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Uptake of arachidonic acid and glucose into isolated human adipocytes

by

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Uptake of arachidonic acid and glucose into isolated fresh human adipocytes

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Both plasma glucose concentration and glucose uptake are deranged in insulin resistance. A high free fatty acid plasma level is a potential cause of insulin resistance, and therefore of type 2 diabetes mellitus animals and humans. The mechanism behind this is still unclear.

The objectives of the present study were: (i) to research the effect of arachidonic acid (AA) as fatty acid representative, on glucose uptake into human isolated adipocytes, (ii) to investigate the uptake of AA into adipocyte membranes and nuclei, as a step to identify the mechanism whereby AA affects glucose uptake, and (iii) to verify the influence of insulin on AA uptake in adipocytes.

The first objective was achieved by exposing adipocytes to AA and measuring the effect on deoxyglucose uptakt. To achieve the second objective, adipocytes

were exposed to ^{14}C-AA; radioactive uptake in membranes and nuclei was determined. The AA uptake into membranes was also determinate by membranes fatty acid profile using gas chromatography; the results of the two methods were compared. Finally, the third objective was achieved by exposing adipocytes to different concentrations of insulin and testing the effect by measuring arachidonic acid uptake by the entire cell.

The results of this study shown that, acute (30 min) exposure of AA significantly stimulates glucose uptake by adipocytes (4.56 ± 0.6 nmole glucose /mg protein /min) compared to the control (3.12 ± 0.25 nmole glucose /mg protein /min).
Secondly, ^{14}C-AA was significantly taken up by the membranes between 20 and 30 minutes of exposure. The uptake into membranes was increased by 49.57 ± 29% and 123 ± 73% compared to the control 100% (1.77 ± 0.06 nmole AA /mg protein) respectively for 20 and 30 min exposure).
AA significantly rose in the nuclei after 30 minutes (147 ± 19% increase) compared to the control 100% (2.25 ± 0.10 nmole AA /mg protein).
The determination of AA uptake by gas chromatography analysis of the membrane fatty acid profile showed that the content of AA increased after 30 min exposure (0.57% AA of total membrane fatty acids) compared to the 10 min exposure (0.29% AA of total membrane fatty acid). Insulin was shown to stimulate 10 and 30 min AA uptake by adipocytes from a non-obese subject. The increases of AA uptake measured for 30 minutes were 20 ± 8%, 21 ± 25% and 31 ± 4% compared to the control (0.58nmole AA / mg protein / min) respectively for the actions of 10nM, 20nM and 40 nM insulin. A similar tendency was observed when the AA uptake was measured for 10 min (81 ± 31% and 208 ± 36% respectively for the action of 10nM and 40nM insulin compared to the control 100% (0.06nmole AA/mg protein/min).
In contrast to this finding, insulin depressed AA uptake by adipocytes from an obese subject (depression of 15 ± 5%, 14 ± 8% and 21 ± 5% respectively for 10nM, 20nM and 40nM insulin, compared to the control 100% (0.74 nmole

AA/mg protein/min). In both situations the effect of insulin seemed dose dependent.

The study demonstrated that AA acid positively modulates glucose uptake into adipocytes exposed for short periods (< 30 min). This was attributed to the probable this FA in the cell membrane, rather than its eventual effect on the DNA. The best method to measure membranes AA over short period of exposure when small amounts of adipocytes (2- 6 ml) are used was by radioactive means. It also suggested that insulin effect's on AA acid uptake into adipocytes was dose dependent. This varies with the body mass index (BMI) of the patient, probably as a result of their cell's insulin resistant state.

DECLARATION

I hereby declare that the work presented here is my original work. To my knowledge this work has not been published or submitted for a degree at the University of Pretoria. The permission right for duplication of the whole thesis or part thereof is reserved to the University of Pretoria

Ana Malipa

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Abbreviations

AA - arachidonic acid

ACBP –acyl-coA-binding protein

Adis – adipocytes

ALA – alpha linolenic acid

A – LBP – adipocyte lipid binding protein

BF_3 – Me - boron trifluoride methanol

BHT – butylated hydroxytoluene

BHSD – beta - hydroxysteroid – dehydrogenase

BGU - insulin–independent glucose uptake

BMI – body mass index

^{14}C-AA – arachidonic acid radioactively labelled with carbon fourteen

cAMP – cyclic adenosine monophosphate

CD – cell differentiation

C/EBP- family of transcriptional factors

CoA – coenzyme A

COX – cyclooxygenase

cpm – counts per minute

Cs – concentration of standard

Ct – concentration of test sample

DAG – diacylglycerol

DHA – docosahexanoic acid

DM – diabetes mellitus

DNA – deoxyribonucleic acid

DMSO – dimethylsulfoxide

DOG – deoxyglucose

EDTA – ethylenediaminetetraacetic acid

EPA – eicosapentanoic acid

EtOH – ethanol

FA – fatty acid

FABP – fatty acid binding protein

FABPpm – plasma membrane fatty acid binding protein

FAT - fatty acid translocase

FFA – free fatty acid

FAFA – albumin free fatty acid

FATP – fatty acid transport protein

G – glucose

GC – gas chromatography

GLA – gamma linoleic acid

GH – growth hormone

GLUT – glucose transporter

HCl – hydochloric acid

HIV – human immunodeficiency virus

HSL – hormone sensitive lipase

IDM – indomethacin

IL – interleukin

Ins – insulin

IR - insulin resistance

ISUG - insulin-stimulated glucose uptake

K-LBP – keratinocyte lipid binding protein

K_m – Michaelis Menton constant

KOH – potassium hydroxide

KRB1 – Krebs Ringer Buffer with glucose

KRB2 - Krebs Ringer Buffer without glucose

LA – linoleic acid

LCFA – long chain fatty acid

L - lipoxygenase

LBP – lipid binding protein

mAspAT – aspartate amino transferase

mRNA – messenger ribonucleic acid

NDGA – nordihydroguaiaretic acid

n-6 – fatty acids with double bond in position 6 n-3 - fatty acids with double bond in position 3

OD - optical density

ODt – optical density of test sample

ODs – optical density of standard

PHL – phloretin

PG – prostaglandin

PI – phosphatidyl inositol

PI3-K – phosphatidyl inositol 3 kinase

PMSF – phenyl methyl sulfonyl fluoride

PK – protein kinase

PPAR – peroxisome proliferator activator receptor

PPRE - peroxisome proliferator response element

PUFA – polyunsaturated fatty acid

SCFA – short chain fatty acid

SD – standard deviation

SDS – sodium dodecyl sulphate

SFA – saturated fatty acid

SREBP – sterol responsive element – binding protein

TATA – nucleotide sequence of thymine (T) and adenosine (A)

T2DM – type 2 diabetes mellitus

TDZ – thiazolidinedione

TNF – tumor necrosis factor

UFA – unsaturated fatty acid

WAT – white adipose tissue

CHAPTER 1

General Introduction

1.1. Motivation for the study

The prevalence of obesity and consequently type 2 diabetes mellitus (T2DM), also known as non insulin dependent diabetes mellitus, is increasing worldwide and it has become a serious public health problem, especially in Western Societies and South Africa is no exception (1, 2, 3). These diseases are not only in themselves, very detrimental to health, but the drugs used to control them, especially T2DM, also have undesirable side effects. Furthermore, their administration is not practical, because some are in the form of injections which should be administered often. Diabetes mellitus (DM) and obesity have drastic implications on economy, because drugs used for their supportive treatment are expensive. This leads to social exclusions as well as serious health consequences such as chronic cardiac and kidney diseases and even loss of limbs and blindness.

Genetic factors, good lifestyle practices (e.g. exercising) (4) and nutritional factors (5) play an important role in the genesis of obesity and T2DM. In this study, fatty acids (FAs) are the nutrients in focus. The main source of FAs in the human organism is dietary fat (6). Since lipolysis in central fat depots in obese subjects is higher (7, 8) and if it remains unchecked, it could also be an important source of FAs not only for normal roles of FAs in the body, but also for the eventual development of insulin resistance (IR) (9, 10, 11). IR is a state where the whole body responds inefficiently to insulin. As a result, uptake of glucose in these cells is impaired and plasma glucose levels rise. The IR state is also accompanied by hyperlipidaemia (12). Studies done in rat skeletal muscles have established a positive relation between saturated fatty acids (SFA) and the development of IR, as well as the utilization of unsaturated fatty acids (UFA) to alleviate the condition (6, 9, 13, 14). Similar strategies have also given useful results in humans (15, 16). Because adipocytes also strongly influence plasma glucose levels in obese subjects (17), studies have been carried out in this cell type in rats, both *in vitro and in vivo,* by different authors (17,19,20). These studies support the results of Storlien *et al*. mentioned above (14). Furthermore, it was demonstrated that 4-8 hours adipocyte exposure to arachidonic acid (AA) (18) improved insulin–independent basal glucose uptake (BGU) as well as the insulin-stimulated glucose uptake (ISGU), confirming the studies by Fong and colleagues (19, 20). The few similar studies done in human adipocytes have given inconclusive results (21).

Two mechanisms by which FAs can affect glucose transport have been proposed: (a) interference of FAs with gene expression of proteins involved in the modulation of glucose transport; and (b) incorporation of FAs into cell membranes, consequently increasing the activity of membrane proteins. This proposal is supported by the findings that FAs affect gene expression of glucose transporters (GLUT4 and GLUT1) (18, 22), and by the fact that the FA content of plasma membranes is related to IR: a higher content of saturated fatty acid in the plasma membrane impairs the action of insulin (11). In contrast, a high content of polyunsaturated fatty acids (PUFA), specifically the omega-3 family, in the membrane improves the action of insulin (18, 23, 24). For example, incorporation of FAs into cell membranes may consequently affect the activity of the Na^+/K^+ ATPase pump (25, 26), an important membrane protein. This could conceivably also occur with membrane proteins involved in the process of glucose uptake.

Furthermore, it has been demonstrated that FA transport through the biological membrane takes place by simple diffusion (27, 28, 29) and facilitated transport (30, 31, 32, 33). Hormones such as insulin can affect the latter process (uptake of FA) (34). Apparently there are no reports about AA uptake in fresh human adipocytes. Therefore, as in skeletal muscle, the modulation of glucose and FA uptake in adipocytes is vital to prevent and treat IR and its consequences.

Due to the period that has been given to this project and financial limitations, only AA, one of the UFA precursor of substances with physiological importance in the organism, was used in this work to represent UFAs. As a step to understand the mechanism whereby FAs rapidly affect glucose uptake, the study might also contribute to the comprehension of nutritional factors on the development, prevention and treatment of IR, as well as to the eventual development of more natural drugs for treating T2DM.

1.2. Purpose of study

This study had the three following aims:

(1) To verify the effect of AA on glucose uptake into fresh human adipocytes over a short period (30 min).

(2) To determine the time-frame in which AA was taken up in subcellular fractions, as a contribution to the identification of mechanisms by which AA affects glucose uptake in fresh human adipocyte.

(3) To verify the effect of insulin on AA uptake into fresh human adipocytes by comparing AA uptake in adipocytes treated with insulin at different concentrations for 10 min and 30 min compared with untreated cells.

To achieve the goal, four studies with respective objectives were carried out, namely:

Study 1: Investigation of the effect of 10 and 30 min AA exposure on adipocyte glucose uptake.

Study 2: Verification of the time dependent uptake of ^{14}C-AA into subcellular fractions (plasma membrane and nucleus).

Study 3: Determination of the FA acid profile of the plasma membrane after 10 and 30 min of adipocyte exposure to AA.

Study 4: Investigation of AA acid uptake over a short period (10 and 30 min) ± insulin at different concentrations.

1.3. Hypotheses

The hypotheses tested in this work are the following:

1. AA stimulates glucose uptake into adipocytes after a short period (10 - 30 min) of exposure.
2. The mechanism by which 10 and 30 min AA exposure affects glucose uptake into adipocytes is based on cell membrane phenomena (plasma membrane and intracellular vesicle membranes).
3. The mechanism by which 10 and 30 minutes arachidonic acid exposure affects glucose uptake into adipocytes is based on the nuclear events (stimulation /repression of genes expression).
4. Insulin stimulates AA uptake in isolated human adipocytes.

The corresponding negative hypotheses of the hypotheses above listed were also considered during the study.

CHAPTER 2

Literature Review

2.1. Introduction

Adipocytes are the main local store of excess of calories (e.g. triglyceride) in the body. The stored triglycerides are hydrolyzed under hormonal control during food deprivation. The free fatty acids (FFAs) are delivered into circulation, and used primarily as an energy source by many tissues. Some FFAs may be used for other functions. Therefore, a crucial role is played by adipose tissue in controlling the flux of FAs to other tissues. FAs enter or leave the cell by simple diffusion. Additionally, it is believed that facilitated transport is also implicated in FA uptake and / or efflux. Recent data reveals that insulin may in part regulate this process by promoting translocation of the FA carriers into the plasma membrane. Abnormal FA metabolism and / or a disarranged manner of their transport can elevate non-esterified FAs in the plasma and play an important role in the aetiology and promotion of obesity and T2DM. In obese subjects, adipose tissue also contributes strongly to the plasma glucose level. Different scientists have demonstrated that unsaturated UFAs can improve insulin sensitivity both in skeletal muscle and adipocytes.

2.2. Diabetes mellitus: incidence and consequences

IR and obesity are lifestyle diseases generally related to comfort. The incidence and prevalence of these two diseases in industrialized countries, to which South Africa also belongs, are high and it continues to rise worldwide. Almost half of South Africans over the age of 15 are overweight or obese (1, 2). Approximately 7 % of people worldwide are obese and 65 % of these suffer from diabetes (3). T2DM and obesity are thus inter-related and have severe health consequences such as: blindness, kidney failure, cardiac problems, loss of limbs, and other severe maladies. T2DM is more frequent (90% of diabetics) than Type 1 diabetes mellitus (insulin dependent diabetes mellitus) (3). Diabetes mellitus is the third highest cause of death in the United States (US). The US Government has given much focus financially in the treatment (3.5 – 7 % of national health expenditure) and research of the disease (35). This is probably also the case in South Africa, although no related statistics have been found.

2.3. Adipogenesis

Adipose cells are produced from the mesoderm. The process of production of mature adipocytes is entitled adipogenesis and it is illustrated in Fig. 1. After birth, white apidose tissue (WAT) rapidly increases by proliferation and increase in size of pre-adipocytes. Adipogenesis is a continuous process during life (36). Environmental factors, especially nutrition, play an important role in regulating this process (37, 38). Several intrinsic factors are also involved in such regulation through stimulating or inhibiting the effect of transcriptional factors.

2.3.1. Phases of adipogenesis:

During adipogenesis, pre-adipocytes display, at first, an exponential growth phase characterized by mitosis. This is followed by growth arrest and differentiation: cells change their shape due to re-organization of extra cellular matrix and cytoskeletal proteins. Then, maturation follows. The cell acquires specialized apparatus that gives it a capacity to:

(a) transport great amounts of glucose in response to insulin, to produce FAs and to accumulate triglycerides;

(b) liberate FA from triglycerides during times of energy deficiency, in response to the stimuli of catecholamine (epinephrine and nor-epinephrine) and cortisol;

(c) synthesize several proteins and non-protein factors, some of which play a role in the endocrine control of energy homeostasis.

Adipocytes have the ability of self-renewal for indefinite periods (39). This may allow liberal adipocyte expansion in the living body.

2.3.2. Control of adipogenesis

Adipogenesis is a controlled process. Hormones, cytokines, nutrients and signalling molecules are involved in the control of adipogenesis by changing the expression and /or activity of a variety of transcription factors, which in turn, regulate the level of adipocyte conversion processes.

2.3.2.1. Transcriptional adipocyte regulation

Several families of transcriptional factor with different modes of activation and function are implicated in the regulation of adipogenesis, of which the peroxisome proliferator activator receptor (PPAR-γ) and a family of transcription factors viz. C/EBP-α are critical (40). These factors act sequentially to generate fully mature adipocytes: Homozygous knockout mice (where both genes are absent) lead to embryonic lethality and abnormal development of adipose tissue (41, 42, 43). PPAR-γ2 and C/EBP-α interact and co-regulate expression of each other: PPAR-γ2 heterozygous gene knockout leads to a rapid reduction of C/EBP-α level (44), whereas, in C/EBP-α null animals, expression of PPAR-γ2 is lower (45). Depending of the nature on the ligand, stimulation of PPAR-γ results in either antimitotic activity or mitotic activity (46) in pre-adipocytes. PPAR-γ was also identified in primary human adipocytes (47).

The importance of C/EBP-β and –δ during adipogenesis has been demonstrated in mice. Embryonic mice fibroblasts lacking either C/EBP-β or -δ have reduced levels of adipogenesis compared with the wild type (48), while its overexpression in adipocytes improves adipogenesis (49, 50). Furthermore, embryonic fibroblasts from C/EBP-β and –δ knockout mice did not differentiate into mature adipocytes (48). "*In vivo*" adipocyte differentiation requires the antimitotic effect of C/EBP-α (51).

Factor-1/sterol responsive element-binding protein-1c (ADD1 SREBP-1c) is another transcriptional factor with a role in adipogenesis. ADD1 SREBP-1c improves immature adipocyte differentiation to the mature adipocyte by inducing PPAR-γ expression, and, by controlling the binding of PPAR-γ by its ligands (52, 53). The dominant-negative form of ADD1 SREBP-1c inhibits adipocyte differentiation, especially the lipogenic pathway (54).

2.3.2.2. Substances that regulate adipogenesis via transcription factors

Several factors are involved in the regulation of adipogenesis. They exert their function either by promoting or blocking the cascade of transcriptional factors that coordinate the adipocyte differentiation process. The equilibrium between stimulatory and inhibitory forces determines the stage of adipogenesis of the pre-adipocyte, i.e. stationary or in mitosis and subsequent differentiation.

A. Stimulatory substances

Factors such as, glucocorticoids, FAs, some prostaglandins, insulin and adiponectin appear to have a stimulating effect on adipogenesis.

In the human, hypercortisolism is linked to obesity and disturbances in fat tissue homeostasis. Glucocorticoids have shown to be potent inducers of adipogenesis in vitro (55) through activation of expression of C/EBP-δ (56) and PPAR-γ (57). Rodent's pre-adipocytes and adipocytes express 11-β-hydroxysteroid-dehydrogenase-1 (11BHSD-1), an enzyme which converts inactive cortisone to active cortisol or corticosterone in rodents (58). Thus, cortisol produced locally in visceral fat might act in a paracrine manner to promote adipogenesis (40). In both rodent and human, overexpression of 11BHSD-1 in adipocytes is related to obesity (59, 60) and to the related metabolic syndrome which includes hypertension, increased visceral fat, IR and dyslipidaemia (58).

Diets high in saturated fatty acids appear to promote hypertrophy and hyperplasia of adipocytes (40). Although the polyunsaturated fatty acids are weaker stimulators of adipocyte mitosis *in vivo*, in culture PUFAs have a more prominent stimulatory effect on pre-adipocyte differentiation than saturated fatty acids do (61). The effect is probably attributed to the ability of PUFAs to act as ligands or precursors of ligands for PPAR-γ (62).

