, M. A., Callender, M., Zhang, H., . . . Siliciano, R. F. (2008). Dose-response curve slope sets class-specific limits on inhibitory potential of anti-HIV drugs. *Nat Med, 14*(7), 762-766. doi: 10.1038/nm1777

Shirai, K., & Jackson, R. L. (1982). Lipoprotein lipase-catalyzed hydrolysis of p-nitrophenyl butyrate. Interfacial activation by phospholipid vesicles. *J Biol Chem, 257*(3), 1253-1258.

Shisana, O. e. a. (2009). South African National HIV Prevalence, Incidence, Behaviour and Communication Survey 2008:. *HSRC Press, Cape Town*.

Shlay, J. C., Visnegarwala, F., Bartsch, G., Wang, J., Peng, G., El-Sadr, W. M., . . . Raghavan, S. (2005). Body composition and metabolic changes in antiretroviral-naive patients randomized to didanosine and stavudine vs. abacavir and lamivudine. *J Acquir Immune Defic Syndr, 38*(2), 147-155.

Shrivastav, S. K., T ; Chrousos,G,P and Kopp,J.B. (2000). HIV-1 Vpr binds and inhibits PPAR-gamma: implications for HIVassociated insulin resistance and lipodystroph,. *Proceedings of the International Meeting of the Institute of Human Virology,*

.

Shulman, G. I. (2000). Cellular mechanisms of insulin resistance. *J Clin Invest, 106*(2), 171-176. doi: 10.1172/JCI10583

Sierra, S., Kupfer, B., & Kaiser, R. (2005). Basics of the virology of HIV-1 and its replication. *J Clin Virol, 34*(4), 233-244. doi: 10.1016/j.jcv.2005.09.004

Silva, M., Skolnik, P. R., Gorbach, S. L., Spiegelman, D., Wilson, I. B., Fernandez-DiFranco, M. G., & Knox, T. A. (1998). The effect of protease inhibitors on weight and body composition in HIV-infected patients. *AIDS, 12*(13), 1645-1651.

Simha, V., & Garg, A. (2006). Lipodystrophy: lessons in lipid and energy metabolism. *Curr Opin Lipidol, 17*(2), 162-169. doi: 10.1097/01.mol.0000217898.52197.18

Simon, J. H., Fouchier, R. A., Southerling, T. E., Guerra, C. B., Grant, C. K., & Malim, M. H. (1997). The Vif and Gag proteins of human immunodeficiency virus type 1 colocalize in infected human T cells. *J Virol, 71*(7), 5259-5267.

Slama, L., Le Camus, C., Serfaty, L., Pialoux, G., Capeau, J., & Gharakhanian, S. (2009). Metabolic disorders and chronic viral disease: the case of HIV and HCV. *Diabetes Metab, 35*(1), 1-11. doi: 10.1016/j.diabet.2008.08.003

Smith, R. F., Humphreys, S., & Hockaday, T. D. (1986). The measurement of plasma acetate by a manual or automated technique in diabetic and non-diabetic subjects. *Ann Clin Biochem, 23 (Pt 3)*, 285-291.

Sone, H., Shimano, H., Sakakura, Y., Inoue, N., Amemiya-Kudo, M., Yahagi, N., . . . Yamada, N. (2002). Acetyl-coenzyme A synthetase is a lipogenic enzyme controlled by SREBP-1 and energy status. *Am J Physiol Endocrinol Metab, 282*(1), E222-230. doi: 10.1152/ajpendo.00189.2001

Spooner, P. M., Chernick, S. S., Garrison, M. M., & Scow, R. O. (1979). Insulin regulation of lipoprotein lipase activity and release in 3T3-L1 adipocytes. Separation and dependence of hormonal effects on hexose metabolism and synthesis of RNA and protein. *J Biol Chem, 254*(20), 10021-10029.

Srivastava, A. K., & Pandey, S. K. (1998). Potential mechanism(s) involved in the regulation of glycogen synthesis by insulin. *Mol Cell Biochem, 182*(1-2), 135-141.