Prostacyclin (PGI), a major metabolite of AA in adipose tissue, binds to the prostanoid G-protein-coupled inositol phosphate (IP) receptors. The subsequent rise in intracellular cAMP mediates the induction of C/EBP-β and –δ by PGI (63) leading to a stimulation of adipogenesis. In addition, PGI_2 might stimulate adipose differentiation by binding to and activating the PPAR-γ nuclear receptor (64). Prostaglandin J2 (PGJ2), also seems to be an adipogenesis promoter through binding to PPAR-γ (65, 66).

Low plasma levels of adiponectin, a protein secreted by adipocytes, has been associated with obesity, IR, T2DM and cardiovascular diseases (67). Adiponectin is overexpressed in certain pre-adipocyte lines, suggesting that, by the stimulus of adiponectin, these cells can rapidly differentiate into mature adipocytes (40).

B. Inhibitory substances

There are several factors with an ability to inhibit adipose tissue development of relevance "*in vivo*", including: inflammatory cytokines, growth hormone (GH), resistin, specific FAs acids and antiretrovirals such as efavirenz, nelfinavir and indinavir.

Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL) -1, -6 and -11, interferon-γ, oncostatin M and ciliary neurotrophic factor, are implicated in the inhibition of adipogenesis (68, 69). The inhibition is the result of decreased expression of PPAR-γ and C/EBP-α. Moreover, TNF-α and IL-1 have shown to repress adipose differentiation via a cascade, which leads to inhibition of PPAR-γ activity (70).

GH negatively affects adipogenesis both "*in vivo*" and "*in vitro*" (36, 40). This takes place by stimulating lipolysis (40). Nevertheless, in an earlier stage of embryonic development, GH stimulates the differentiation of stem cells into adipocytes (71).

Resistin, another protein secreted by adipose tissue, appears to be associated with obesity and IR in rodents (72). Insulin sensitivity increases in resistin knockout mice (73). Resistin has been also implicated in "*in vitro*" inhibition of adipogenesis (74), but the physiological relevance of this observation is still to be determined.

Antiretroviral therapy leads to a positive prognosis of human immunodeficiency virus (HIV) infection, although it has been associated with IR, dyslipidaemia, peripheral lipo-atrophy and visceral adiposity (75). *In vitro* studies using the protease inhibitors nelfinavir and indinavir, have decreased pre-adipocyte conversion and lipogenesis, while increasing apoptosis and lipolysis (76, 77, 78). The level of pro-inflammatory cytokines in adipocytes of patients with HIV-associated lipo-atrophy is increased (79). This suggests that the effects of protease inhibitors on adipogenesis might result in the local overproduction of cytokines. Furthermore, efavirenz, a non-nucleoside reverse transcriptase inhibitor, has prevented the storage of lipids during "*in vitro*" differentiation of adipocytes by down-regulation of the transcription factor SREBP-1c (80).

Diets rich in medium-chain fatty acids have been shown to reduce the numbers and size of rodent adipocytes (81). However, this finding contrasts with the observation in human adipocytes where most fatty acids stimulate triglyceride storage (40). AA was shown to inhibit adipocyte differentiation via protein kinase A (PKA) (82). In the presence of non-steroidal cycloxygenase (COX) inhibitors, AA also decreases adipogenesis (83, 84). The inhibitory effects of FAs on adipose differentiation are exerted via decreases in PPAR-γ, C/EBP-α and SREBP-1c gene expression (85).

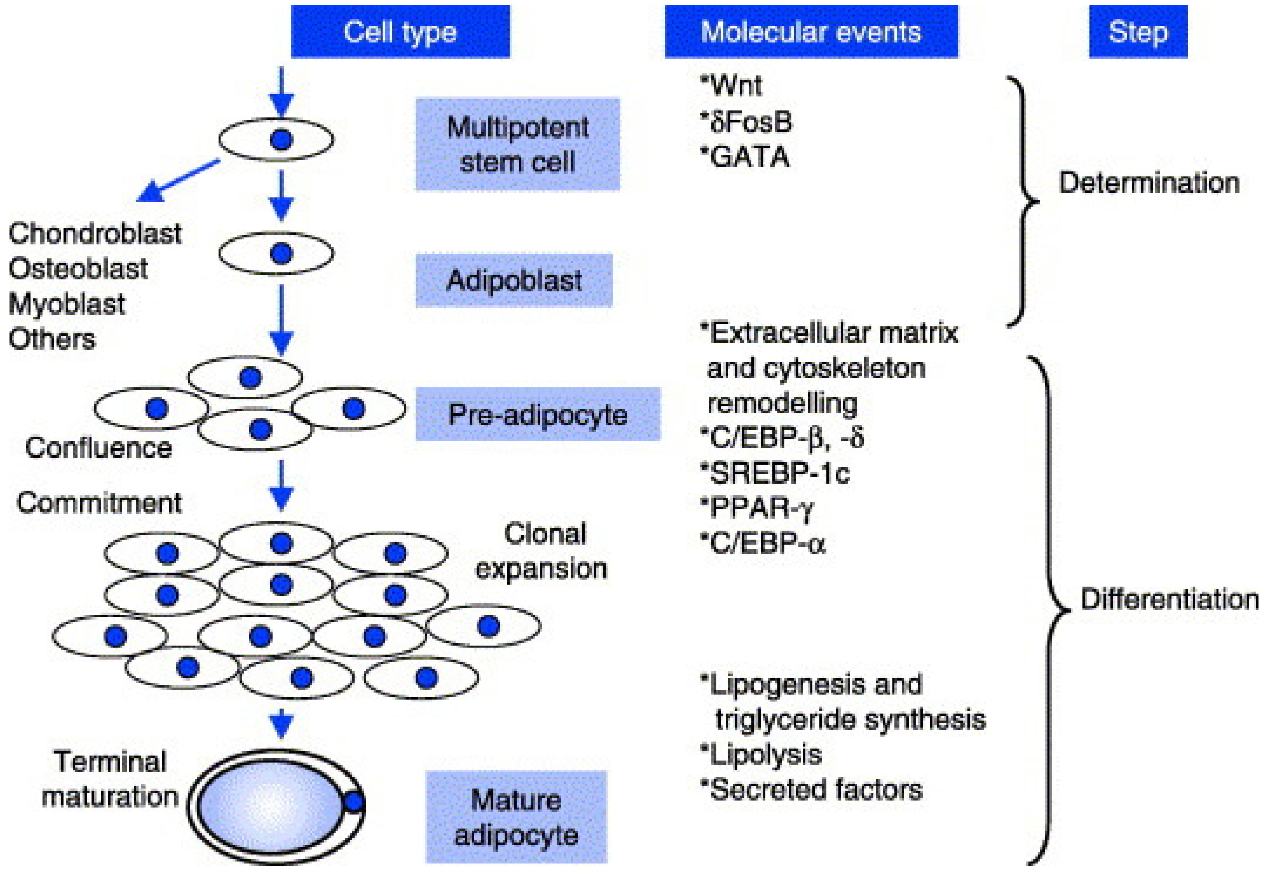


Fig. 2.1: Representation of different phases of adipogenesis according to Fève (2005) (40).

2.4. Types of FAs:

FAs are compounds composed of a carboxylic group linked to hydrocarbon chains of different lengths. They are classified according to:

(i) The number of carbon atoms: long chain FAs (LCFA), those with more or equal to 14 carbons; and short chain FAs (SCFA), those that have less than 14 carbons.

(ii) The number of double bonds present in the hydrocarbon chain: SFAs, are FAs with no double bonds; and USFAs, if they have double bonds. Monounsaturated FAs have one double bond, and PUFAs have more than one.

(iii) The position of the first double bond from the methyl-terminal of the FA e.g. omega-3 or n-3 are fatty acids where the first double bond from the methyl-terminal of the FA is localised at carbon three; omega-6 or n-6 where the first double bond from the methyl-terminal is in position six.

(iv) The need and capacity of the body to synthesise them: The body cannot synthesize essential FAs or their synthesis is lower than their need in the body. The opposite of this group are the non-essential FAs.

2.5. Synthesis of UFAs in humans

Humans have the capacity to synthesize a variety of SFAs and some UFAs.

Palmitic acid, a SFA, is the first to be synthesized. From this, other FAs are synthesized by elongation and desaturation process, and major products are stored in the endoplasmic reticulum. Because mammals, including humans, do not have the enzymatic capacity that is responsible to insert a double bond in the position n-3 and n-6 of the fatty acid (n-12 and n-3 desaturase activities) (86), they cannot produce linoleic acid (LA) (18:2 n-6) and α- linolenic acid (ALA) (18:3, n-3) from precursors. These FAs are considered essential for the human, and must be provided in the diet. LA is found in large amount in seeds of most plants except coconuts, cacaos, and palms. ALA is abundant in flaxseed and chloroplasts of green leafy vegetables.

Normal human adults synthesize enough AA (20:4,n-6) for his needs, if its precursors, LA and ALA, are included in the diet in sufficient amounts to cover their needs (87). Thus, in the condition described before, AA is not an essential FA. But during growth (pre- and postnatal), AA is considered essential because the synthesized amount does not meet the need. Therefore, beside the inclusion of LA and ALA in the diet, AA should be supplemented. The synthesis of AA and other eicosanoids, e.g. eicosapentaenoic acid (EPA) (20:5,n-3), involves a series of elongation and saturation enzymes. The synthesis of 22:6,n-3 dicosahexanoic acid(DHA) requires synthesis of 24:6,n-3 in the endoplasmic reticulum followed by chain shortening via one cycle of β-oxidation (88). The desaturation steps, especially n-6 desaturase, are generally slower than the elongation steps, therefore, these desaturase steps are rate limiting of the pathway (89).

2.6. Function of FAs in the body

FAs and /or their derivatives play a variety of roles in the body. They can be used as: (a) metabolic fuels, (b) components of cell membranes, (c) precursor of eicosanoids (local acting substances, e.g. prostaglandins), (d) as second messengers in intracellular signal transduction, and (e) gene regulators of adipose tissue development (62, 90 – 96).

(a*) Role of FAs as fuel and energy stores*

Under normal conditions, adipocytes store more than 95% of total body triglycerides (96). This stored lipid is the main source of FA for the body during fasting. In two different studies (in 1993 and 1997), Raclot and his colleagues (97, 98) demonstrated that both in rodents and humans, SFAs are preferentially stored and they are also more difficult to mobilize from adipose depots than UFAs.

After a meal, the level of both glucose and FAs rise in the circulation (99). LCFAs are transported in the plasma in the form of triglycerides bound to lipoprotein, while circulating SCFAs are bound to albumin. Through the action of lipoprotein lipase, FFAs are formed from lipoproteins in the circulation and bound to albumin. They traverse the endothelial cell layer by an undefined manner and interact directly with the plasma membrane to stimulate FA uptake by the cells (100). Depending on the dietary FA class and the necessity of FAs in the body, some FAs from the diet are immediately used. Excess FAs are stored in the esterified form with glycerol in adipocytes.

In the case of FA need, triglycerides are metabolized by the action of hormone sensitive lipase, producing FFAs and glycerol. FAs are then exported to other tissues where, through the process of β-oxidation, they produce energy. The most readily mobilized FAs are EPA and AA (101).

In 1993, Boden and colleagues (102) observed that insulin suppresses oxidation and release of FAs from adipocytes as well as the reesterification of FAs in the circulation.

(b) The role of fatty acids in membrane composition

The biological membrane is a structure that limits and compartmentalizes the cells. It is basically composed of a phospholipid bilayer with some steroids (e.g. cholesterol, in mammals including humans) and a variety of immersed proteins that function as receptors, enzymes, transporters or ion channels (103). The plasma membrane (the membrane that individualizes the cell) has a small amount of carbohydrates attached to the outside (87). One of the phospholipid components are FAs. The FA composition of the membrane defines the properties of the respective membrane, for example, its fluidity, flexibility and permeability. These properties crucially affect the activity of receptors, enzymes (such as ATPases) or ion channels in the membrane (25, 26). Manipulation of dietary lipid content in both experimental animals and humans affects the FA composition of membranes (87, 90, 104, 105). As a result, the cell changes the way that it responds to different stimuli.

(c) Eicosanoid synthesis

Eicosanoids are derivatives of 20-carbon essential FAs, e.g. AA, in the body. These substances have biological effects. AA is one of the major (it may account for as much as 25 % of all phospholipid PUFAs present in mammalian cells (22). It is synthesized in the liver of mammals from dietary LA (18:2) by elongation and desaturation (88, 106). AA is transported in plasma to the various tissues bound to serum albumin or lipoproteins (106). The level of AA in serum is low relative to other FAs acids except in obesity and diabetes where levels can be significantly elevated over normal matched controls (95, 107). Additionally, many cells possess a high affinity arachidonyl-CoA synthetase (22) that facilitates selective accumulation of AA even when other FA species are in excess.

As result of different stimulating factors e.g. hormones and stress, AA is mobilized from the membrane by the action of phospholipase A_2 and it is used to produce derivatives with various physiological roles. Cyclooxygenase (COX, existing as two isoenzymes: COX 1 and COX 2) is the main enzyme that transforms AA to a variety of PGs and thromboxanes. Indomethacin (IDM) inhibits COX by competing with AA (18). Other important biological metabolites of AA are formed through the activity of different lipoxygenase (L): L-5, L-12 and

L-15. These enzymes catalyse the transformation of AA to lipoxins in leukocytes. Lipoxins seem to have a bronchoconstrictor and vasoconstrictor action. L-5 is the only lipoxygenase responsible for the synthesis of leukotrienes. This is one of the substances involved in immunologic events (88). Nordihydroguaiaretic acid (NDGA) is a selective inhibitor of the lipoxygenases.

Eicosanoids are also synthesized using AA from the diet (26). In this case, the enzymes involved are the same (COX and L) and, the products are also similar to those produced during metabolism of membrane AA.

The derivatives of AA have the ability to transduce signals via: (i) Gs protein, so elevating cAMP levels, (ii) Gi protein, with consequent reduction of cAMP, and (iii) the phosphoinositide (PI) signaling system. The fact that PGs affect different signal transducer pathways explains the variety of PG effects (89, 108).

(d) Second messengers

PUFAs themselves are also implicated in the second messenger signaling process within the cell. PUFA derivatives affect: (i) diacylglycerol (DAG) release from PI during the course of inositol signaling. In turn, DAG affects the activity of PKC, an important enzyme that regulates the activity of other enzymes by phosphorylating them (89, 109, 110); (ii) the activity of PKC directly (11); (iii) the proteins Gs and Gi that modulate cAMP levels; (iv) the insulin receptor which influences the PI-3 kinase system.

(e) Modulation of gene transcription

FAs and their derivatives (eicosanoids) can interact with specific nuclear receptors thereby regulating gene expression (110). The regulatory effect of FAs might be either stimulation or repression of certain genes. The nature of the effect depends on the transcriptional factor, and the respective binding element involved (111).

Types of PPARs with a role in FA metabolism, their distribution and function

PPARs are nuclear hormone receptors which use derivatives of LCFA (e.g. prostaglandins) as their ligand (113). Three functional receptors are known, namely, PPAR-α (NR1C1), PPAR-β (NR1C2) and PPAR-γ (NR1C3) (113). Although these PPARs are encoded by separate genes, their structure is similar - six structural regions (A-F) grouped in four functional domains: A ligand-binding domain (E/F), a DNA-binding domain (region C) and two domains which modulate function (region A/B and D) (114). PPARγ and PPARα are involved in lipid metabolism.

Localization and function of PPAR-γ and PPAR-α

The PPARs are encountered in all body tissues in different quantities. PPAR-γ is abundant in white adipose tissue and large intestine; while the kidney, liver and small intestine have moderate amounts, and in the muscle there is very little (114). As reviewed by Guo and Tabrizchi (114), seven isotypes of PPAR-γ (1 – 7) have been identified.

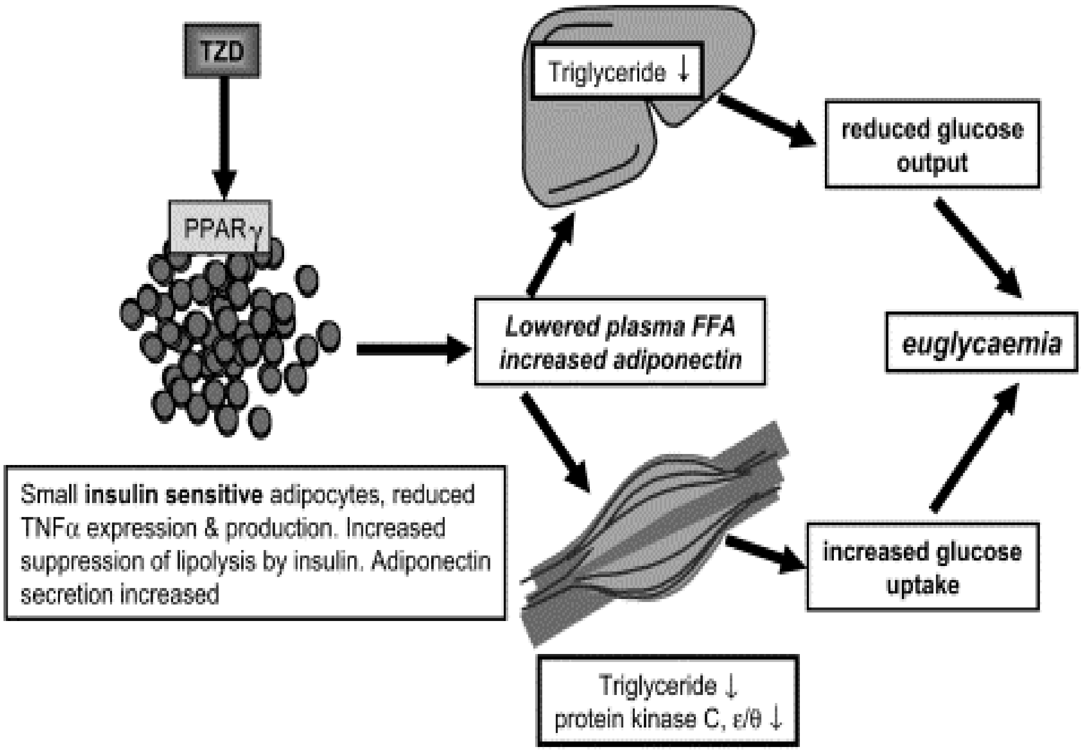
In adipose tissue, PPAR-γ has been shown to contribute to the control of adipocyte differentiation (111, 115, 116). This receptor also influences the storage of FAs by inducing lipoprotein lipase and FA transporters, as well as inhibiting cytokines and COX2- expression (116, 117). Furthermore, PPAR-γ appears to play a role in the both development and treatment of IR. This is supported by the fact that, on the one hand, PPAR-γ is involved in the development of IR via cytokines (70, 118), and, on the other hand, the crucial function of this receptor in the mechanism of drug action (e.g. thiazolidinediones (TZDs), that are used to treat IR. TZDs improve glycaemia by lowering glucose levels and insulin sensitivity in both rats and humans with T2DM diabetes by activation of the PPAR-γ receptor in adipocytes both "*in vitro*" and "*in vivo*" (120). The mechanism by which TZDs alleviates T2DM is summarised in Fig. 2.2. The activation of PPAR-γ in adipocytes leads to two positive consequences for type 2 diabetics. Firstly, TZDs stimulate adipogenesis (40). It increases the number of small insulin sensitive adipocytes as well as the expression of certain adipose-tissue-specific genes important to sustain triacylglycerol synthesis and storage, e.g. lipoprotein lipase, fatty acid binding protein (FABP), specially the aP2 and phosphoenolpyruvate carboxykinase-C (facilitator of glyceroneogenesis). Also, PPAR-γ activation affects metabolism of fat cells (120) such as increasing insulin-stimulated glucose transport and reducing the rate of FFA release, both of which have important implications in IR. Several reasons have been suggested by Smith (119) to explain the reduced levels of FFA in circulation: (i) an increased number of small insulin sensitive adipocytes; (ii) high rate of re-esterification of FAs acids, a consequence of overexpression of glycerol kinase in adipocytes, and (iii) suppression of lipolysis through reduction of expression of TNFα gene and its activity, as well as by positively affecting the insulin receptor substrate-1 (IRS-1). It has been well documented that the beneficial effect of TZD on insulin sensitivity in liver and skeletal muscle is due to the action of this drug on adipose tissue (119). The lower FFA plasma levels lead to a reduction in the glucose output and triglyceride content in the liver as well as empowering glucose uptake and insulin signalling in the muscle by decreasing FFA-induced inhibition of PKC. In summary, TZD is helpful in the treatment of T2DM but it might cause obesity. This predisposes the patients to other consequences of obesity, such as cardiovascular problems (114).

Another important response element in lipid metabolism is PPAR- α. It is found in relatively high concentrations in the liver, lower concentrations in the kidney and brown adipose tissue and least concentrations in the heart and intestine. This receptor controls the synthesis of lipids in the liver (111). PPAR-α regulates expression of genes implicated in glucose and lipid metabolism as well as in FA, FABPs and fatty acyl-CA synthesis (114).

Endogenous and exogenous ligands of PPARs

Various FAs have been shown to activate PPARs, these include: γ-linoleic acid (GLA), AA, LA, ALA, EPA, DHA, oleic acid, elaidic acid, palmitic and stearic acid (112, 117, 120, 121). The n-3 PUFAs (EPA and DHA) have been shown to have rapid effects on gene expression. Changes in levels of mRNA encoding numerous lipogenic enzymes can be detected within 9 hours after feeding animals with diets rich in n-3 PUFAs (122, 123). Prostaglandins, metabolites of PUFA, can also ligate with PPAR-γ, thus inducing adipogenesis (66). TZDs (troglitazone, rosiglitazone, pioglitazone), are drugs that improve IR by binding and activating PPAR-γ (119). Fenoprofen and ibuprofen are also ligands of PPAR-γ (124).

There is strong evidence that FFAs can modulate gene expression by binding either cytoplasmic or nuclear steroid hormone receptors (125). In turn, the steroid hormone, either bound or unbound to the receptors, can influence the synthesis and activities of diverse enzymes involved in releasing, uptake or synthesis of FAs (125).



Activation of PPARγ in fat increases the number of small, insulin sensitive adipocytes. This, together with reduced TNFα expression and activity, decreases FFA release into the circulation. Liver triglyceride and glucose output is reduced. Reduced FFA supply to muscle increases glucose uptake and potentiates insulin signalling by reduced activation of inhibitory PKC isoforms. Beneficial effects of reduced FFA are reinforced by a simultaneous increase in adipose-derived adiponectin secretion.

Fig. 2.2. . Mechanism for reduction of hepatic and muscle IR by activation of PPAR-γ in adipose tissue, according to Smith (119).

2.7. FA transport into adipocytes and its control by insulin

The ability of FAs to cross the adipocyte plasma membrane is critical not only for the maintenance and mobilization of stored energy reserves but also for the ability of the cell to respond to the changes in extracellular FA concentration in order to mantain homeostasis and all functions that FAs play in the body (126). Specialized membrane proteins and cytoplasmic proteins carriers are utilized to facilitate the process (106). Hormonal and feedback regulation have been reported to be involved in the regulation of FA (34).

2.7.1. Types of FA transport through the adipocyte plasma membrane

For a long time, FA transport through the membrane was considered an entirely passive (flip-flop diffusion) and unregulated process because of the hydrophobic property of FAs and the nature of the plasma membrane (27, 28, 29). Since the first reports of Abumrad and colleagues in 1981 about membrane proteins capable of binding FA, it is believed that facilitated transport of these amphipatic compounds can also take place during uptake and efflux (31, 32).

The evidence of involvement of plasma membrane proteins in the uptake of FAs was observed in adipocytes and other cell types with high rates of FA metabolism, e.g. hepatocytes and skeletal myocytes (34). FA uptake via a saturated pathway was shown to be higher than 90% in the adipocyte. This requires plasma membrane rafts (34, 127). The precise involvement of the protein carriers of FAs is not yet fully understood. The consensual belief is that some protein (either in the membrane or in the cytosol) has a dual function to allow FA uptake and / or its efflux (transport from intracellular environment to extracellular) for the following reasons: (i) the high expression of a protein with the capacity to bind FAs or its derivatives in cells involved in lipid metabolism, and (ii) the positive correlation observed between phenomena of recruitment of FA transporters from the cytosolic vesicles where they are stored for the plasma membrane (due to certain stimuli such as insulin) and FA uptake (128, 129, 130).

2.7.1.1. Protein carriers of FAs in the adipocyte plasma membrane

Five plasma membrane proteins have been proposed to facilitate FA uptake in the adipocyte. They are: (i) plasma membrane fatty acid binding protein (FABPpm) (131), (ii) fatty acid translocase (FAT) (132), (iii) 22-kDa 3T3-L1 adipocyte plasma membrane caveolin (133), (iv) the scavenger receptor FAT/CD36 (132), and (v) fatty acid transport protein (FATP 1 and 4) (30).

i. FABPpm

FABPpm was first isolated both in liver cells and adipocytes (132, 133). This plasma membrane protein was shown to be identical to mitochondrial aspartate aminotransferase (mAspAT), a protein which binds to the inner mitochondrial membrane and is associated with the α-ketoglutarate dehydrogenase complex (134 – 136).

ii. FAT

FAT is a 88 kDa plasma membrane glycoprotein in adipocytes. In humans, FAT is associated with CD36 (132). It is part of an extracellular lipid binding domain, therefore facilitating the clearance of oxidized lipoprotein particles (132, 137).

iii. Scavenger receptor FAT /CD36

FAT/CD36 facilitates LCFA transport across the plasma membrane (137, 138). This protein is localised specifically in lipid rafts in the plasma membrane (139, 140). Rafts are membrane micro-domain enriched in sphingolipids and cholesterol and form a liquid-ordered subdomain with specific type of protein, while caveolae are distinct rafts that form invaginations into adipocytes (141). This is critical for LCFA binding since its disruption abolishes binding of LCFAs to FAT/CD36 (143, 144). This observation was similarly done in human skeletal muscle cells (145). Sulfo-N-succinimidyl oleate is a specific inhibitor of FAT/CD36 (138). Certain scientists have used this inhibitor to study the function of FAT/CD36.

iv. 22-kDa 3T3-L1 adipocyte plasma membrane caveolin

Trigatti and colleagues first identified the 22-kDa 3T3-L1 adipocyte plasma membrane caveolin. This protein was shown to be capable of binding a photoreactive FA analogue with high affinity, and possibly contribute to its transport, as reviewed by Bernlohr in 1997 (145). Caveolin, also known as plasmalemmal vesicles, form invaginations in the plasma membrane of many different cell types, including adipocytes.

v. FATP

Both in humans and mice, six types of FATP have been identified (34, 126). FATP1 and FATP4 are the FATPs present in the adipocyte (30, 138, 146, 147). These fatty acid transporters are present in plasma membrane and microsomal fraction (96) as well as endoplasmic reticulum (143).

FATP1 has been the most studied. This protein weighs 63 kDa, encodes 646 amino acids and its Michaelis Menten constant (K_m) for oleic acid is 200 nM (30). The structure of FATP 1 (Fig.2.3) was predicted by Lewis *et al* in (2001) (148), and it is believed that the other members of the family also share the same structure.

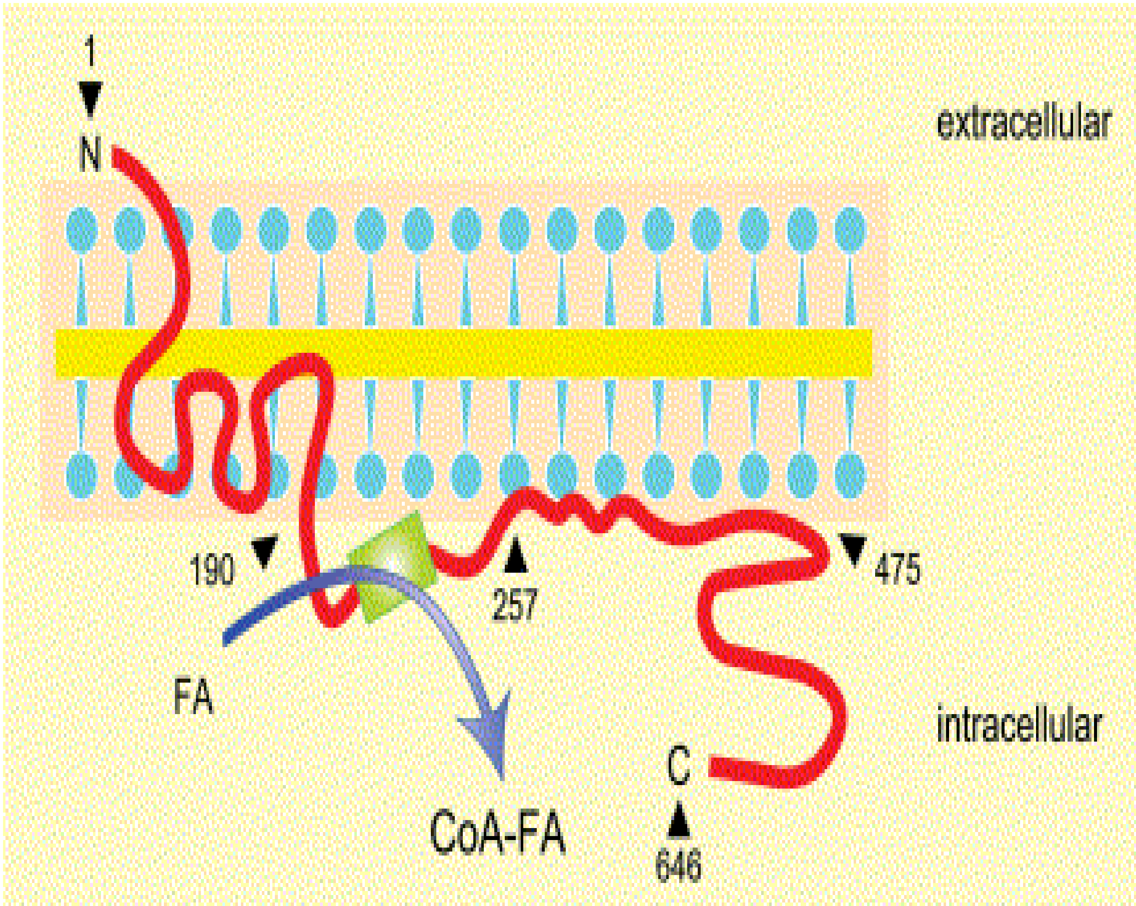


Fig. 2.3: Membrane topology of FATP as proposed by Lewis *et al.* (148). FATP is a transmembrane protein, with a short segment of the amino terminus facing the extracellular side of the membrane bilayer, while the C-terminus is located in the cypoplasm. Amino acid residues 1-190 of FATP1 are integrally associated with the membrane. Amino acid residues 190-257 are cytosolic and it is the AMP-binding motif that mediates acyl CoA-synthetase activity (149). Amino acid residues 258 - 475 are peripherally associated with the inner leaflet of the plasma membrane. There are homodimeric complexes in FATPs that interact to form a cytoplasmic loop (150).

Mechanism of FATP action and model of FA uptake in the membrane

The mechanism for LCFA uptake by FATPs for the other kinds of FA transporters listed above is poorly understood as yet. Given that the uptake of FA shorter than 10 carbon atoms is not affected by FATP expression, the activity of the fatty acid transport system in the membrane is utilized for LCFA uptake (30, 151). Nevertheless, specific binding sites for LCFA within the FATP structure are still to be identified (34). There is evidence that the LCFA transported by FATP1 are preferentially driven directly to triglyceride synthesis because FATP1 has shown to have acyl-CoA synthetase activity (149, 152). However this supposition was challenged by studies on the yeast FATP gene (153) where the FATP1 was shown to have an independent function to acyl CoA synthetase.

A model for LCFA uptake

The uptake of LCFA into adipocytes can be compared with what occurs in myocytes (34) illustrated in Fig. 2.4.

FAs destined for adipocytes circulate as triglyceride bound to lipoprotein particles. Hydrolysis of lipoprotein triglycerides occurs under the action of lipoprotein lipase. The liberated FAs are bound by albumin and transported across the endothelial cell layer by an unknown mechanism (128). The concentration of FFAs outside the cell is in the nanomolar range because of the high concentration of serum albumin in the extracellular space and its binding constants for FAs (154). Thus, the dissociation of FAs from albumin outside the plasma membrane, is facilitated by membrane-associated proteins such as FAT/CD36 associated with the raft in the plasma membrane, an important structure for LCFA binding and uptake (140). FAT/CD36 might deliver LCFAs directly to FATPs for transport across the plasma membrane, as suggested by Stahl in 2004 and Stahl *et al* 2001 (138, 147). On the other hand, FAT/CD36 can also interact with FABPpm at the plasma membrane to facilitate the uptake of LCFA (137). Alternatively, FA may be protonized and integrated into the outer phospholipid bilayer. This consequently creates a concentration gradient toward the inner leaflet, and FAs might flip-flop across (31). Once the FA is on the inner side of the cell membrane, it might be directed to two principal pathways. (a) Synthesis of triglycerides: at the plasma membrane level LCFAs might be activated by FATP to acyl CoA, which are then bound to cytoplasmic acyl-CoA-binding protein (ACBP) and channelled into triglyceride synthesis or, (b) other lipid metabolic pathways, for example: after LCFAs enter the cell, they might subsequently activate carnitine and the acyl-CoA transporter protein involved in translocation of FAs to the mitochondria (where β-oxidation occurs) and to the peroxisomes (site of synthesis of FAs and their derivatives), respectively (34).

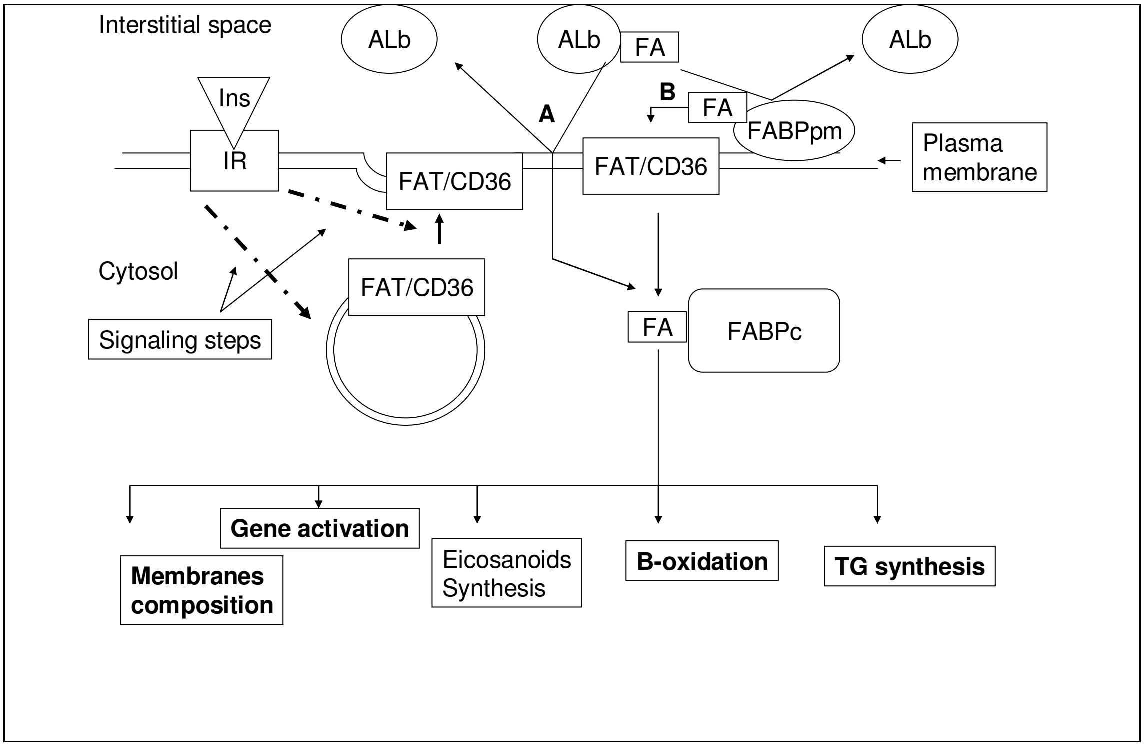


Fig. 2.4: Transport of FAs through the plasma membrane and trafficking in the cell. IR: Insulin receptor, FA: fatty acid, FABP: fatty acid binding protein, Ins.: Insulin, FAT/CD36: fatty acid translocase CD36, ALb: albumin, TG: triglycerides, A: simple diffusion and B: facilitated transport. Designed according to Bernlohr et al. (1999) and Bonen *et al.*) (126, 130).

Regulation of expression of FATPs and regulation of FA uptake

Several sources of evidence point to PPAR as playing an important role for the regulation of FATP expression. This postulate is supported by the fact that a

PPAR binding site was identified in the FATP1 promoter (155) as well as by the positive regulation of FATP observed when ligands activate PPAR-γ (156). PPAR-γ also appears to be involved in adipogenesis in human tissue (47). Fatty acids and their derivatives are ligands for PPARs (66) therefore promoting a positive feedback regulation of expression of their transporters. This allows the cells to import LCFAs, since they are present in the plasma.