Stanley, C. A. (2009). Regulation of glutamate metabolism and insulin secretion by glutamate dehydrogenase in hypoglycemic children. *Am J Clin Nutr, 90*(3), 862S-866S. doi: 10.3945/ajcn.2009.27462AA

Stanley, C. A., Fang, J., Kutyna, K., Hsu, B. Y., Ming, J. E., Glaser, B., & Poncz, M. (2000). Molecular basis and characterization of the hyperinsulinism/hyperammonemia syndrome: predominance of mutations in exons 11 and 12 of the glutamate dehydrogenase gene. HI/HA Contributing Investigators. *Diabetes, 49*(4), 667-673.

Stanley, C. A., Lieu, Y. K., Hsu, B. Y., Burlina, A. B., Greenberg, C. R., Hopwood, N. J., . . . Poncz, M. (1998). Hyperinsulinism and hyperammonemia in infants with regulatory mutations of the glutamate dehydrogenase gene. *N Engl J Med, 338*(19), 1352-1357. doi: 10.1056/NEJM199805073381904

Stewart, J. E., & Schotz, M. C. (1974). Release of lipoprotein lipase activity from isolated fat cells. II. Effect of heparin. *J Biol Chem, 249*(3), 904-907.

Suarez, S., Baril, L., Stankoff, B., Khellaf, M., Dubois, B., Lubetzki, C., . . . Hauw, J. J. (2001). Outcome of patients with HIV-1-related cognitive impairment on highly active antiretroviral therapy. *AIDS, 15*(2), 195-200.

Summers, S. A., Garza, L. A., Zhou, H., & Birnbaum, M. J. (1998). Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide. *Mol Cell Biol, 18*(9), 5457-5464.

Takano, A., Haruta, T., Iwata, M., Usui, I., Uno, T., Kawahara, J., . . . Kobayashi, M. (2001). Growth hormone induces cellular insulin resistance by uncoupling phosphatidylinositol 3-kinase and its downstream signals in 3T3-L1 adipocytes. *Diabetes, 50*(8), 1891-1900.

Tamura, S., Fujita-Yamaguchi, Y., & Larner, J. (1983). Insulin-like effect of trypsin on the phosphorylation of rat adipocyte insulin receptor. *J Biol Chem, 258*(24), 14749-14752.

Taylor, C. E., & Greenough, W. B., 3rd. (1989). Control of diarrheal diseases. *Annu Rev Public Health, 10*, 221-244. doi: 10.1146/annurev.pu.10.050189.001253

Taylor, S. I., & Jungas, R. L. (1974). Regulation of lipogenesis in adipose tissue: the significance of the activation of pyruvate dehydrogenase by insulin. *Arch Biochem Biophys, 164*(1), 12-19.

Tilg, H., & Moschen, A. R. (2008). Inflammatory mechanisms in the regulation of insulin resistance. *Mol Med, 14*(3-4), 222-231. doi: 10.2119/2007-00119.Tilg

Tirosh, A., Potashnik, R., Bashan, N., & Rudich, A. (1999). Oxidative stress disrupts insulin-induced cellular redistribution of insulin receptor substrate-1 and phosphatidylinositol 3-kinase in 3T3-L1 adipocytes. A putative cellular mechanism for impaired protein kinase B activation and GLUT4 translocation. *J Biol Chem, 274*(15), 10595-10602.

Todesco, T., Zamboni, M., Armellini, F., Bissoli, L., Turcato, E., Piemonte, G., . . . Bosello, O. (1993). Plasma acetate levels in a group of obese diabetic, obese normoglycemic, and control subjects and their relationships with other blood parameters. *Am J Gastroenterol, 88*(5), 751-755.

Trauner, D. A., Nyhan, W. L., & Sweetman, L. (1975). Short-chain organic acidemia and Reye's syndrome. *Neurology, 25*(3), 296-298.

Tremblay, F., Brule, S., Hee Um, S., Li, Y., Masuda, K., Roden, M., . . . Marette, A. (2007). Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. *Proc Natl Acad Sci U S A, 104*(35), 14056-14061. doi: 10.1073/pnas.0706517104

Tremblay, F., Lavigne, C., Jacques, H., & Marette, A. (2007). Role of dietary proteins and amino acids in the pathogenesis of insulin resistance. *Annu Rev Nutr, 27*, 293-310. doi: 10.1146/annurev.nutr.25.050304.092545

Tremblay, F., & Marette, A. (2001). Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. *J Biol Chem, 276*(41), 38052-38060. doi: 10.1074/jbc.M106703200

Tsiodras, S., Mantzoros, C., Hammer, S., & Samore, M. (2000). Effects of protease inhibitors on hyperglycemia, hyperlipidemia, and lipodystrophy: a 5-year cohort study. *Arch Intern Med, 160*(13), 2050-2056.

Tsiodras, S., Perelas, A., Wanke, C., & Mantzoros, C. S. (2010). The HIV-1/HAART associated metabolic syndrome - novel adipokines, molecular associations and therapeutic implications. *J Infect, 61*(2), 101-113. doi: 10.1016/j.jinf.2010.06.002

Turner, B. G., & Summers, M. F. (1999). Structural biology of HIV. *J Mol Biol, 285*(1), 1-32. doi: 10.1006/jmbi.1998.2354

Tweeddale, H., Notley-McRobb, L., & Ferenci, T. (1998). Effect of slow growth on metabolism of Escherichia coli, as revealed by global metabolite pool ("metabolome") analysis. *J Bacteriol, 180*(19), 5109-5116.

Ullrich, N. F., Purnell, J. Q., & Brunzell, J. D. (2001). Adipose tissue fatty acid composition in humans with lipoprotein lipase deficiency. *J Investig Med, 49*(3), 273-275.

Um, S. H., D'Alessio, D., & Thomas, G. (2006). Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell Metab, 3*(6), 393-402. doi: 10.1016/j.cmet.2006.05.003

UNAIDS, W. H. O. (2011). Global summary of the AIDS epidemic.

Unger, R. H. (2003). Minireview: weapons of lean body mass destruction: the role of ectopic lipids in the metabolic syndrome. *Endocrinology, 144*(12), 5159-5165. doi: 10.1210/en.2003-0870

Unger, R. H., Clark, G. O., Scherer, P. E., & Orci, L. (2010). Lipid homeostasis, lipotoxicity and the metabolic syndrome. *Biochim Biophys Acta, 1801*(3), 209-214. doi: 10.1016/j.bbalip.2009.10.006

USAID Health Policy Initiative. (2009). Computer Program for Making HIV/AIDS Projections and Examining the Demographic and Social Impacts of AIDS.

van der Valk, M., Bisschop, P. H., Romijn, J. A., Ackermans, M. T., Lange, J. M., Endert, E., . . . Sauerwein, H. P. (2001). Lipodystrophy in HIV-1-positive patients is associated with insulin resistance in multiple metabolic pathways. *AIDS, 15*(16), 2093-2100.

van Leth, F., Phanuphak, P., Ruxrungtham, K., Baraldi, E., Miller, S., Gazzard, B., . . . Lange, J. M. (2004). Comparison of first-line antiretroviral therapy with regimens including nevirapine, efavirenz, or both drugs, plus stavudine and lamivudine: a randomised open-label trial, the 2NN Study. *Lancet, 363*(9417), 1253-1263. doi: 10.1016/S0140-6736(04)15997-7

Van Obberghen, E., Baron, V., Delahaye, L., Emanuelli, B., Filippa, N., Giorgetti-Peraldi, S., . . . Giudicelli, J. (2001). Surfing the insulin signaling web. *Eur J Clin Invest, 31*(11), 966-977.

van Vonderen, M. G., Blumer, R. M., Hassink, E. A., Sutinen, J., Ackermans, M. T., van Agtmael, M. A., . . . Reiss, P. (2010). Insulin sensitivity in multiple pathways is differently affected during zidovudine/lamivudine-containing compared with NRTI-sparing combination antiretroviral therapy. *J Acquir Immune Defic Syndr, 53*(2), 186-193. doi: 10.1097/QAI.0b013e3181c190f4

Vannier, C., & Ailhaud, G. (1989). Biosynthesis of lipoprotein lipase in cultured mouse adipocytes. II. Processing, subunit assembly, and intracellular transport. *J Biol Chem, 264*(22), 13206-13216.

Vannier, C., Amri, E. Z., Etienne, J., Negrel, R., & Ailhaud, G. (1985). Maturation and secretion of lipoprotein lipase in cultured adipose cells. I. Intracellular activation of the enzyme. *J Biol Chem, 260*(7), 4424-4431.

Vigano, A., Mora, S., Testolin, C., Beccio, S., Schneider, L., Bricalli, D., . . . Brambilla, P. (2003). Increased lipodystrophy is associated with increased exposure to highly active antiretroviral therapy in HIV-infected children. *J Acquir Immune Defic Syndr, 32*(5), 482-489.