The negative regulators of FATP1 mRNA levels reported in adipocytes are: insulin, endotoxin, tumor necrosis factor (TNF) and interleukin (IL)1 (157, 158). Although, it is reported that insulin stimulates FA uptake (18, 97), the process by which insulin stimulates FA uptake in target tissues is tissue-specific (137). Insulin (10 nM for 30 min) improved palmitate uptake in cardiac myocytes by inducing the translocation of FAT/CD36 from an intracellular depot to the plasma membrane (137). In contrast, insulin increases LCFA uptake in adipocytes within 60 min by inducing recruitment of FATPs from an intracellular perinuclear compartment to the plasma membrane (18, 96). This finding leads to the conclusion that LCFA uptake and glucose uptake are similar in the way that they are regulated by the same hormone although FATP1 and GLUT4 are localized in different intracellular vesicles. In addition, insulin was reported to profoundly suppress FA export from adipocytes (102, 159). This was accompanied by increased expression of the FABPpm gene and the amount of its protein in the plasma membrane (159).

Protein FA carriers in adipocyte cytosol

In the cytosol, lipid-binding proteins (LBPs) have also been identified (160). The first discovery of these proteins was done by Ockner *et al* in 1972 (161). Fatty acid-binding proteins (FABP) belong to the intracellular lipid-binding proteins having molecular masses around 15kDa found in the animal kingdom (162, 163). Several subfamilies of LBPs have been identified. WAT contains two of them: (i) adipocyte lipid binding protein (A-LBP or aP2), and (ii) keratinocyte lipid binding protein (K-LBP) also known as epidermal-type (E-LBP) in the proportion of 99:1 (128). In most cases, the expression pattern of the LBPs, including the specific LBPs encountered in adipocytes, is similar in all vertebrates (164).

Beside the role of LBPs on transport and direction of FAs to different metabolic pathways (an aspect directly related to the present work), diverse functions have been proposed for these proteins, including maintenance of cellular uptake of FAs, protection of the cell from damage by an excess of these amphipathic molecules; creating a large cytosolic pool of FAs and participation in the regulation of gene expression and cell growth (145, 165).

Action of cytosolic FABP in adipocytes

FABPs are responsible for maintaining the cellular uptake of FAs. This is possible because they increase the concentration gradient of fatty acid, due to minimizing unbound FA in the cell (145). Notwithstanding this fact, there is evidence that A-FABP is not rate-limiting in cellular FA uptake (165).

A-LBP is a transporter between intracellular compartments. This is supported by the fact that FFAs accumulate in cytoplasm when their transport to storage or export is disrupted, and the reduction in lipolysis observed when there is a lack of A-LBP (149). Furthermore, A-LBP and E-LBP interact (bind and activate) with hormone sensitive lipase (HSL), and the rate of lipolysis in adipocytes depends on the total LBP concentration. However, it is independent of the particular type of LBP (167 -169).

FABPs direct FAs to different pathways in the adipocyte. The routes are as follows:

1- Transport of FAs from the plasma membrane to acyl CoA synthetases, present on the inner parts of the plasma membrane, readily for utilization for triacylglycerol synthesis.

2- During lipolysis, transport of FAs from the droplet surface where the active HSL resides, to the plasma membrane for export. This function also contributes to avoid inhibition of HSL by the products of lipolysis.

3- Transport of FAs or their metabolites to nuclear sites (PPAR).

4- Transport of FAs for delivery to mitochondria for β-oxidation.

Structure of FA binding proteins:

The FABPs have a common structure, characterised by a β-barrel structure formed by two orthogonal five-stranded β-sheets (170). The binding pocket is located inside the barrel, and usually has one or two conserved basic amino acid side chains that bind the carboxylate-group of the FA ligand. The opening of the binding pocket is framed on one side by the N-terminal helix-turn-helix domain (85, 171).

Mechanism of FABP action

All identified FABPs bind LCFA, though they have differences in selectivity of the type of ligand, the binding affinity, and the binding mechanism (85). The binding process is usually as observed with oleic acid, a U-shaped entity. Some fatty acids, such as DHA, are bound in a helical conformation (172, 173). The dissociation constant for LCFA is in the nano- to micromolar range (85).

The mechanism and kinetics of FA binding and release differs. Most FABP-types exchange FAs with membrane structures by collision transference, facilitated through electrostatic interactions between the basic amino acid side chains in the helix-turn-helix that is part of the ligand portal region in the FABP (174).

Control of FABP gene expression:

Often FABPs are overexpressed in tissues which have high capacity of biosynthesis, storage, or breakdown of lipids e.g.: hepatic, adipose and muscle (cardiac and skeletal) tissue (175). In these tissues, the content of the respective FABP type's is between 1% and 5% of all soluble cytosolic proteins (160). The FABP content in these tissues increases considerably when they are exposed to prolonged elevated extracellular lipid levels, as observed during endurance training or pathological nutrient changes seen in DM (175, 176).

All FABP genes have a TATA box, followed by a conserved gene structure, which includes three introns of variable length, separating the coding sequences. The FABP genes have enhancer elements which control the expression of respective FABPs (177).

The regulation of the peroxisome proliferator response element (PPRE) by FAs is important because it was shown to be involved in up-regulation of L and A-LBP, by a mechanism not yet elucidated (178-180).

2.8. Relation of FAs to T2DM

Problems of FA transport (uptake and/or efflux) and their disturbed metabolism that increases the concentration of plasma non-esterified FAs can play a central role in the pathogenesis of obesity and non-insulin-dependent DM (181, 182). A cross-sectional study by Pohl *et al* 2004 (34) has found an inverse relationship between fasting plasma free FA concentration and insulin sensitivity. Furthermore, McGarry *et al*., (183) described a strong relationship between accumulation of triglyceride and IR in skeletal muscle.

Studies conducted in rats demonstrated that both the amount and type of FAs ingested alter insulin sensitivity in target tissues (i.e., muscle, adipose tissue and liver) associated with T2DM and obesity (6). This was also observed in humans (184). Chronic exposure to n-6 UFAs caused a reduction in insulin-stimulated glucose uptake (ISGU) in 3T3-L1 adipocytes. This was a result of a decrease in the cellular amount of GLUT4 by inhibition of GLUT4 gene expression (22, 184). *In vitro* studies demonstrated that n-3 UFAs also reduce the metabolic effect of insulin in rat adipocytes (185, 186). Diets high in saturated fat (range 40 to 75% of total kilocalories) reduce whole body ISGU, (184, 187). This was also observed by Hunnicutt *et al* in 1994 (188) when isolated rat adipocytes were treated for 4 hours with 1 mM palmitate.

IR develops in most cases where the visceral triglyceride store of subjects is increased. As a consequence, lipolysis is high and provides another source of mostly SFAs to the body (9, 11). In obese IR subjects a large amount of FAs released by intravascular lipase and by HSL go into the circulation. Thus, FFAs could promote and perpetuate the IR state (9, 189, 190, 191). In contrast, Storlien and his colleagues in 1986 and in 1991 (6, 23) demonstrated that diets rich in UFAs (especially omega-3), improve insulin sensitivity in skeletal muscle. The same trend was observed in adipocytes by Grunfeld *et al*. in 1981 (107).

Changes in membrane phospholipid composition may affect several metabolic processes, including the effect of insulin (13). For example, studies related to Na^+-K^+ pump activity versus membrane properties has also demonstrated the positive correlation between Na^+-K^+ ATPase localized in the membrane with membrane fluidity, defined by its phospholipid composition (25, 26).

As discussed in 2.5. e), FAs can ligate with the PPAR DNA transcription factor so activating or suppressing certain genes. In 1994 Tebbey *et al* (22) demonstrated that AA (50 µM) could reduce (by approximately 91%) the cellular content of GLUT4 mRNA in 3T3-L1 adipocytes after 48 hour of exposure. Two mechanisms were identified by this group of investigators to be involved in this phenomenon: (i) reduction of gene transcription by 50% and (ii) a decrease in the half-life of GLUT4 mRNA from 8.0 hours to 4.6 hours. Additionally, Long & Pekala (192) proposed a third mechanism by which AA and various LCFAs alter the occupation of a PPRE in the GLUT4 promoter by a complex protein that is still to be identified. In contrast, Tebbey *et al* (22) observed that AA increased the cellular amount of GLUT1 mRNA by 65% by stimulating both transcription and stability of mRNA. Thus, although AA had no effect on total cellular GLUT4 content, significant enhancement of glucose uptake was observed as a result of increasing total GLUT1 transporter and its increased activity in response to insulin (22). In the case of treatment with AA for less than 4 hours, the improvement of glucose uptake resulted from the recruitment and consequent increase in glucose transporters in the plasma membrane (18).

According to Shulman (193), defects in the adipocyte lead to increased FFA delivery to liver and muscle, where they might induce IR. This researcher reports that the level of intracellular oxidation of fatty acid is also correlated with IR. High levels of intracellular diacylglycerol and fatty acyl CoAs were shown to activate a serine / threonine kinase cascade. The activation of this cascade, possibly initiated by protein kinase (PK) C, led to phosphorylation of serine / threonine sites on the insulin receptor. As a consequence of this phosphorylation, the insulin receptor reduces its ability to associate and activate PI 3-kinase (PI 3-K). All these processes have as final result a decreased activation of glucose transporter activity.

Taken together, there are three mechanisms by which FFAs could promote T2DM:

1. The action of excessive FA oxidation products or intermediary metabolites. Two possible mechanisms are possible: (a) inhibition of pyruvate dehydrogenase activity, the rate-limiting enzyme of glycolysis, by excess acetyl-CoA. This has the consequence of inhibiting glucose uptake; (b) reduction of ability of the insulin receptor to associate and activate PI 3-kinase, due to activation of phosphorylation of serine / threonine sites on the insulin receptor by excess of intracellular diacylglycerol and fatty acyl-CoA; This results in decreased activation of glucose transporters and other related downstream events (193, 194).

2. The modulating effect of FFAs, both saturated and unsaturated, and their derivates on PPAR-γ with consequent stimulation of adipogenesis (Fig.2.5), especially the increase of large insulin resistant adipocytes (40), and alteration of expression of the genes related to the glucose transporter (GLUT4 rather than GLUT1) and other genes related to lipid metabolism (40, 61).
3. Effect of FAs on membrane fluidity. This may influence membrane protein activity and thus affecting not only the insulin receptor, but also FA and glucose transporters. The higher the SFA content of the membrane, the less is its fluidity and consequently the more impaired is the activity of proteins localized in the membranes (11, 25, 26, 193).

The probable mechanisms (Fig. 2.6) by which FAs could alleviate IR, and therefore T2DM, are the following:

1. Effect of FAs on membrane fluidity, especially PUFAs: PUFAs increase fluidity of membranes, thereby improving the activity of all membrane proteins (11, 25), specifically, in this case, glucose transporters, FATs as well as the insulin receptor with its consequent transduction of signals. Two distinct pathways involving the activation of insulin receptor were identified as significant in insulin-induced glucose transport: (a) the dependence of PI3-kinase activation followed by involvement of PKB and PKC (195, 196). These events culminate with induction of the fusion of the vesicles containing GLUT4 and those with GLUT1 to the plasma membrane (22, 197); and (b) secondly, there is a PI3-kinase independent transduction signal pathway. This involves tyrosine phosphorylation of proto-oncogene c-Cbl (CAP/cbl) and results in the activation of a small

GTP-binding protein that induces overexpression of the glucose transporters in the plasma membrane (198).

2. Stimulatory effects of FAs, both saturated and unsaturated, and their derivates on PPAR-γ. This might affect glycaemia in two ways: (a) overexpression of glucose transporter genes (GLUT4 rather than GLUT1) (119); (b) by stimulating adipogenesis, the number of small insulin-sensitive adipocytes increases (40). Associated with low TNFα expression and activity, this decreases FFA delivery into the plasma. The liver triglyceride and glucose output decrease. Reduced FFA delivery to the muscle with consequent deceleration of the inhibitory activity of PKC serves to potentiate insulin signalling and glucose uptake. Increased secretion of adiponectin (40, 67) from adipocytes and its action emphasizes the beneficial effects of a lower systemic FFA concentration. Increased adipogenesis may also increase adiponectin synthesis which seems to be beneficial to IR (84).

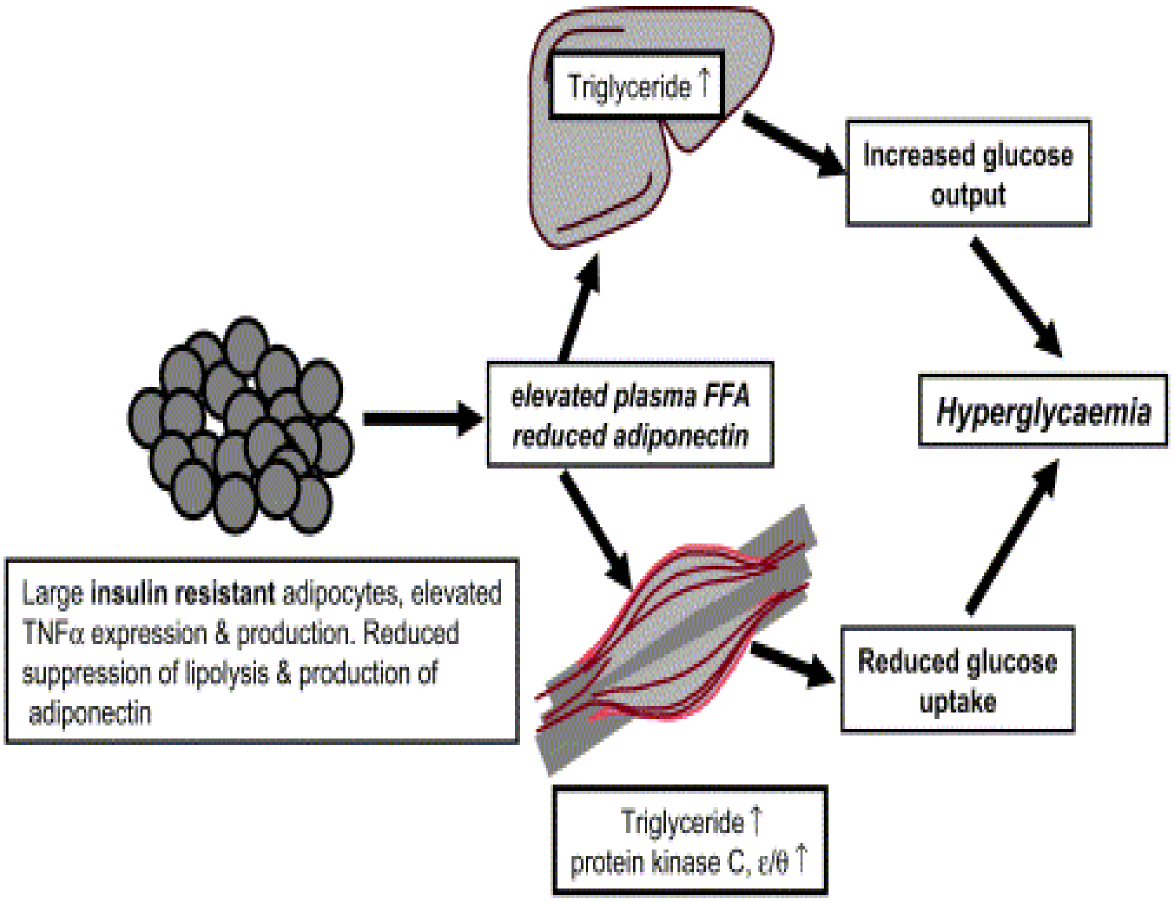
2.9. Glucose transporters in adipocytes

Adipocytes express GLUT1 and GLUT4 carriers (199, 200), but GLUT4 is more abundant than GLUT1 (199). These two transporters primarily reside in different intracellular vesicles (199, 201). In the absence of insulin, GLUT1 transporters are equally distributed between the plasma membrane and the cytoplasmatic low-density microsomes, whereas GLUT4 transporters are encountered only in intracellular vesicles (202, 203). This sequestration of GLUT4 functions as a reserve mechanism by which adipocytes rapidly may greatly increase glucose uptake and utilization in response to insulin stimulation (204).

Exposure of adipocytes to insulin has been shown to strongly increase GLUT4 (10 to 20 – fold) in contrast to GLUT1 (1.5 to 3 fold) in the plasma membrane (203).

2.10. Modulation of the level of glucose transporter expression

Expression of various transporter isoforms appears to be regulated at both pretranslational and posttranslational stages. Both in rat and human adipocytes, during fasting or DM, decreased insulin-stimulated glucose transport is observed. This may be due to decreased GLUT4 synthesis, resulting from depressed mRNA levels (200, 205). Re-feeding fasted rats or treatment of diabetic rats with insulin increases GLUT4 mRNA levels and restores GLUT4 protein levels. In contrast, GLUT1 mRNA and protein levels are unaltered during diabetes. However, with insulin treatment, GLUT1 mRNA levels in adipocytes increase while GLUT1 protein remains unchanged (203). In addition, it has been reported that adipocyte vesicles containing GLUT4 carriers possess an associated protein which specifically recognises and interacts with a cognate protein in the target membrane (207). Disorders of the plasma membrane protein in adipocytes leads to inhibition of insulin-induced translocation of GLUT4 to the plasma membrane but does not affect the recruitment of GLUT1 in adipocytes (195). There are therefore differences in the regulation of the two glucose transporter isoforms within adipocytes in response to insulin: the cellular content of GLUT4 and its translocation to the plasma membrane being rapidly and greatly affected by insulin while this hormone eventually affects only the posttranscriptional regulation of GLUT1 rather than its translocation to the plasma membrane.



Chronic elevations of plasma FFA as a consequence of insulin resistance in adipose tissues increase liver glucose production by stimulating glycogenolysis and gluconeogenesis. In skeletal muscle, FFA reduce glucose uptake and oxidation. In both tissues increased FFA promote triglyceride accumulation. Products of triglyceride catabolism, including diacylglycerol, can activate specific isoforms of protein kinase C in muscle that inhibit insulin signalling. Adverse effects of elevated FFA are amplified by reduced expression and secretion of adiponectin from adipose tissue.

Fig. 2.5: Contributing effect of adipogenesis on IR according to Smith) (120).