Vigouroux, C., Gharakhanian, S., Salhi, Y., Nguyen, T. H., Chevenne, D., Capeau, J., & Rozenbaum, W. (1999). Diabetes, insulin resistance and dyslipidaemia in lipodystrophic HIV-infected patients on highly active antiretroviral therapy (HAART). *Diabetes Metab, 25*(3), 225-232.

Vigouroux, C., Maachi, M., Nguyen, T. H., Coussieu, C., Gharakhanian, S., Funahashi, T., . . . Bastard, J. P. (2003). Serum adipocytokines are related to lipodystrophy and metabolic disorders in HIV-infected men under antiretroviral therapy. *AIDS, 17*(10), 1503-1511. doi: 10.1097/01.aids.0000072646.21517.64

Wajchenberg, B. L. (2000). Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr Rev, 21*(6), 697-738.

Waki, H., & Tontonoz, P. (2007). Endocrine functions of adipose tissue. *Annu Rev Pathol, 2*, 31-56. doi: 10.1146/annurev.pathol.2.010506.091859

Walli, R., Herfort, O., Michl, G. M., Demant, T., Jager, H., Dieterle, C., . . . Goebel, F. D. (1998). Treatment with protease inhibitors associated with peripheral insulin resistance and impaired oral glucose tolerance in HIV-1-infected patients. *AIDS, 12*(15), F167-173.

Wand, H., Calmy, A., Carey, D. L., Samaras, K., Carr, A., Law, M. G., . . . Emery, S. (2007). Metabolic syndrome, cardiovascular disease and type 2 diabetes mellitus after initiation of

antiretroviral therapy in HIV infection. *AIDS, 21*(18), 2445-2453. doi: 10.1097/QAD.0b013e3282efad32

Wand H, L. M., Emery S, Cooper DA, Carr A. (2005). Increase in limb fat after nucleoside analogue cessation is not associated with decreased visceral fat and has different risk factors. *Antivir Ther., 10*(L5).

Wang, C. N., O'Brien, L., & Brindley, D. N. (1998). Effects of cell-permeable ceramides and tumor necrosis factor-alpha on insulin signaling and glucose uptake in 3T3-L1 adipocytes. *Diabetes, 47*(1), 24-31.

Wang, C. S., Hartsuck, J., & McConathy, W. J. (1992). Structure and functional properties of lipoprotein lipase. *Biochim Biophys Acta, 1123*(1), 1-17.

Wang, H., & Eckel, R. H. (2009). Lipoprotein lipase: from gene to obesity. *Am J Physiol Endocrinol Metab, 297*(2), E271-288. doi: 10.1152/ajpendo.90920.2008

Weinzimer, S. A., Stanley, C. A., Berry, G. T., Yudkoff, M., Tuchman, M., & Thornton, P. S. (1997). A syndrome of congenital hyperinsulinism and hyperammonemia. *J Pediatr, 130*(4), 661-664.

Weiss, R. A. (1993). How does HIV cause AIDS? *Science, 260*(5112), 1273-1279.

Welsh, G. I., Wilson, C., & Proud, C. G. (1996). GSK3: a SHAGGY frog story. *Trends Cell Biol, 6*(7), 274-279.

Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuzawa, Y., Pratley, R. E., & Tataranni, P. A. (2001). Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab, 86*(5), 1930-1935.

Weyrich, P., Neuscheler, D., Melzer, M., Hennige, A. M., Haring, H. U., & Lammers, R. (2007). The Par6alpha/aPKC complex regulates Akt1 activity by phosphorylating Thr34 in the PH-domain. *Mol Cell Endocrinol, 268*(1-2), 30-36. doi: 10.1016/j.mce.2007.01.011

WHO., U. (2009). AIDS epidemic update. *Joint UN Programme on HIV/AIDS,World Health Organization; Geneva: 2009*.