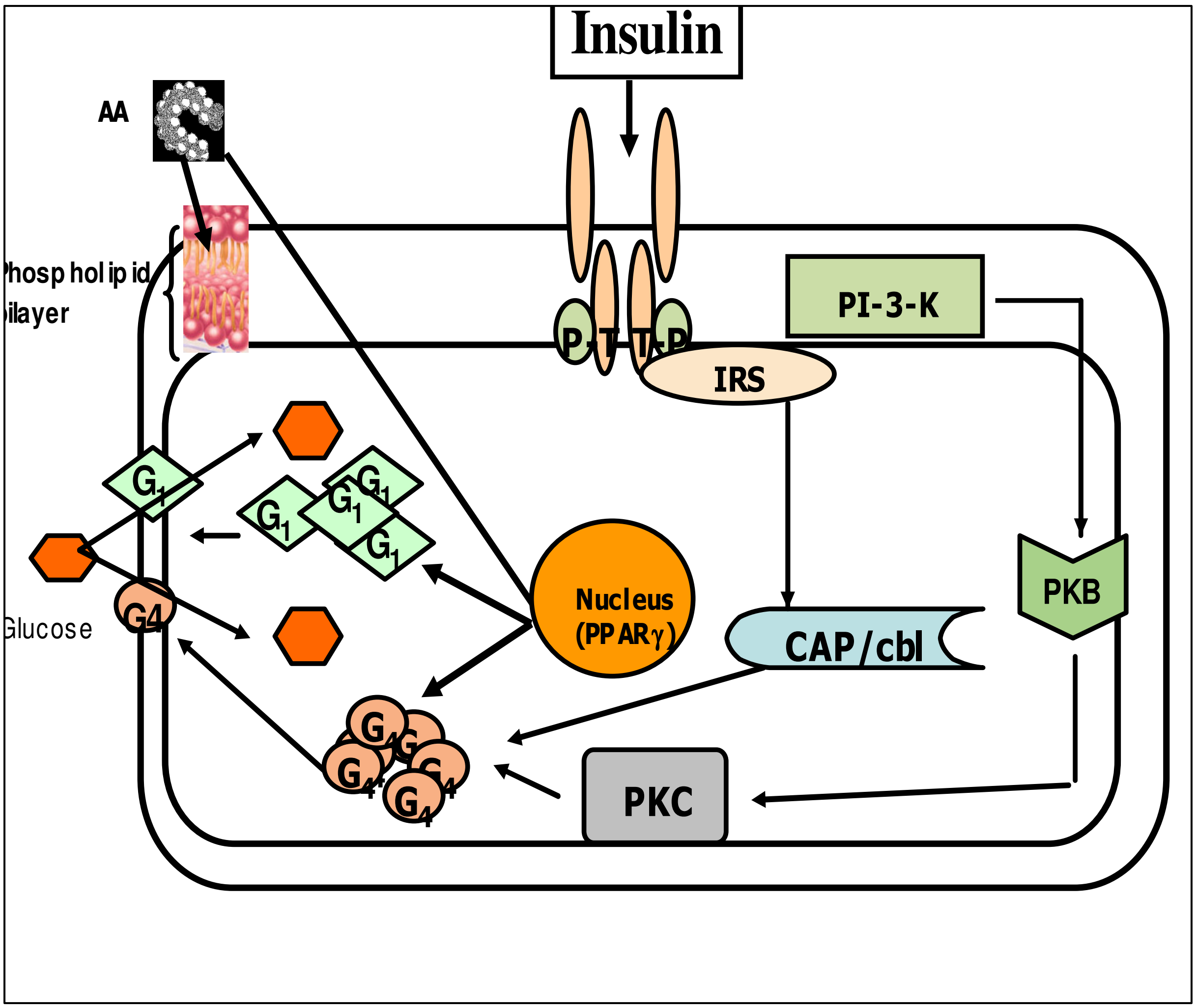


Fig. 2.6. Insulin signaling pathway to affect glucose transport in adipocytes and proposal of the mechanism by which FAs affect glucose transport. From Haag & Dippenaar (10) with modification according to Fong *et al*. (19) and Tebbey *et al*. (22): AA: arachidonic acid; IRS: insulin receptor substrate; PK: protein kinase; PI: phosphatidylinositol; CAP/cbl: proto-oncogene c-Cbl protein; G: glucose transporter.

CHAPTER 3

General experimental procedures

3.1. Materials

Collagenase CLS type I was purchased from Worthington Biochemical Corporation, Lakewood, USA; polyamide nylon filter with 400µm pore size was obtained from Neolab, Heidelberg, Germany. [1-^{14}C] AA and 2-deoxy-D-[2,6-^{3}H] glucose was purchased from Amersham Bioscience UK limited. Arachidonic acid, glucose, fatty acid free bovine serum albumin (FAFA), insulin, sucrose and all other chemicals were obtained from Sigma, St Louis, USA.

Samples of visceral or omental fat (± 100g) were obtained from 29 non- diabetic women undergoing abdominal hysterectomy in the Pretoria Academic Hospital, Eugene Marais Hospital and Femina Clinic. Ethical approval for the procedures was obtained from the Ethical Committee, Faculty of Health Sciences. Consent forms were signed by the patients prior to the procedures.

3.2. Adipocyte isolation

A. Solutions / Reagents

i. Krebs Ringer Buffer without glucose (KRB2)

The substances listed below were dissolved in double distilled and deionized water to yield the following concentrations: 25 mM Tris, 125 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 2.5 mM [$MgSO_4 7(H_2O)$], 1 mM [$CaCl_2$ 2(H_2O)]. The mixture was brought to pH 7.4 with 1 N HCl. It was stored at 4℃ and used within a week.

ii. Krebs Ringer Buffer with glucose (KRB1)

With exception of glucose, KRB1 had basically the same composition as KRB2. To prepare KRB1, 72 mg glucose was added to 100 ml of KRB2, to yield a final glucose concentration of 4mM. This buffer was also kept at 4℃ and used within a week.

iii. Collagenase solution

A solution of 3.6 mg / ml collagenase type I in KRB1 was prepared and kept at room temperature for immediate use.

B. Procedure of adipocyte isolation

Adipocytes were prepared using the method described by Schurmann & Joost (208) and Rodbell (207), with slight modifications. The principle of this method is that collagenase digests extracellular connective tissue, thus liberating cells. The procedures are illustrated in Fig. 3.1. Intra-abdominal fat tissue obtained from abdominal hysterectomy, was immediately rinsed in KRB1 to keep the cells alive. It was processed within 30 min at room temperature.

Firstly, ± 100 g fat tissue was dissected to remove as much as possible connective tissue and blood vessels. Five ml KRB1 was added to each of six sterile polypropylene tubes. Then, 5 g dissected fat was added to each tube and made up to 15 ml with KRB1 at 37 °C. The fat was minced finely with scissors. Subsequently, 5 ml of 3.6 mg/ml collagenase type I solution was added, giving a final collagenase concentration of 0.9 mg/ml. The suspension was incubated in a water bath at 37 °C for 90 min, gently mixing by inversion every 15 min.

After incubation, the collagenase solution was immediately removed by aspiration using a Hamilton syringe. Cells were resuspended in 15 ml KRB2 at 37℃, and filtered through a nylon membrane (400 µm pore size). After that, the cells were washed twice with 15 ml KRB2. Subsequently, adipocytes were centrifuged at room temperature for 30 sec at 400xg in a P-Selecta Mixtasel bench centrifuge and the oil layer discarded. Cells were suspended in 10 ml KRB2 and kept at 37℃ for 40 min to return to basal conditions (209). Then, the KRB2 was discarded and cells observed with light microscopy. Finally, isolated adipocytes were suspended in KRB2 to yield an approximate lipocrit of 30% and kept at 37℃ for immediate use.

3.3. Protein determination

Quantification of protein was done in triplicate using the spectrophotometric method of Lowry and co-workers (210), with small modifications. The principle of this method is that, in an alkaline medium, Cu^{2+} is reduced in the presence of protein (peptide bonds) and then Cu^{+} reduces the Folin reagent producing a stable blue product which absorbs at 650 mn.

A. Solutions

i. Solution A

Solution A was made by mixing three solutions: 10% Na_2CO3 / 0.5M NaOH, 1% Na^+ /K^+ -tartrate and 5% $CuSO_4$ at a fixed ratio of 10:1:0.1, respectively. A quantity [0.5 (X) ml + 10 ml] was produced immediately prior to the assay; X is number of protein determinations to be done.

ii. Folin solution

Folin-Ciocalteu's phenol reagent was diluted in double distilled and deionized water in a ratio of 1:9. A volume of 1.5 (X) ml + 10 ml was prepared (X is the number of determinations to be done).

iii. Standard solution

A solution of 0.1 mg/ ml bovine serum albumin (BSA) was prepared using 2% SDS (sodium dodecyl sulphate) as solvent, the same in which the sample to be tested was dissolved.

iv. Blanks

To zero the spectrophotometer, blanks were prepared using 2% SDS, the solvent used to dissolve the standard in 3.3. and the sample to be tested.

v. Sample

The crude membrane, nuclear or adipocyte sample were dissolved in 1 ml 2% SDS at 80℃ for 10 min.

B. Protein assay procedure

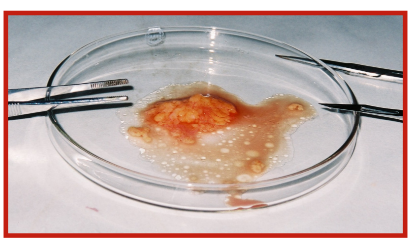
The samples were first diluted four times with 2% SDS, in order to have the approximate concentration (0.1 mg/ml) of the standard used.

In 4ml glass or polystyrene tubes, the following were mixed: 0.5 ml of double distilled and deionized water, 0.1 ml of diluent or standard or sample to be tested (according to the respective group: blank, control or test group), and 0.5 ml of solution A (1: 0.2: 1 proportion, respectively).

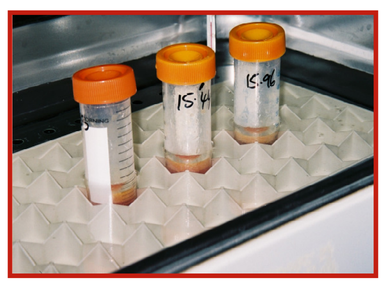
After 10 min at room temperature, 1.5 ml of Folin solution was added to each tube, vortexed, and incubated for 10 min in a water bath at 50 ºC to form a blue color, as indicator of the presence of protein. Solutions were allowed to cool at room temperature, and then the optical density (OD) was read at 650 nm, in an ultraviolet - visible light RS spectrophotometer within 30 minutes.

The average OD for each sample was used to calculate protein concentration using the formula $Ct = Cs \times ODt / ODs$, where Ct is concentration of the test, ODt is optical density of test, Cs is concentration of the standard and ODs is optical density of the standard. Sample dilution was also considered to calculate the amount of protein

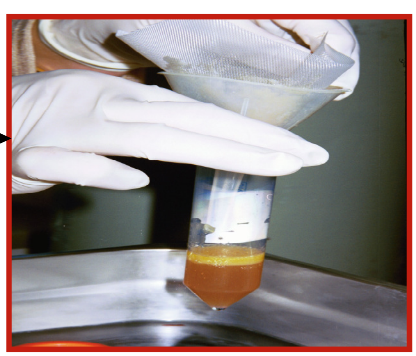
Fat from hysterectomy dissected



Incubated with collagenase 37 deg



Filtered through nylon mesh



Adipocytes washed with KRB

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CHAPTER 4

Effect of arachidonic acid on glucose uptake

4.1 Introduction

The dietary FA profile as well as the plasma level of FFA has been related to development of IR both in animals (6, 101, 185, 186, 211) and humans (212). Obesity (24, 212, 213) and the plasma levels of FAs (190, 214, 215) are positively correlated with IR and T2DM. After muscle and liver, adipocyte stores also strongly contribute to the plasma glucose levels in obese subjects (17). Therefore, many authors have studied the effect of FAs on glucose uptake into this tissue. Short period (< 30 min) exposure of 3T3-L1 adipocytes in culture to 100 µM palmitate decreased insulin-stimulated glucose uptake (107, 137). The opposite effect was observed when fresh adipocytes were exposed to SFA including palmitate (1 mM to 3 mM) for the same period (188, 216). In contrast, prolonged treatment (> 4 h) of adipocytes with SFAs (palmitate, myristate and stearate, all at 1 mM) has been shown to induce IR (188).

Although it has been reported that rats fed UFAs (oils rich in omega-3 and omega-6 FA) for three weeks had an impaired adipocyte ISGU (185, 216), most evidence points to a stimulatory effect of both monounsaturated FAs (specifically oleate) (217, 218), and PUFAs (185, 211, 219) on ISGU.

Looking specifically at AA, only long-term (more than an one hour) FA effects have been reported. It has been shown that exposure of 3T3-L1 adipocytes to AA for 48 hours increased ISGU by stimulating translocation of GLUT1 and GLUT4 to the plasma membrane (18). It has also been reported that AA stimulates BGU (18 - 20). Similar studies conducted in human adipocytes, using both isomers of conjugated linoleic acid (21, 218) yielded results inconsistent with those above authors (18). Additionally, it has recently been concluded that the trans-10, cis-12 GLA isomer decreases ISGU (219). Furthermore, it has been demonstrated that the stimulatory effect of palmitate on glucose uptake in rat adipocytes is achieved by activation of insulin receptor kinase and recruitment of GLUT4 to the plasma membrane (220).

The levels of glucose transporters in the cell, translocation of glucose transporters from cytoplasmatic vesicles (storage of glucose transporters) to the plasma membrane, as well as the activity of the glucose transporters have been reported to be involved in the mechanism whereby FA affects glucose transport in to adipocytes. Defective recruitment of GLUT4 to the plasma membrane was observed after rats were fed diets high in fat (55% of calories of which 30% were saturated) (220). On the other hand, palmitate (220) and AA (18, 22) have been shown to stimulate glucose transport by translocation of glucose transporters (GLUT1 and/or GLUT4) to the plasma membrane. During chronic (48 h) exposure of AA, expression of GLUT1 and GLUT4 genes has also been implicated in the mechanism: an increase of GLUT1 mRNA and reduction of intracellular levels of GLUT4 mRNA was observed (22). In addition, AA was shown to enhance the ability of GLUT4 to respond to insulin (22). In contrast, in 1996 Fong and colleagues (19) observed that AA enhanced BGU without altering glucose transport in response to insulin. This indicates that GLUT1 was the affected isoform. This group of researchers also observed that long-term (8 hours) 200 μM AA exposure to 3T3-L1 adipocytes enhanced glucose transport also by stimulating GLUT1 gene expression and by inducing translocation of GLUT1 to the plasma membrane through a PKC independent mechanism (19). The observation that phlorizin could improve IR in adipocytes of diabetic rats (221) and, the observation that transgenic mice fed on diets high in fat develop IR while they had overexpression of GLUT4 (222), are further studies that suggest that the activity of the glucose transporters can also play a role in defective glucose transport. The short-term (2 hours) AA effect on glucose transport in 3T3-L1 adipocytes has also been ascribed to modulation by the intrinsic activity of GLUT-1(19).

Because of the inconsistency of results observed in the studies listed above relating to the effect of fatty acids on glucose uptake, specifically in human fresh adipocytes, it was decided to further research the effect of AA on glucose uptake over a short period (> 30 min).

4.2. Materials and methods

4.2.1. Solutions / reagents needed

i. Krebs Ringer Buffer without glucose (KRB2): see Chapter 3

ii. Krebs Ringer Buffer with glucose (KRB1): see Chapter 3

iii. Two percent sodium dodecyl sulphate (2% SDS):

A. Stock solutions

The following stock solutions were prepared and stored at -70℃ until the day of experiment.

i. Phloretin (PHL): 52 mM PHL in ethanol (EtOH) (14 mg PHL / 1 ml ETOH).

ii. AA: 328 mM AA in EtOH (10 mg AA /100µl ethanol).

iii. 2-Deoxy-D-[2,6-^{3}H] glucose (DOG): 10 nM DOG in KRB2 (8.2 mg DOG / 5 ml KRB2).

B. Solutions for daily use

i. albumin free FA (FAFA in KRB2) : 1% FAFA in KRB2 (45 mg FAFA / 4.5 ml KR2).

ii. PHL: 50 µl 52 mM PHL (PHL stock solution) plus 1250 µl KRB2, final concentration 10.4 mM PHL.

iii. AA: 6 µl 328 mM AA (AA stock solution) in EtOH plus 1986 µl 1% FAFA-KRB2, final concentration 1 mM AA.

iv. DOG: 1.5 ml 10 nM DOG (DOG stock solution) plus 3.6 ml KRB2 plus 10µl ^{3}H-DOG, final concentration 4.17 mM DOG. Specific radioactivity (cpm/nanomole DOG) was determined by counting radioactivity of 100 µl of 4.17 mM DOG.

v. Adipocyte suspension (Adis): adipocytes four times diluted in KRB2 to give a final lipocrit of 30%.

vii. FA-blank: 6 µl EtOH plus 1986 µl 1% FAFA-KRB2

4.2.2. The effect of AA on glucose uptake

The reaction was carried out in 4 ml polypropylene tubes in a water bath at 37°C. Firstly, 350 µl KRB2 and 300 µl adipocytes (30 % lipocrit) were placed in the tube. The cells were pre-treated with 80µl 1 mM ^{14}C-AA in ethanol-FAFA for 10 and 30 minutes. Subsequently, 100 µl 4.17 mM ^{3}H-deoxyglucose (approximately 88 cpm /nanomole) in KRB2 was added. ^{3}H-deoxyglucose uptake was performed in a 37 ºC water bath for 6 minutes. The final concentrations in the reaction mix were: 96 µM AA, 0.52 % FAFA, 0.03% ethanol, 502 µM ^{3}H-deoxyglucose. To terminate the reaction, 35 µl 10.4 M PHL in DMSO-KRB2 was added (final concentration 200 µM PHL) and incubated for 5 minutes at 18 °C. Then, the cells were washed twice with KRB2 and lysed in 2% SDS at 80 °C for 10 minutes. After cooling at room temperature, protein was determined using the modified Lowry method (219). Radioactivity in the samples was counted using a model Beckman L-17 scintillation counter. Deoxyglucose uptake was expressed in nanomole deoxyglucose /mg protein /min. Blanks were performed under the same conditions as the test except that PHL was added in the beginning of the incubation. The value was subtracted from to the test to correct for unspecific AA uptake.

4.2.3. Statistics

Results are expressed as means ± standard deviation of at least four samples in two representative experiments. Comparisons between groups (control and test both, blank subtracted) were done using ANOVA in Statistix for Windows using Bartlett's post-hoc test. A P value less than 0.05 was considered statistically significant.

4.3. Results

The results of the influence AA on glucose uptake are presented in the Fig. 4.1. The effect of AA on glucose uptake was dependent on time of exposure: Ten minutes of incubation of adipocytes with 100 μM AA insignificantly inhibits glucose uptake with 1.04 ± 3.1% compared to the control, whereas 30 min adipocyte stimulation with 100 μM AA significantly improved glucose uptake (46.09 ± 5.4 %) compared to the control.

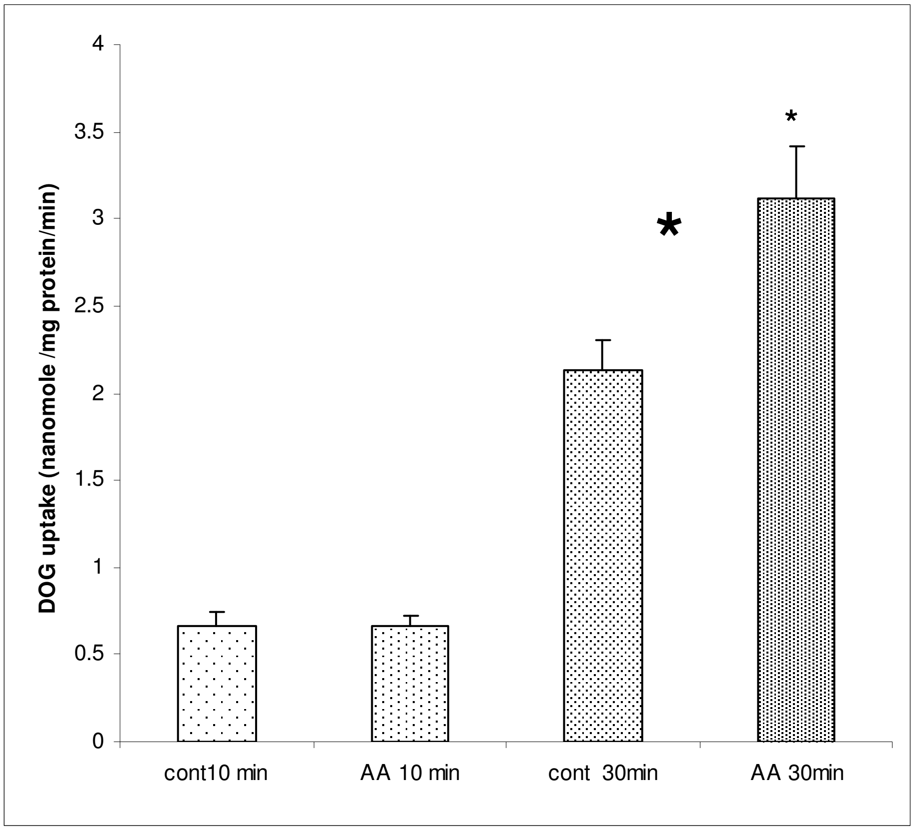

Fig.4.1: Influence of arachidonic acid on glucose uptake into fresh human adipocytes: Adipocytes were pre- treated with 100 µM AA for 30 minutes. Then ^{3}H-deoxyglucose (0.52 mM) uptake was performed for 6 min. ^{3}H-deoxyglucose uptake is expressed in nanomole/ mg protein/minute. ANOVA with Bartlett's post-hoc test was used to analyze the data, $P < 0.05$ was considered to be significant. A significant increase of deoxyglucose uptake was observed after 30 minutes of exposure. The experiment was repeated three times (n=3) and a representative experiment is shown here.

4.4. Discussion

High levels of plasma SFAs have been correlated with the development of IR and T2DM. However, brief *in vitro* exposure (< 30 min) of adipocytes to SFAs have been reported to stimulate insulin-dependent glucose uptake in adipocytes (188, 216), while prolonged (> 1 hours) exposure impaired ISGU (107, 188). The dietary effect of UFAs (both monounsaturated and polyunsaturated) on glucose uptake is also controversial. Some research groups found that dietary omega-3 and omega-6 FAs impaired the ISGU in adipocytes (185, 216). In contrast, there is evidence that oleate (217), and PUFAs (219) stimulate ISGU. Similar results were reported by different research groups (6, 13, 186) in their studies in muscle where they have shown that the substitution of omega-3 PUFAs for other types of fatty acids prevents IR.

In the present study it has found that 10 min of AA exposure had no significant effect on glucose uptake, whereas 30 min AA exposure stimulated glucose uptake into fresh human adipocytes significantly (Fig 4.1). Because AA is metabolized to eicosanoids, for example PGs (product of COX)), the effect observed could be due to the action of this metabolite (19), since IDM (a COX inhibitor) was not used in this experiment and because the use of IDM has seemed to abolish the effect of AA on glucose uptake (193). The effect might be also due to activation of a PKC- dependent or independent mechanism (195, 196, 217) with consequent stimulation of GLUT4 and/or GLUT 1 translocations to the plasma membrane (22). Because the minimal period required to express the glucose transporter genes was reported to be 30 min (137), the possibility of AA or its metabolite activating the expression of the GLUT1 gene (19, 20) may also be possible. In addition, the possibility of AA enhancing the fluidity of the membrane with consequent stimulation of the activity of membrane proteins (specifically, GLUT 1 and GLUT4 in this case) (11, 19, 24, 221) and the insulin receptor may also be reason for our observations.

The extent of the effect on glucose uptake observed in this study isrelatively small. This might be due to the loss of radioactive glucose by efflux from the adipocytes after the end of the reaction and PHL, which stops efflux, should be added to the KRB2 used for washing adipocytes in future experiments. Washing the exposed cells in cold FAFA in with 200 µM PHL (a glucose transporter blocker) has been reported to significantly prevent the efflux of radioactivity (127, 131, 223, 224). To our knowledge there is no literature available about short-term exposure of human adipocytes to AA. Therefore, comparison of this work is difficult. Notwithstanding the difference in procedures, part of the results of the present study (the stimulatory effect of AA) are in accordance with the observation done by Nugent and coworkers (18), who demonstrated that long-term (4 to 48 hours) exposure of AA led to enhancement of insulin-stimulated glucose uptake in 3T3-L1 adipocytes. The study also confirms the results of Fong *et al* in 1996 (19) that demonstrated that AA enhances the activity of GLUT1 at an early stage, whereas longer exposure increases the cellular levels of GLUT1. The present study also confirms the findings of Fong and colleagues in 1999 (20) that have shown the stimulatory effects of AA exposure on BGU. In the present study it was seen 10 min AA exposue had no significant effect on glucose uptake by adipocytes. Tebbey *et al* observed a depressor effect at 10 min AA exposure (22). He reported that long period exposure (>24 hours) of AA down- regulates GLUT4 gene and decreases the stability of its mRNA in 3T3-L1 adipocytes. A similar observation was made by Liu *et al* 1998 (226). This group of researchers concludes that AA synergistically with cycloheximide, inhibits insulin-stimulated glucose transport. Additionally, rats supplemented with omega-6 for 4 weeks have exhibited a depression of expression of GLUT4 genes in their adipocytes (186). The reports of these two groups suggest that longer exposure of AA to these cells reduces ISGU.

In conclusion, the effects of AA on ISGU by fresh human adipocytes remains unclear. For more conclusive results, more experiments of the same nature are recommended. For further answers, experiments including blockers of AA metabolism should also be done.

CHAPTER 5

Arachidonic acid uptake into subcellular fractions

5.1 Introduction

As was well detailed in the last chapter, the action and concentration of FAs have been correlated with insulin sensitivity, and therefore with DM in both rats and humans (6, 22, 186, 188).

FA transport into and out of the cell is known to be through simple diffusion (27 - 29) and highly regulated protein mediated transport (31, 32). In addition, it has been reported that the uptake of LCFA (oleic acid) into 3T3-L1 adipocytes, mediated by FAT/CD36, requires a membrane raft (144). Abnormalities in transport (uptake and / or efflux) of FAs and their disturbed metabolism that lead to an increase in the concentration of plasma free FAs have been suggested as part of the cause of obesity and T2DM (9, 10, 181, 226). In 2002, Luiken and coworkers (159) have observed that fatty acid (15 μM palmitate) transport from and to adipocytes is increased in streptozotocin-induced type 1 diabetes mellitus rats. This is concomitant with an increment of FABPpm expression in the plasma membrane (159). It was also observed that genetically obese and insulin resistant rats (progressing to T2DM) have enhanced FA transport in their adipocytes (27, 159). Additionally, 4 hours of 3T3-L1 adipocyte exposure to 800 μM AA has significantly increased the membrane fluidity and glucose uptake by the cell (18).

Once in the cell, the FAs affect glucose uptake through their actions in the different parts of the cell, specifically, in the membrane and nuclei. FAs act by the following mechanisms:

1) Affecting the composition of membrane phospholipids. This influences the fluidity of the membrane that, in turn, affects the activity of all proteins in the membrane, including glucose transporters, and insulin receptors (11,

19, 24, 227). This action has two consequences: (i) increased or decreased glucose transport as result of increased or decreased activity of the glucose transporters (GLUT1 and GLUT4); (ii) modulating signal transduction via activation of PI3-kinase (195, 196) or through a PI3-kinase independent-pathway (225). Depending on the influence of the signal transduction, this may lead to stimulation of translocation of GLUT1 and GLUT4 from intracellular vesicles to the plasma membrane (22, 197). The phenomenon of glucose transporter translocation is, in turn, responsible for improved glucose transport through the plasma membrane.

2) Activating the PPAR-γ, which has the consequence of stimulating GLUT4 rather than GLUT1 gene expression (119). In contrast, Fong *et al* in 1996 and in 1999 (19, 20) has shown that longer AA exposure increases the cellular levels of GLUT1 in adipocytes. In addition, PPAR-γ can stimulate adipogenesis, thus increasing the number of small insulin-sensitive adipocytes (40). PPAR-γ also affects the expression of other genes involved in energy homeostasis (40).
3) Affecting stability of mRNA of the glucose transporter, for example, long exposure (> 24 hours) of AA exposure have been shown to enhance the stability of GLUT1 mRNA and lower the stability of GLUT4 mRNA (22, 225).

In the first chapter it was observed that 10 minutes of exposure of fresh adipocytes to 100 µM AA does not have a significant effect on glucose uptake while with 30 minutes of exposure, glucose uptake is stimulated by an undefined mechanism. This corroborates the results of earlier experiments of M. Haag (personal communication - 2006). It was decided to investigate the time frame of uptake of AA into subcellular fractions, especially membrane and nuclei, using a radioactive method. In chapter 4 of the present investigation, it was reported that nuclear events only play a role in glucose uptake after 30 min of exposure (time when AA rose in the nucleus) of the cells to the FA. In concordance with this finding, we decided to give more attention to FA uptake into the cell membrane. Determination of the FA profile of the membrane using GC (non-radioactive method) was additionally used to measure AA uptake.

5.2. Materials and Methods

5.2.1. Measurement of radioactive arachidonic acid uptake

5.2.1.1. Exposure of adipocytes to AA

A. Solutions / Reagents

i. Adis

Adipocytes were isolated by collagenase and resuspended as described ins section 3.2 of this study.

ii. Radioactive AA solution

20 µl of [1-^{14}C] AA (56.0 mCi / m mole) was added to 15 µl of 328 mM AA. This was diluted with 9.44 ml of 1% FAFA in KRB2, to yield a final concentration of 0.52 mM AA. The specific radioactivity (cpm /nanomole AA) was determined by counting the radioactivity of a 100 µl of the 0.54 mM ^{14}C-AA solution.

iii. IDM

Stock solution: 254 mM IDM in DMSO (6 mg IDM in 66 µl DMSO).

Working solution: 2 mM (66 µl of 254 mM IDM in DMSO plus 8.31 ml KRB2) at room temperature.

iv. Nordihydroguaiaretic acid (NDGA)

Stock solution: 301 mM NDGA (6 mg NDGA dissolved in 66 µl DMSO).

Working solution: 2 mM NDGA (66 µl of 301 mM NDGA plus 9.93 ml KRB2).

v. PHL solution

Stock solution: 51 mM PHL (14 mg PHL was diluted in 1 ml DMSO), aliquots of 80 µl were stored at – 70ºC.

Working solution: 5 mM PHL (70 µl of 51 mM PHL stock solution in DMSO was diluted with 630 µl KRB2 at room temperature).

B. Exposure of adis to AA

Exposure to ^{14}C-AA was done in a 50 ml polypropylene flask in a water bath at 37℃. To prevent AA metabolism, 9 ml adipocytes (30 % lipocrit) diluted with 1.7 ml KRB2 were pre-treated for 5 min with 500 µl of 2 mM IDM and the same amount of 2 mM NDGA, giving a final concentration of both IDM and NDGA of 85 µM at this stage. Then, 2.8 ml of 0.52 mM [1-^{14}C] AA –FAFA-EtOH was added, so that the pre-treated adipocytes were exposed to a final concentration of 100 µM AA for different times (0 min, 10 min, 20 min and 30 min). At these times of incubation, 3 ml aliquots were transferred to 4 ml experimental tubes, prior to which 157 µl 5.1 mM PHL had been added to terminate AA uptake, the final PHL concentration being 253 mM. The medium was immediately removed and the cells were washed twice with 1 ml KRB2 at 37℃. Subcellular fractionation procedures were subsequently followed. To correct for non-specific uptake of AA, a blank was treated under the same conditions as the test, but

PHL was added in the tubes at beginning of the experiment and its value subtracted from the test.

5.2.1.2 Preparation of subcellular fractions

The subcellular fractions were prepared by ultracentrifugation of a cellular homogenate at 4℃. Phenyl methyl sulfonyl fluoride (PMSF) was used to prevent proteolysis through all membrane preparation processes.

a) Solutions

i . Adis pre-treated with AA

Adipocytes were exposed to AA and washed as described in 5.3.2. for immediate use.

ii. TES buffer: 250 mM sucrose, 20 mM Tris and 1mM EDTA, adjusted to pH 7.4 with HCl. The buffer is kept at 4℃ and used within a week.

iii. PMSF solution: 100 µM PMFS in isopropanol. This solution is kept at room temperature and used within a month. 2µl PMSF was added to each ml buffer immediately before use.

b) Subcellular fractionation procedures

Adipocytes pre-treated with AA and washed were once again washed once with 3 ml cold (4℃) TES buffer containing 0.2µM PMSF. All subsequent steps were carried out at 4ºC. A model L-17 Beckman centrifuge was used.

Cells were resuspended in 8 ml TES buffer and immediately homogenized by 10 strokes at the maximum setting of a Potter homogenizer. The homogenate was centrifuged first at 800 g for 10 min to pellet **nuclei.** This was resuspended in 5 ml TES and centrifuged again under the same conditions. The supernatant of the first centrifugation was ultracentrifuged at 10,000xg for 20 min. The pellet (**crude membranes**) was resuspended in 5 ml TES and centrifuged again for 20 min at 10 000 g. Finally, to maximize lysis, both nuclei and membranes were dissolved in 2% SDS and heated at 80℃ for 10 min. Then, they were left to cool overnight at room temperature. Aliquots of 100 µl were taken for scintillation counting and protein determination.

5.2.1.3. AA uptake into subcellular fractions

^{14}C radioactivity was counted in a Beckman scintillation counter. Protein determination was performed according to the Lowry method as described in 3.3. The counting and protein determination were done in triplicate for each sample. The results were expressed as nmole AA uptake / mg protein / min, after subtraction of the blank value.

5.2.1 4. Statistics

Results of at least three measurements of the combination of two representative experiments were expressed as means ± standard deviation. Comparisons between groups were done using the T-students test in the Windows program. A P value less than 0.05 was considered statistically significant.

5.2.2. Fatty acid profile of the membrane

5.2.2.1. Materials and Methods

A. Solutions / Reagents

The solutions needed to expose adipocytes to AA were the same as those described in 5.2.1.1, except that non-radioactive AA was used.

B. Exposure of adipocytes to AA

To 3 ml of adipocytes (30% lipocrit) in a 4ml plastic tube and equilibrated at 37℃. Then 125 µl of 2 mM IDM and the same amount of 2 mM NDGA were added yielding 71 µM of both IDM and NDGA to prevent AA metabolism. After 5 min of incubation with the inhibitors, 280 µl of 0.52 mM AA (8 µl of 328 mM AA in DMSO plus 4.79 ml of 1% FAFA in KRB2) was added. At this stage, the concentrations of other chemical / substances in the reaction mix were: adipocytes (23% lipocrit), 38 µM AA, 71 µM IDM, 71 µM NDGA, 0.16% FAFA and 0.05% DMSO. AA uptake was performed for 10 min and 30 min. AT these times of incubation, 157 µl of 5.1 mM PHL was added to yield a final concentration of 217 mM PHL in this reaction stage. The reaction of PHL to stop AA uptake was done at 15℃ for 4 min. Then, the medium was immediately removed and the cells washed twice with 1 ml KRB2 at room temperature. Subcellular fractionation procedures were subsequently followed. Controls were performed for 10 min under the same conditions as the test but PHL was added at beginning of the experiment.

C. Membrane preparations

Adipocytes that have been exposed to AA and washed were used to prepare crude membranes as described at in detail at C. of 5.2.1.2. Each portion (control, test: 10 min and 30 min exposure) was worked up separately.

D. Fatty acid extraction and methylation

Fatty acid extraction from the plasma membrane was done according to the Folch method (229) with minor modifications. Briefly, phospholipids were hydrolysed in the presence of butylated hydroxytoluene (BHT), an antioxidant agent. The extract was dried under nitrogen in a heating block at 40 ºC. Transmethylation of FAs was done using boron trifluoride-methanol (BF3-Me). Finally transmethylated FAs were dissolved in hexane for gas chromatography. Only glass tubes were used and the mixing process was done by capping the tube and vortexing it for 1 minute.

E. Solutions / Reagents

The following reagents were used to extract FAs from the membrane phospholipids:

(i) Internal standard (300 mM pentadecanoic acid in heptane)

(ii) Hydrolysing solution (3 g KOH + 50 mg Butylated hydroxy toluene (BHT) + 5 ml H_2O + methanol (MeOH) up to 50 ml)

(iii) HCl (32%)

(iv) Petroleum-ether

(v) Hexane

(vi) Boron –trifluoride-methanol (BF_3-MeOH)

(vii) $MgSO_4$ powder.

F. Fatty acid extraction and methylation

The reaction was done in a 25 ml glass extraction tube. Defrosted crude membranes (500 µl), prepared as described in C. of 5.2.2.1 were placed into the extraction tube. Then, 125 µl internal standard and 6.5 ml hydrolysing solution were added. The mixture was vortexed under nitrogen and heated for 30 min in a 60 °C water bath. This leads to hydrolysis of the phospholipids, releasing the FAs. Subsequently, 5 ml distilled water was added. The mixture was vortexed again under nitrogen and heated again under the same conditions. After that, it was left for 10 min at room temperature to cool. Subsequently, the suspension was acidified with 1.5 ml of 32% HCl to acidify FA anions. To extract FAs, 2.5 ml petroleum-ether was added and the tube vortexed for 1 min. The upper liquid phase of the extract was transferred to another 25 ml long extraction tube using a pasteur pipette. To maximize the FA extraction, the water phase was again acidified with 1.5 ml of 32% HCl, and the extraction was repeated twice more. Subsequently, the extracts were dried in the same tube under nitrogen using a block heater at 40°C. Then, to methylate FAs, they were mixed with 5 ml of BF_3-methanol and heated for 5 min in a 60 °C water bath. During the incubation, the mix was vortexed three times for 1 min. Thereafter, 2.5 ml hexane was added and the tube vortexed for 1min. The supernatant was transferred to a V bottom tube. For purification, 5 ml of saturated NaCl was added to the bottom layer. It was then mixed by vortex for 1 min and 2.5 ml hexane was added and vortexed again for 1 min. The supernatant from this mix was also poured into a V bottom tube as mentioned above. An amount equal to ¼ of the volume of sample of

$MgSO_4$ powder was added to the sample in the V tube to remove water. The liquid phase was decanted to another V bottom tube and centrifuged 800 rpm for 1min in a P-selecta Mixtasel centrifuge. Finally the supernatant which contained methylated FAs in hexane and 20 µM methylated internal standard was transferred to the GC vials, ready to be analyzed immediately or, stored overnight at 4ºC.