Wijekoon, E. P., Skinner, C., Brosnan, M. E., & Brosnan, J. T. (2004). Amino acid metabolism in the Zucker diabetic fatty rat: effects of insulin resistance and of type 2 diabetes. *Can J Physiol Pharmacol, 82*(7), 506-514. doi: 10.1139/y04-067

Williams, K., Rao, Y. P., Natarajan, R., Pandak, W. M., & Hylemon, P. B. (2004). Indinavir alters sterol and fatty acid homeostatic mechanisms in primary rat hepatocytes by increasing levels of activated sterol regulatory element-binding proteins and decreasing cholesterol 7alpha-hydroxylase mRNA levels. *Biochem Pharmacol, 67*(2), 255-267.

Williams. A , K. G., LindequeC, Reinecke and Meyer.D. (2012). Qualitative serum organic acid profiles of HIV-infected individuals not on antiretroviral treatment. *Metabolomics, 8*, 804–818.

Wishart, D. S., Tzur, D., Knox, C., Eisner, R., Guo, A. C., Young, N., . . . Querengesser, L. (2007). HMDB: the Human Metabolome Database. *Nucleic Acids Res, 35*(Database issue), D521-526. doi: 10.1093/nar/gkl923

Witvrouw, M., Pannecouque, C., Switzer, W. M., Folks, T. M., De Clercq, E., & Heneine, W. (2004). Susceptibility of HIV-2, SIV and SHIV to various anti-HIV-1 compounds: implications for treatment and postexposure prophylaxis. *Antivir Ther, 9*(1), 57-65.

Wu, P. Y., Hung, C. C., Liu, W. C., Hsieh, C. Y., Sun, H. Y., Lu, C. L., . . . Chien, K. L. (2012). Metabolic syndrome among HIV-infected Taiwanese patients in the era of highly active antiretroviral therapy: prevalence and associated factors. *J Antimicrob Chemother, 67*(4), 1001-1009. doi: 10.1093/jac/dkr558

Wyatt, C. M., Winston, J. A., Malvestutto, C. D., Fishbein, D. A., Barash, I., Cohen, A. J., . . . Klotman, P. E. (2007). Chronic kidney disease in HIV infection: an urban epidemic. *AIDS, 21*(15), 2101-2103. doi: 10.1097/QAD.0b013e3282ef1bb4

Xu, C., Bailly-Maitre, B., & Reed, J. C. (2005). Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest, 115*(10), 2656-2664. doi: 10.1172/JCI26373

Yan, Q., & Hruz, P. W. (2005). Direct comparison of the acute in vivo effects of HIV protease inhibitors on peripheral glucose disposal. *J Acquir Immune Defic Syndr, 40*(4), 398-403.

Yang, C., Watson, R. T., Elmendorf, J. S., Sacks, D. B., & Pessin, J. E. (2000). Calmodulin antagonists inhibit insulin-stimulated GLUT4 (glucose transporter 4) translocation by preventing the formation of phosphatidylinositol 3,4,5-trisphosphate in 3T3L1 adipocytes. *Mol Endocrinol, 14*(2), 317-326.

Yang, Y., Ikezoe, T., Nishioka, C., Bandobashi, K., Takeuchi, T., Adachi, Y., . . . Taguchi, H. (2006). NFV, an HIV-1 protease inhibitor, induces growth arrest, reduced Akt signalling, apoptosis and docetaxel sensitisation in NSCLC cell lines. *Br J Cancer, 95*(12), 1653-1662. doi: 10.1038/sj.bjc.6603435

Yin, J., Gao, Z., Liu, D., Liu, Z., & Ye, J. (2008). Berberine improves glucose metabolism through induction of glycolysis. *Am J Physiol Endocrinol Metab, 294*(1), E148-156. doi: 10.1152/ajpendo.00211.2007

Yokota, T., Nagashima, M., Ghazizadeh, M., & Kawanami, O. (2009). Increased effect of fucoidan on lipoprotein lipase secretion in adipocytes. *Life Sci, 84*(15-16), 523-529. doi: 10.1016/j.lfs.2009.01.020

Yu, C., Chen, Y., Cline, G. W., Zhang, D., Zong, H., Wang, Y., . . . Shulman, G. I. (2002). Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem, 277*(52), 50230-50236. doi: 10.1074/jbc.M200958200

Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z. W., Karin, M., & Shoelson, S. E. (2001). Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science, 293*(5535), 1673-1677. doi: 10.1126/science.1061620

Zammarchi, E., Filippi, L., Novembre, E., & Donati, M. A. (1996). Biochemical evaluation of a patient with a familial form of leucine-sensitive hypoglycemia and concomitant hyperammonemia. *Metabolism, 45*(8), 957-960.