G. Gas chromatographic analysis of FAs

GC of the FA methyl ester preparation was done on a Shimadzu gas chromatograph- 17A. The machine has a hydrogen flame ionization detector at 260°C, a non-polar fused silica capillary column (3,000 mm length) at 80ºC and an injection port at 260ºC. Nitrogen was used as carrier gas at a flow rate of 0.9 ml / min, and oxygen for combustion. The temperature gradient program used was: heating to 100 °C for 5 min, then increasing by 4 °C at a time up to 224 °C, and remaining there for 10 min. Identification of fatty acid methyl esters was done by comparison with retention times of internal standard (pentadecanoic acid) and standard data of a known FA mix.

H. AA uptake into the membrane

The quantification of AA and other FAs in the membrane was done using the GC windows data analysis (GC analysis editor 1) program that calculates the amount of the FA using the area under the respective peak compared with the concentration of the internal standard added (300 mM pentadecanoic acid). The test and standard, spiked with methylated AA, were used to determine the retention time of AA under the conditions of our gas chromatograph.

5.3. Results

A. FA uptake into subcellular fractions (radioactive method)

The result of the method where ^{14}C-AA radioactivity was counted (Fig 5.1 and 5.2) showed that AA was significantly taken up into both adipocyte crude membranes (23 ± 73%) and nuclei (47 ± 23%) after 30 min exposure to 100 µM AA, compared to the controls, 188% ± 35 and 137 ± 35, respectively.

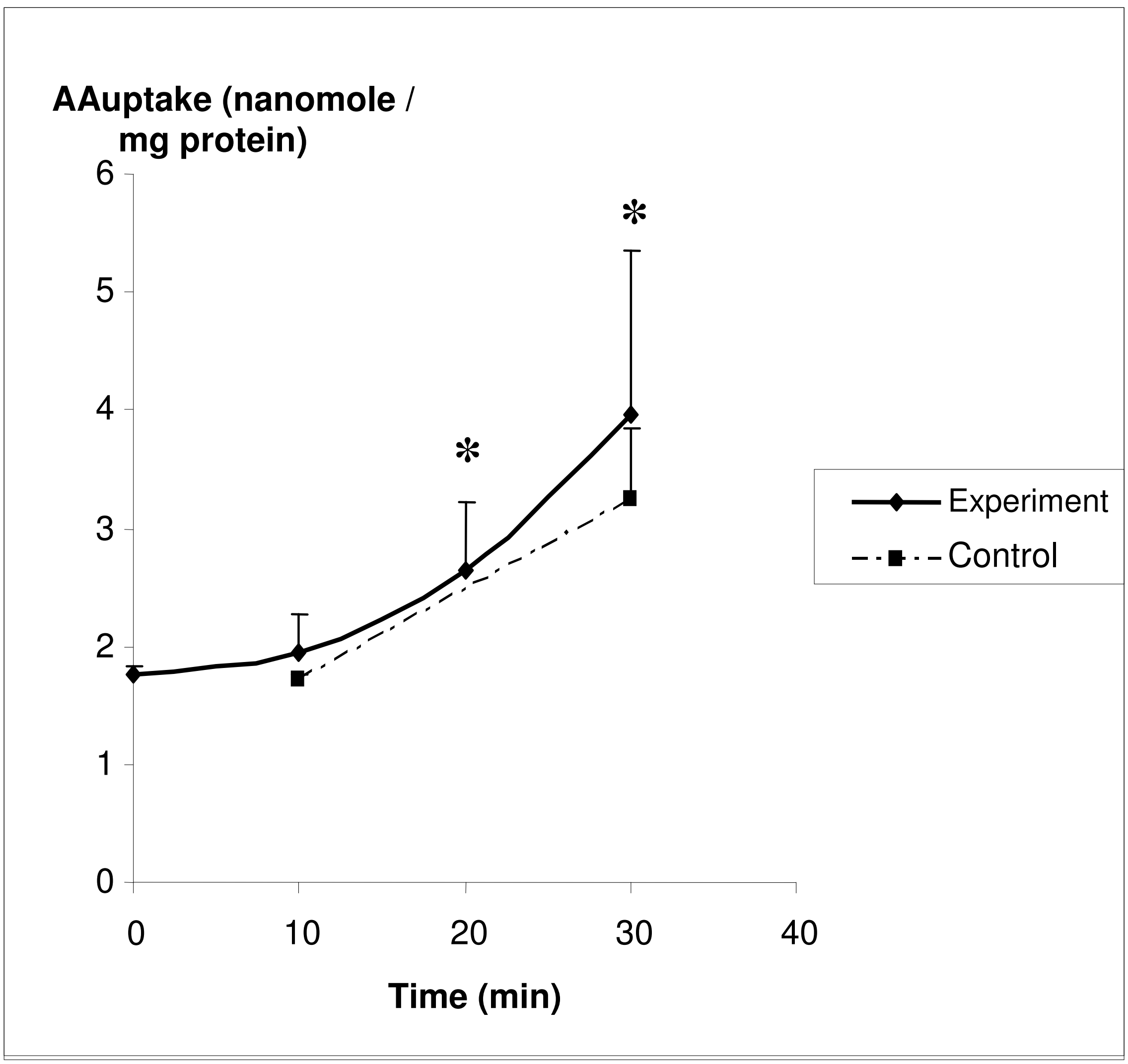
AAuptake (nanomole / mg protein)
6
5
4
3
2
1
0
0
10
20
30
40
Time (min)
*
*
Experiment
Control

Fig. 5.1. Time dependence of ^{14}C-AA uptake into crude membranes of fresh human adipocytes of a non-obese subject (Body Mass Index (BMI) 25): Adipocytes were preincubated for 5 minutes with 100 µM of IDM and NDGA. Subsequently the cells were treated with 100 µM AA. Adipocyte ^{14}C-AA uptake was performed for 0, 10, 20 and 30 minutes. Crude membranes were prepared. ^{14}C-AA uptake into membranes was quantified and expressed in nanomole AA / mg protein. Controls (PHL treated) at 10 and 30 minutes were performed under the same conditions. Comparisons between the uptake at zero minutes and uptake at different times were done using students-T test. P < 0.05 was considered to be significant: the uptake was significantly increased at 30 minutes. Six independent experiments were conducted in triplicate. Data of two experiments were combined and presented as results: mean ± SD.

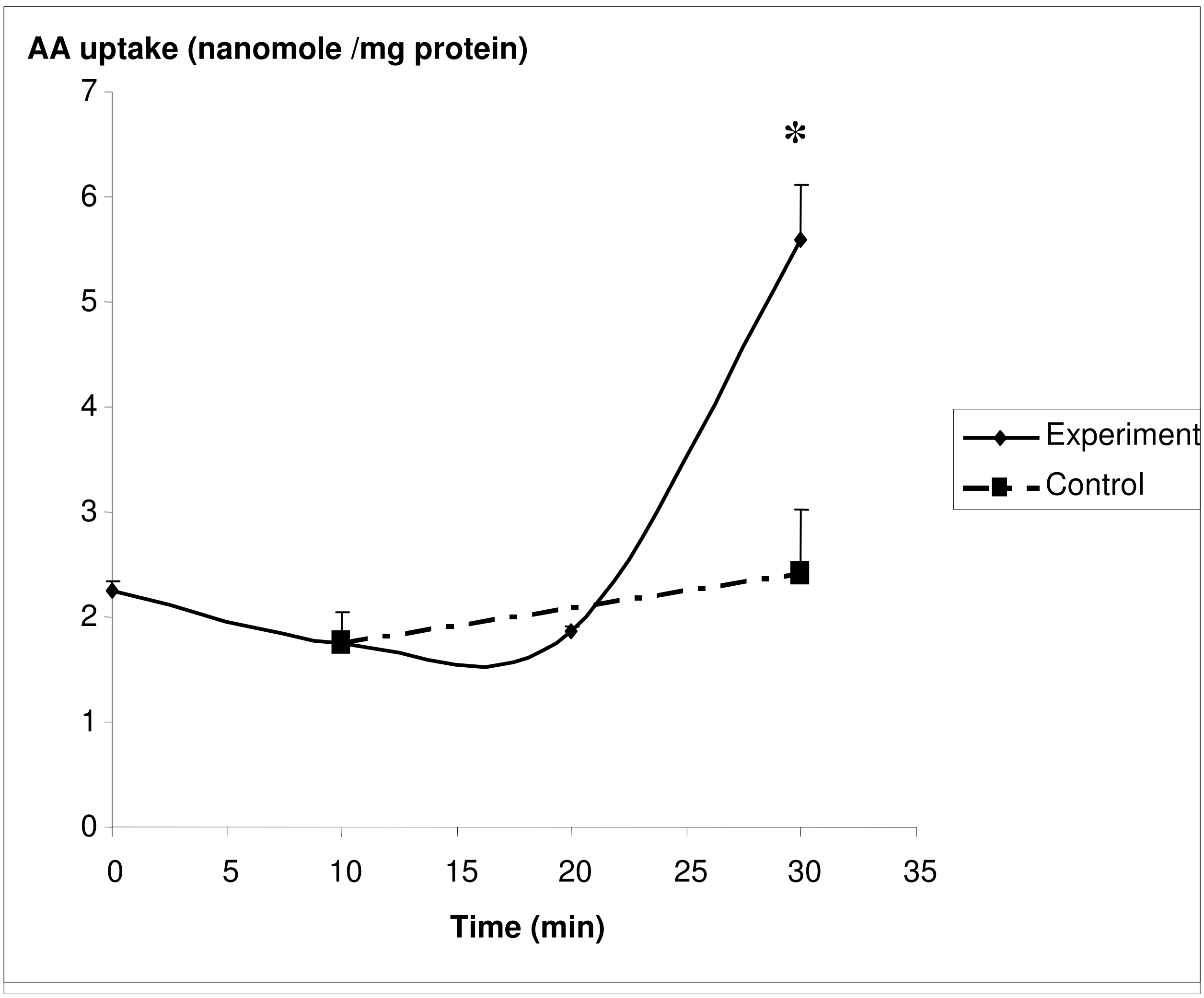

Fig. 5.2.: Time dependence of ^{14}C-AA uptake into nuclei of fresh human adipocytes of a non-obese subject (BMI 25): Adipocytes were preincubated for 5 minutes with 100 µM of IDM

and NDGA. Subsequently the cells were treated with 100 µM AA for different times (0, 10, 20 and 30 minutes). Nuclei were prepared. ^{14}C-AA uptake into nuclear fraction was quantified and expressed in nanomole/mg protein. Controls (PHL treated) at 10 and 30 minutes were performed under the same conditions. Comparisons between the zero minute uptake and uptake at different times were done using students T-test. $P < 0.05$ was considered to be significant. The uptake was significantly increased only at 30 minutes. Six independent experiments were conducted in triplicate. Data of two experiments were combined and presented as results: mean ± SD.

B. Fatty acid uptake into subcellular fractions (GC method: FA membrane profile)

In order to investigate the AA uptake into crude membranes by the GC method (FA profile of the membrane), it was necessary to optimize the assay conditions first. Then, the FA profile of adipocyte crude membranes exposed for 10 and 30 minutes to 100 µM AA was investigated. The results (chromatograms in **appendix 1**) were processed and presented in Table 5.1. The percentage of AA content increased from 0.3 % to 0.57 %, between 10 and 30 min. This corresponds to a significant increase of 90 % compared to the AA content at 10 min. These results confirm, in part, the observation made in the investigation of AA uptake into crude membranes using the radioactive method. The relatively high percentage of AA in the control (2.2 %) is, however, inexplicable. These trends stay the same when the results are expressed in nanomole/mg protein.

10 min AA control	10 min AA exposure	30 min AA exposure

Fatty acid	FA (nmole)	FA (%)	FA (nmole/ mg protein)	FA (nmole)	FA (%)	FA (nmole/ mg protein)	FA (nmole)	FA (%)	FA (nmole/ mg protein)
C14:0	1363.78	19.16	15.7078	3396.38	31.62	46.4995	3010.84	32.13	52.6737
C15:0	19.89	0.28	0.22907	119.29	1.11	1.63313	35.31	0.38	0.61768
C16:0	5202.10	73.10	59.917	6054.47	56.37	82.8912	5159.12	55.06	90.2572
C16:1	176.82	2.48	2.03653	180.03	1.68	2.46471	145.09	1.55	2.53836
C18:0	16.16	0.23	0.18617	9.27	0.09	0.12693	15.46	0.17	0.2705
C18:1n9t	4.84	0.07	0.05579	7.53	0.07	0.10315	3.02	0.03	0.05276
C18:1n9c	25.91	0.36	0.29843	22.22	0.21	0.30427	17.17	0.18	0.3004
C18:2n6t	8.24	0.12	0.09491	8.36	0.08	0.11441	6.91	0.07	0.12087
C20:0	7.60	0.11	0.08756	5.32	0.05	0.07278	10.44	0.11	0.18259
C18:3n6	49.03	0.69	0.56475	23.08	0.21	0.31601	38.43	0.41	0.67229
C18:3n3	84.87	1.19	0.97754	6.67	0.06	0.09133	11.01	0.12	0.19263
C20:4n6	**157.29**	**2.21**	**1.81164**	**31.47**	**0.29**	**0.43081**	**53.48**	**0.57**	**0.93562**
Total	7116.54	100.00	81.9672	9864.08	91.83	135.048	8506.28	90.78	605.179

Table 5.1. Fatty acid profile of the crude membranes after exposure to AA and control membranes as determined by GC, percentage of the total and FA per mg protein.

5.4. Discussion and conclusion

The main problem in this experiment was that the AA content of the control membrane was higher than those exposed to AA. In the present study, it was demonstrated with a radioactive method that a significant amount of AA was taken up in adipocytes after 30 minutes of exposure: AA was detectable in the crude membrane fraction after 10 minutes but a significant increase was registered after 30 minutes of exposure. In the nuclei, AA content rose only after 30 minutes of exposure. These results suggest that the effect of short-term (30 min) 100 µM AA exposure on the enhancement of basal glucose uptake into fresh human adipocytes of non-obese subjects may take place done by a membrane based mechanism. Eventual participation of gene expression is also possible. It is very difficult to compare the results of the present experiment with the literature because there is no literature available on the effect of AA on glucose uptake into fresh adipocytes over this period (less or equal to 30 min). Although the time of exposure in the present experiment differs with that used by others, in general, the results of the present study agree with earlier research findings made in 3T3-L1 adipocytes by Nugent *et al* (18). It is also important to mention here that fatty acid uptake into intact cells is difficult to measure because the FAs are rapidly incorporated into metabolism (127, 130). The data of this experiment are, however considered to be reliable, since the metabolism of AA was minimized by pretreatment of the adipocytes with IDM (18) and NDGA (18, 229). But, because carnitine-acyl-transferase was not inhibited, it is likely that some amount of FA could have moved into mitochondria. However, because the AA uptake in the present work is done in crude membranes, which include plasma membranes and mitochondria and other citosolic organelles except the nucleus, the arachidonic acid eventually taken up by mitochondria was measured together with plasma membranes.

The finding that ^{14}C-AA was significantly taken up into the crude membrane fraction after 30 minutes was, to a certain extent, confirmed by the results of the investigation of the content of membrane AA (fatty acids profile) by GC of the membranes exposed to AA over 30 min.

In summary, the results of the present study suggest that: (i) over the short term (less than 30 minutes) AA uptake into adipocytes is best monitored by the radioactive method using ^{14}C-AA; (ii) The action of the AA on the membranes is suggested to be primarily involved on the mechanism whereby the FA stimulates glucose uptake into adipocytes, since AA was significantly incorporated into the membrane between 20 to 30 minutes of exposure; (iii) and, only after 30 minutes of exposure the effect of arachidonic acid might also be attributed to modulation of gene expression. For more accurate results, it would also prudent to conduct further studies where the plasma membrane is purified from cytosolic organelles.

Because of the interest in the effect of AA on glucose transport and, the investigation of AA uptake into adipocytes, it was decided to verify the effect of insulin on AA uptake into adipocytes.

CHAPTER 6

Influence of insulin on arachidonic acid uptake

6.1 Introduction

Insulin influences both glucose and FA acid transport into and out of the cells. Thus, in IR individuals, FAs are easily released from adipocytes but they have more difficulty in entering the cells (96). This has the effect of worsening the condition of insulin resistant subjects, because disorders related to abnormal function of fatty acids in the body are also developed.

In 3T3- L1 adipocytes, insulin has been suggested as a negative regulator of FATP1 mRNA levels (157). However, there is evidence that this hormone stimulates FA uptake (18, 96). It has been demonstrated that the LCFA uptake into adipocytes shares many similarities with the hormonal regulation of glucose uptake: LCFA uptake is enhanced as result of translocation of FATP1 from the intracellular pool to the plasma membrane. However, FATP 1 and GLUT4 are localized in different intracellular vesicles (18, 31, 96). In contrast, insulin was shown to profoundly suppress FA transport from the adipocytes (102). This observation was also supported by Luiken and colleagues in 2002 (160), who showed that adipocytes from streptozotocin-induced diabetic rats increased their FA transport across the plasma membrane, releasing FAs, with a simultaneous increase of FABPpm expression and increased amounts of this fatty acid transporter in the plasma membrane.

AA uptake into subcellular fractions of adipocytes over the short-term (less than 30 minutes) was investigated in the last chapter, due to the lack of data about the short-term influence of insulin on AA uptake into fresh human adipocytes. In the present chapter the short-term influence of insulin (0 nM, 10 nM, 20 nM and 40 nM) on this process in a non-obese and obese subject is investigated. AA uptake was only measured at 10 and 30 minutes because of the limited amount of fat that a patient can donate.

6.2 Material and Methods

6.2.1. Solutions / Reagents

6.2.1.1. Adis

A suspension of adi's in KRB2 was prepared on the experimental day as described in 3.2.2. and kept at 37℃.

6.2.1.2. ^{14}C AA solution

To expose the adipocytes to 100 µM AA, a solution of 0.52 mM AA containing a trace of ^{14}C-AA (56.0 mCi / m mol) was prepared as described by Grunfeld et al 1998 ( ) with minor modifications (6 µl of 328 mM AA in DMSO plus 3.67 ml of 1% FAFA in KRB2 plus 20 µl ^{14}C-AA in EtOH at 56.0 mCi / mmole). Specific radioactivity (cpm/ nanomole) of 100 µl 0.52 mM AA was determined by scintillation counting.

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6.2.1.3. Insulin

Stock solution: 4 µM insulin (3 mg insulin was dissolved in 125 ml KRB2). Aliquots of 1ml were stored at -70℃.

Working solution: 0, 69, 138 and 275 nM insulin. Firstly, 687 µl of 4 µM insulin was added to 9.12 ml KRB2 to yield a final concentration 275 nM insulin. Then, part of this solution was diluted two and four times to yield 138 nM and 69 nM, respectively.

6.2.1.4. Indomethacin (IDM)

Both 254 mM IDM stock solution and 2 mM working solution were prepared as described in 5.2.1.1.A.(iii).

6.2.1.5. NDGA

NDGA both 301 mM stock solution and 2 mM working solution were prepared according to 5.2.1.1. A (iv).

6.2.1.6. PHL

70 µl of 51 mM PHL stock solution in DMSO was diluted with 630 µl KRB2 at room temperature, 5 mM final concentration PHL, as described in detail in 5.2.1.1.A.(v).

6.2.2. Exposure of adipocytes to ^{14}C AA and insulin

Firstly, four aliquots of 0.9ml of adi's were each placed in 4 ml polypropylene tubes at 37°C. At zero minutes, 100 µl of a mix of equal volumes of 2 mM IDM and 2 mM NDGA was added, giving a final concentration 100 µM each. The incubation to prevent AA metabolism took 5 min. Then, 170 µl of insulin (275 nM, 138 nM and 69 nM) was added to the cells for 20 minutes, the final concentration of insulin at this stage was 40 nM, 20 nM and 10 nM insulin, respectively. Subsequently, 280 µl of 0.52 mM ^{14}C-AA in FAFA-EtOH was added. The final concentrations in the reaction mix were: adipocytes (19% lipocrit), 100 µM AA, 69 µM IDM, 69 µM NDGA, 0.19% FAFA, 0.05% DMSO and 32 nM, 16 nM and 8 nM insulin. The adipocytes were exposed to AA for 10 minutes. Thereafter, 59 µl 5 mM PHL was added to give a final concentration of 200 µM PHL. The tubes were kept at 18 °C for 5 min. Cells were washed three times with 1 ml KRB2 at room temperature, resuspended in 1ml of 2% SDS and finally heated at 80ºC for 10 min to lyse the cells. After cooling at room temperature, they were vortexed and 100 µl aliquots were mixed with 3 ml scintillation liquid. They were kept in the dark at room temperature overnight before scintillation counting. 100 µl of the remaining samples was used for protein determination by the Lowry method (210) (see 3.3). To analyze results, zero blank and control experiments were carried out simultaneously. In the zero blanks group insulin was excluded and PHL was added at the beginning of experiment. Control group procedures were similar but insulin was excluded.

6.2. 3. Effect of insulin on AA uptake

Scintillation counting of ^{14}C (100 µl 3 times for each sample) was carried out for 20 min per vial (detail in 5.2.1.3). Activity of FA transporters was expressed as nmole AA /mg protein/ min. The effect of insulin was measured comparing the AA uptake of the control (without insulin) with the test (exposure for insulin different concentration). Unspecific activity (blank) was subtracted from both.

6.2.4. Statistics

At least four measurements per sample of one representative experiment of the effect of insulin on 30 min AA uptake for both a normal and a obese subject were used to calculate mean ± SD. In the study of the influence of insulin on 10 min AA uptake, four measurements per sample of a experiment done on a non-obese subject were used as results. Comparisons between groups (control and test, both blank subtracted) were done using ANOVA with Bartlett's post-hoc test in the Windows Statistix programme. A P value of less than 0.05 was considered statistically significant.

6.3. Results

The influence of different concentrations of insulin on 10 minutes of 100 µM AA uptake was determined in a non-obese subject (Fig 6.1): 10 nM and 40 nM insulin increased the AA uptake by 81 ± 31 % and 208 ± 36 %, respectively, in relation to the control (0.06 nmale AA/ mg protein/ min). Insulin (20 nM) decreased AA acid uptake by 62 ± 2 % compared to the control. The increment observed was significant at 40 nM insulin.

The effect of insulin on 30 minutes AA uptake was performed in both obese and non-obese subjects. It is clear that insulin acts in a dose-dependent manner to increase arachidonic acid uptake into adipocytes from a non-obese subject (Fig.6.2). The increases of AA uptake were 20 ± 8 %, 21 ± 25 % and 31 ± 4 % compared to the control (0.058 nmole AA/ mg protein/ min), respectively for the action of 10 nM, 20 nM and 40 nM insulin. No saturation was observed at the relative high concentration of 40 nM insulin. In contrast, in the obese subject (Fig. 6.3), insulin decreased the AA uptake in a seemingly dose dependent manner. The decreases observed were in order of 15 ± 5 %, 14 ± 8 % and 21 ± 5 % compared to the control (0.074 nmole AA/ mg protein/ min), respectively for the action of 10 nM, 20 nM and 40 nM insulin.

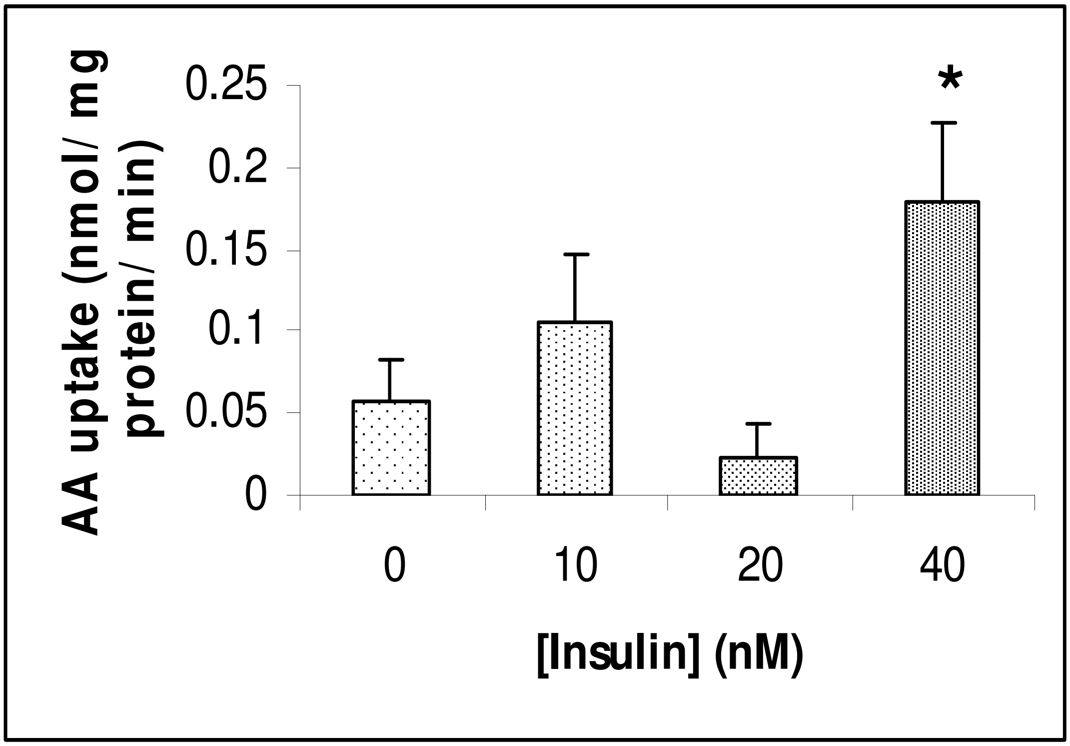

Fig.6.1. Influence of insulin concentration on 10 min AA uptake into fresh human adipocyte of a non-obese subject (BMI = 23.5 kg / m^2): The uptake of AA was performed as described in the Materials and Methods. Adipocytes were preincubated for 5 minutes with 100 µM of IDM and NDGA. Subsequently the cells were treated for 20 minutes with insulin (0, 10, 20 and 40 nM). Then, adipocytes were exposed to 100 µM AA for 10 minutes. AA uptake was expressed in nanomole AA / mg protein /minute. Comparisons between the control (zero nM) and uptake at different concentrations were done with ANOVA with Bartlett's post-hoc test. A significant increase was observed at 40 nM insulin. Four independent experiments were conducted, n = 4. Data from a representative experiment are presented: mean ± SD. P< 0.05 was considered as significant .

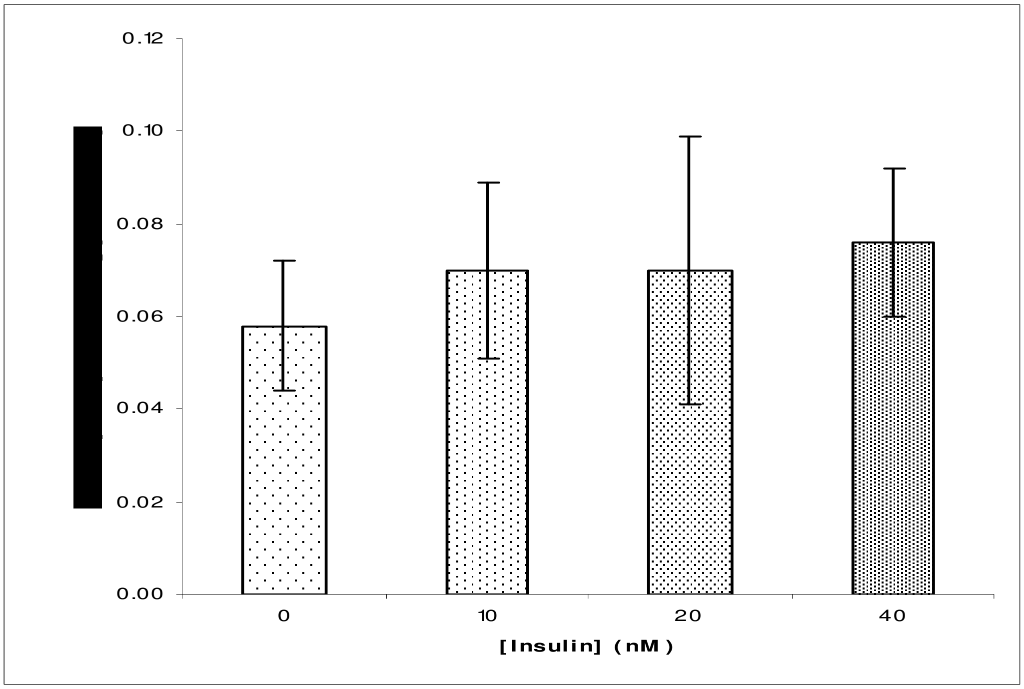

Fig.6.2. Influence of insulin concentration on AA uptake measured for 30 minutes into fresh human adipocytes from an-obese subject (BMI = 24.5 kg / m^2). Adipocytes were preincubated with 100 µM of IDM and NDGA. Subsequently the cells were pre-treated with for 20 minutes with insulin (0, 10, 20 and 40 nM). AA uptake was performed for 30 minutes and expressed in nanomole /mg protein /minute. Comparisons between the control (zero nM) and uptake at different concentrations were done with ANOVA with Bartlett's post-hoc test. No significance differences were seen. A P value of less than 0.05 was considered significant. Three experiments were conducted, n = 3. Data from one representative experiment is presented: mean ± SD. No significant differences between groups were found.

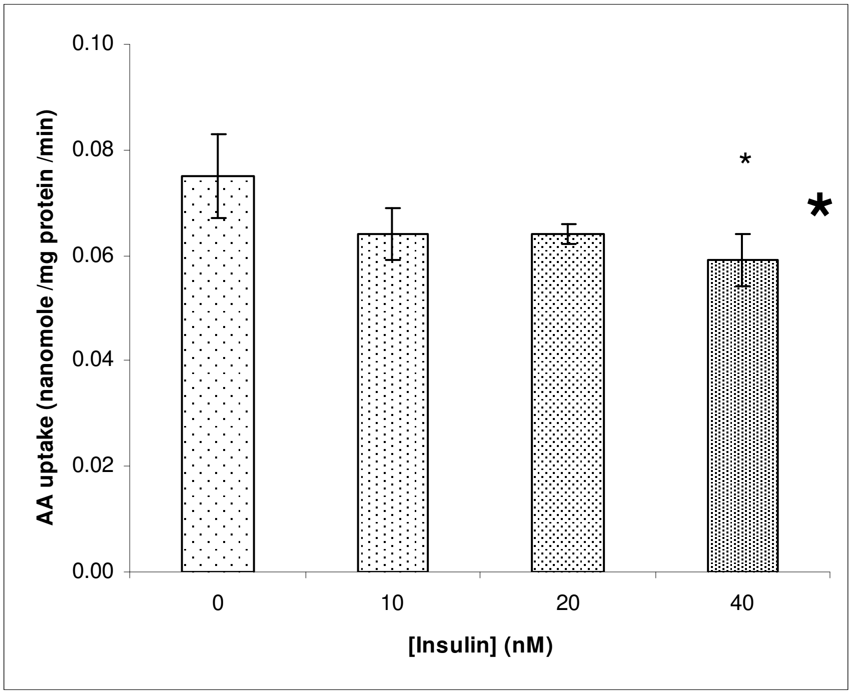

Fig. 6.3. Influence of insulin concentration on AA uptake measured for 30 minutes into fresh human adipocytes from a obese subject (BMI = 30.5 kg / m^2). Adipocytes preincubated with 100 μM of IDM and NDGA. Subsequently, cells were sensitized with insulin (0, 10, 20 and 40 nM) for 20 minutes. AA uptake was performed for 30 minutes and expressed in nanomole /mg protein /minute. Comparisons between the control (zero nM) and uptake at different insulin concentrations were done with ANOVA, with Bartlett's post-hoc test. A significant difference was observed only at 40 nM. Three experiments were conducted, n = 3. Data from one representative experiment is presented: mean ± SD, $P < 0.05$ was considered as significant.

6.4. Discussion and conclusion

Insulin has been shown to influence FA transport into and out of cells. Thus, subjects with IR could develop disorders related to lack or reduced function of FAs acids in the body.

In a non-obese subject, insulin stimulated AA uptake into adipocytes in a seemingly dose dependent manner at both 10 minutes (Fig.6.1) and 30 minutes (Fig.6.3) of exposure at 10nM which is within the normal physiological range. The maximal insulin concentration (40 nM) was not enough to have a saturation effect. Although the methods used were different, this study agrees with the finding of Hamilton & Kamp (31) in their studies using 3T3-L1 adipocytes. Insulin has been shown to stimulate FATP1 translocase to the plasma membrane of adipocytes (31, 96). Insulin was also reported to stimulate the translocation of FAT/CD36 from intracellular vesicles to the plasma membrane of myocytes, resulting in enhanced palmitate uptake (138). Adipocytes also express FAT/CD36 (138, 139). Therefore, beside the more probable mechanism that involves FATP1 translocation, the translocation of FAT/CD36 protein could also be involved in increased arachidonic acid uptake observed in the present study.

Furthermore, it was also observed that in an obese subject (Fig.6.3.) insulin decreased AA uptake (30 minutes) by adipocytes in a dose-dependent manner. This could result from the fact that the cells from this obese subject are already insulin resistant, thus depressing AA uptake. The time of insulin exposure could also play a role since it has been reported that prolonged exposure to high concentrations of this hormone in fact depresses glucose uptake by cells (22). This could conceivably also happen with AA.

In conclusion, in the present experiment it has been demonstrated that the effect of insulin on AA uptake is also influenced by the BMI of the adipocyte donor. Thus, insulin stimulates FA uptake into adipocytes of non-obese subjects, whereas in IR obese subjects, insulin depresses the FA uptake.

Chapter 7

General conclusion

The motivation for the present study was:

(i) Inconsistency of results relating to the effect of FAs on glucose uptake in human adipocytes.

(ii) Lack of information about the probable part of the cell involved in the mechanism by which unsaturated fatty acids affect glucose uptake over short periods (less than 30 minutes).

(iii) The lack of literature about the influence of insulin on FA uptake in fresh human adipocytes.

The three following objectives were delineated:

Objective one: to research the effect of AA, as representative FA, on deoxyglucose uptake into adipocytes. To achieve this, isolated human adipocytes were successively exposed to AA and deoxyglucose and deoxyglucose measured.

Objective two: examination of AA uptake into subcellular fractions of adipocytes (membranes and nuclei). This was done in order to observe in which part of the cell AA acts to influence glucose uptake into adipocytes. To achieve the objective, adipocytes were exposed to AA and subcellular fractions obtained; then AA uptake into membranes and nuclei was determined.

Objective three: investigation the influence of insulin on AA uptake into the adipocyte. To achieve this objective, adipocytes were exposed to insulin and subsequently to AA, and AA uptake measured.

Results from this study have shown that the 100 µM AA stimulates glucose only after 30 minutes of exposure. Since no changes of AA uptake were observed within 10 minutes of exposure; the stimulatory effect of AA on glucose uptake was more probably the result of the action of the FA in membranes than in stimulating DNA transcription. AA was significantly taken up by crude membranes after 20 minutes of exposure, while in the nuclei AA was only significantly found after 30 minutes. Both the method of counting radioactivity of ^{14}C-AA taken up by the crude membranes as well as investigating the content of AA in the membranes by its GC FA profile are suitable for analysis of FA uptake into membranes at 0.17 - 0.34 mg protein / ml, prepared from a small amount of adipocytes (2 to 6 ml) for a short period (less than 30 min) of exposure. The action of insulin on AA uptake into human isolated adipocytes over a short period of exposure was dependent on the BMI of the patients, probably a result of the insulin sensitivity of their cells. Insulin was shown to stimulate both 10 min and 30 min AA uptake into adipocytes from a non-obese subject in a dose dependent manner, while in adipocytes from an obese subject, insulin depressed AA uptake over the period of study, also in a dose-dependent manner.

For more conclusive results, we suggest that a similar study be repeated in the future, in which:

1. The solution to wash adipocytes after their exposure to the factors investigated in the present study should also contain 1% albumin and 200

µM PHL to minimize the efflux of radioactivity from the adipocytes, that contributes to obtaining more exact results.

2. In the study of AA uptake, plasma membranes should be purified. This would allow the exact determination of the part of the adipocyte were AA acts to improve glucose uptake.In this study it was not possible to do this because the crude membrane fraction included the plasma membranes, mitochondria and other cytosolic membranes.

3. To come to an accurate conclusion in the study of the influence of insulin on AA uptake, obese and non-obese subjects should be tested for their IR status.

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Appendix 1:

Manuscripts submitted for publication:

Malipa,ACA, Meintjes,R & Haag,M Glucose and arachidonic uptake into fresh human isolated adipocytes" . Cell Biochemistry & Function

Appendix 2:

Participation in Conferences:

Malipa, A.C.A., Meintjes,R.A., Matlala,E.,Laurie,R., Mouton,A.,Groot,M., Dreyer,G. & Haag,M.2006 "Rapid uptake of arachidonic acid into isolated human adipocytes" Physiological Society of Southern Africa, Durban,October,2006

Haag,M., Laurie,R., Matlala, E. & Malipa,A. "Rapid effects of fatty acid on adipocyte glucose uptake". 19th World Congress of Diabetes, Cape Town, December 2006. Published abstract in Diabetic Medicine 2006; 23 (Suppl.4):479

Appendix 3:

Chromatograms of Chapter 5

Chromatogram 1: AA uptake into control crude membranes after 10 min. exposure of adipocytes to vehicle

Chromatogram 2: AA uptake into crude membranes after 10 min. exposure of adipocytes to AA

Chromatogram 3: AA uptake into crude membranes after 30 min. exposure of adipocytes to AA