Zhang, B., MacNaul, K., Szalkowski, D., Li, Z., Berger, J., & Moller, D. E. (1999). Inhibition of adipocyte differentiation by HIV protease inhibitors. *J Clin Endocrinol Metab, 84*(11), 4274-4277.

Zhang, H., Wei, J., Xue, R., Wu, J. D., Zhao, W., Wang, Z. Z., . . . Jiang, J. D. (2010). Berberine lowers blood glucose in type 2 diabetes mellitus patients through increasing insulin receptor expression. *Metabolism, 59*(2), 285-292. doi: 10.1016/j.metabol.2009.07.029

Zhang, K., & Kaufman, R. J. (2004). Signaling the unfolded protein response from the endoplasmic reticulum. *J Biol Chem, 279*(25), 25935-25938. doi: 10.1074/jbc.R400008200

Zhang, Q., Xiao, X., Feng, K., Wang, T., Li, W., Yuan, T., . . . Wang, H. (2011). Berberine Moderates Glucose and Lipid Metabolism through Multipathway Mechanism. *Evid Based Complement Alternat Med, 2011*. doi: 10.1155/2011/924851

Zhang, W., Thompson, B. J., Hietakangas, V., & Cohen, S. M. (2011). MAPK/ERK signaling regulates insulin sensitivity to control glucose metabolism in Drosophila. *PLoS Genet, 7*(12), e1002429. doi: 10.1371/journal.pgen.1002429

Zhou, L., Yang, Y., Wang, X., Liu, S., Shang, W., Yuan, G., . . . Chen, J. (2007). Berberine stimulates glucose transport through a mechanism distinct from insulin. *Metabolism, 56*(3), 405-412. doi: 10.1016/j.metabol.2006.10.025

APPENDICES

(A) Cell Culture Reagents

1. Composition of HAM's F12

Reagents	Volume	Final Concentration
Ham's F12 medium	500	
Fetal calf serum (FCS)	50	10%
200 mM Glutamine	5	2mM
100 x Antibiotic/antimycotic solution	5	1%
Gentamicin	2.5	0.5
G418	.5	400 ug/ml
Total volume	563	

G418-sulphate

0.4 g was dissolved in 1 ml dH2O and filtered using a 0.2 μM syringe filter.

2. Serum Free Medium

Reagents	Volume (ml)	Final concentration
DEMEM	500	
200 mM Glutamine	5	2mM
100 x Antibiotic/antimycotic solution	5	1%
Gentamicin	2.5	0.5
Total volume	5.12	

3. 10 X Lysis buffer solution

Reagents	Volume	Final concentration
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5	445 ml	50mM
Trintron X-100	5 ml	1%
Glycerol	50ml	10%
Sodium Chloride	1.461 g	50mM
Toltal Volume	500 ml	

1x lysis buffer was prepared with 1 tablet of protease inhibitor and 1 tablet of phosphatase inhibitor in 10 ml of 10 x

lysis buffer stock solution

4. 5 x Laemmli buffer

Reagents	Volume	Final concentration
1 M Tris, pH 6.8	1.25 ml	25 nM
Sodium dodecyl sulphate (SDS)	1 g	2 %
1 % Bromophenol blue	0.33 ml	0.002 %
Glycerol	5 m	10 %
2-Mercaptoethanol	2.5 ml	5 %
H_2O	1.25 ml	
Total volume	10.33 ml	

5. Freezing medium for CHO-IR cells

Reagents	Volume (ml)	Final concentration
Dimethyl sulphoxide (DMSO)	10	10%
FCS	40	
Total Volume	50	

6. Phosphate buffered saline (PBS)

1 tablet of PBS was dissolved in 200 ml dH_2O and the mixture was sterile by autoclave.

7. 1mg /ml Insulin stock

Insulin was prepared in 100 mM acetic acid + 0.1% bovine serum albumin (BSA)

(1) 100 mM acetic acid

    a. 0.294 ml 17 M acetic acid + 49.706 ml dH2O

(2) 0.1% BSA

    a. Add 0.05 g BSA into 50 ml acetic acid to become 0.1% BSA

(3) 5 mg insulin was dissolved in 5 ml of 100 mM acetic acid + 0.1% BSA = 1 mg/ml

9. Working Insulin solution

100 ng/ml (17 nM) insulin = 10 μl of 1 mg + 990 μl PBS

10 ng/ml (1.7 nM) insulin = 100 µl of 100 ng/ml (17 nM) insulin + 900 µl PBS

10. Drugs stock solutions

Drugs were prepared using a standard formula:

Mass drug (g) = concentration (M) x molecular weight (MW) x volume (L)

a) Indinavir, MW = 711.88

b) Nelfinavir, MW = 663.89

c) Ritonavir, MW = 720.95

d) Tenofovir, MW = 287.21

e) Stavudine, MW = 224.21

f) Sodium salicylate, MW = 160.10

g) Berberine chloride, MW = 371.82

h) IKK-16,MW = 520.09

11. Calculation of cell number

CHO-IR cells were counted using a haemocytometer to determine the total number of cells in the original flask. Then, 10 µl cells suspension was loaded into the haemocytometer. The number of cells in 5 of 10 of the 0.1 mm3 blocks was counted as follows:

Cells cm-3 = Number of cells counted x conversion factor (1000)

Number of squares counted

100 000 cells per well of each 6-well plate was required to achieve 70-85 % confluence on day 2/3.

(B) Electrophoresis and Western blot analysis

a) 1.5M Tris pH 8.8

Prepared by 54.46 g of Tris dissolve in 300ml dH2O

b) 0.5M Tris pH 6.8

12 g Tris dissolved in 200ml dH_2O

c) 10 % Ammonium persulphate (APS)

0.1 g APS was dissolved in 1 ml dH2O

d) 10 % SDS

5 g SDS was dissolved in 50 ml dH2O

e) 0.1 % Ponceau S solution

0.1 g Ponceau was dissolved in 100 ml of 5 % acetic acid.

f) 5 % BSA

2.5 g BSA was dissolved in 50 ml TBST

g) 5 % Milk solution

2.5 g skimmed milk powder was dissolved in 50 ml TBST.

1. **4% Stacking Gel**

Reagents	Volume (ml)	Final concentration
0.5 M Tris-HCl, pH 6.8	2.52	0.126 M
30 % Acrylamide/bis	1.32	4 %
dH_2O	6.00	
10 % SDS	.1	0.1 %
10 % APS	.05	0.05 %
TEMED	.01	0.1 %
Total volume	11.35	

2. **7.5% Resolving gel**

Reagents	Volume (ml)	Final concentration
1.5 M Tris-HCl, pH 6.8	2.5	.375
30 % Acrylamide/bis	2.5	7.5
dH_2O	4.85	

10 % SDS	.1	0.1 %
10 % APS	.05	0.05 %
TEMED	.01	0.05%
Total volume	10ml	

3. 10X Running Buffer

Reagents	Volume (ml)	Final concentration
Tris	30.3 g	.025
Glycine	144 g	.0195
SDS	10 g	0.1%
dH$_2$O	Make up to 1 Litter	
Total volume	1L	

1 x Running buffer
100 ml of 10 x stock solution was added to 900 ml dH2O

4 X Transfer buffer

Reagents	Volume (ml)	Final concentration
Tris	3.03 g	.25M
Glycine	14.4g	1.92M
Methanol	200 ml	
H$_2$O	800 ml	
Total Volume	1 L	

5 Tris bufferd saline +.05%Tween 20(TBST). PH 7.4

Reagents	Volume (ml)	Final concentration
Sodium chloride	8g	
Potassium chloride	.2 g	
Tris	3 g	
Tween 20	.5 ml	
dH$_2$O	1L	
Total Volume	IL	

(C) Lipoprotein Lipase essay Reagent

Reagent A

Reagent	Volume	Final concentration
Sodium Phosphate, Monobasic	6.89 g	100mM
Sodium Chloride	4.38 g	150mM
Triton$_1$ X-100,	2.5 ml	.5%
Total Volume	500 ml	

Adjusted the PH7.2 with 1M sodium hydroxide.

Reagent B: Acetonitrile

Reagent C: 50 mM p-Nitrophenyl Butyrate (*P*-NPB)

10.2ul of 98% solution of p-Nitrophenyl Butyrate (MW209.2) in 1 ml of acetonitrile.

Reagent D

I mg of lipoprotein lipase dissolved in 1 ml of Reagent A.