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Regulation of gene expression in pre-adipocytes and adipocytes: role of alpha tocopheryl-phosphate

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Abstract

The molecular function of vitamin E has been the object of intensive investigations during at least the last 50 years (Zingg and Azzi 2004). After the original molecular understanding of vitamin E as an antioxidant at the level of cells and organs, it has become clear that, beyond and above this, vitamin E could act in *in vitro* cell systems as well as *in vivo* as a regulator of gene expression and cell signal transduction. More recent work has also shown that vitamin E can undergo phosphorylation in cells and experimental animals. The phosphorylated form of vitamin E, α-TP, was recently also found in human plasma and tissues. It has been also described that supplementation with α-TP in humans is able to increase the amounts found in plasma (Zingg, Libinaki et al. 2010).

α-TP, naturally present in the body or after supplementation, appears to be in amounts (nanomolar concentrations) not compatible with a storage form of vitamin E. The hypothesis has been then formulated that α-TP is a signaling molecule, similar in nature to other lipid-phosphate compounds such as inositol phosphates or sphingosine phosphate. Although very stable, α-TP can be somewhat hydrolyzed both in cells and in the body. However, the amounts of free α-T produced after administration to cells or to experimental animals are not sufficient to explain the superior function of α-TP relative to α-T(Zingg, Meydani et al. 2010). In fact, in a number of experiments ranging from cell signaling to protection against atherosclerosis or heart infarction, α-TP was shown, at the same concentrations, to be more effective than α-T (Libinaki, Tesanovic et al. 2010). The function assigned to α-T, alternative to the antioxidant one, has been to modulate the expression of a number of genes both in the sense of up and down regulation. In the attempt to understand the molecular function of α-TP a gene array analysis of the two molecules, α-T and α-TP has been made. Also in this case it was shown that, at the same concentration, α-TP appeared to be, in some cases, several folds more potent than the non-phosphorylated compound. The analysis of the genes over expressed in the presence of α-TP by THP-1 cells indicated that a surprising number of them belonged to a group of genes involved in cell lipid storage (See figure 6.1) and in particular, REDD-1, TRB3, C8FW [anti-G-Protein-Coupled Receptor Induced Protein GIG2 (C8FW) (TRIB1)], Sestrin-2, and Insulin Induced Gene 1, INSIG(Zingg, Libinaki et al. 2010).

As a proof of concept, the transcription/translation of the VEGF gene was analyzed by quantitative RT-PCR and by Western blot, and the results were fully consistent with the gene array outcome.

In the course of this study, NIH3T3-L1 pre-adipocytes have been employed, cells that have the ability to differentiate from fibroblasts to adipocytes (see Figure 5.2). In parallel experiments α-TP appears to be more effective than α-T in inhibiting the proliferation of NIH3T3-L1 at the same concentrations and incubation time. This experiment confirms that α-TP acts as such under these conditions, and not as a precursor of free α-T; in fact the parent compound is much less active, excluding the possibility that the effects of α-TP be caused by its conversion to α-T.

α-T is not able to induce lipid accumulation both in NIH3T3-L1 pre-adipocytes and in adipocytes. On the other hand, α-TP was capable of inducing a significant lipid accumulation in adipocytes and it had only a small effect in NIH3T3-L1.

To evaluate the effect of α-T and of α-TP on the gene expression, we performed RT-PCR using specific primers for the genes which are most relevant in the process of fat accumulation in cells, namely Sestrin 2, TRB3 and INSIG.

These genes were shown to be strongly up-regulated in THP-1 cells by α-TP .

- Sestrins potentiate AMPK and inhibit activation of target of mTOR increased lipolysis and increased fatty acid oxidation may result. At the same time inhibition of fatty acid synthesis is also occurring.
- TRB3 has been shown to suppress adipocytes differentiation by negative regulation of PPAR alpha and to facilitate the proteasome degradation of acetyl-CoA-carboxylase.
- INSIG1 plays an important role in the SREBP-mediated regulation of cholesterol biosynthesis, its action resulting in a decreased expression of HMG-CoA-reductase and in increased degradation of the enzyme.

These genes are more relevant in the lipid metabolism.

All these genes pathways, should increase lipid degradation and inhibition of lipid synthesis, but more studies are, however, needed to substantiate this hypothesis.

In all cases, it is surprising that the effects of α-T are opposite to those of α-TP. In particular a clear dose-dependent up regulation of Sestrin by α-TP is observed in NIH3T3-L1 pre-adipocytes as well as a clear suppression by α-T in the same cells. Also TRB3 and INSIG transcription is up-regulated by α-TP and down regulated by α-T.

It appears therefore that in NIH3T3-L1 pre-adipocytes, the presence of α-T diminishes the transcription of fat catabolic enzymes while α-TP activates the transcription of the same genes therefore making this cell metabolically more active and less prone to accumulate lipid.

When the NIH3T3-L1 differentiated to adipocytes the opposite picture becomes visible. Sestrin transcription is clearly diminished by α-TP. The transcription of the other two genes appears also to be down regulated although to a lesser extent. On the other side α-T was capable of stimulating transcription of genes.

Consequently, also in adipocytes α-T appears to have an effect opposite to that of α-TP the former being efficient in up regulating the gene set intended to limit lipid accumulation.

The simplest conclusions that can be drown from the experiments presented here is that the presence of α-TP (but not α-T) in NIH3T3-L1 pre-adipocytes appears to activate a transcriptional gene set potentially preventing fat accumulation in these cells. In undifferentiated adipocytes, α-TP appears to be responsible for activation of those potentially protective genes. Using cell lines *in vitro* may not give reliable indications of the complex metabolic disorder resulting in obesity. However, *in vitro* results are important to understand, at least in part, the complexity of the disease. The use of high concentrations (micromolar) of α-TP may be criticized on the basis of the amounts physiologically found in plasma (nanomolar). However, the presence of large amounts of divalent cations needed in the incubation media for cell activities and survival makes the actual concentration of the free α-TP several orders of magnitude lower than the added amounts due to the sequestration properties of the divalent metal ions. Consequently, the used amounts of α-TP are comprised within a physiological range. Altogether, describing and understanding the effects of the two physiological compounds α-T and α-TP on single genes or a set of genes may be useful to focus upon some details of the complex picture of the control of pathological fat accumulation.

Clinical studies are, however, needed to substantiate this hypothesis.

CHAPTER 1
INTRODUCTION

1.1 Introduction

In 1905, Englishman William Fletcher determined that if special factors (vitamins) were removed from food, disease ensued. Fletcher was researching the causes of the disease Beriberi when he discovered that eating unpolished rice prevented Beriberi and eating polished rice did not. William Fletcher believed that there were special nutrients contained in the husk of the rice. The next year, English biochemist Sir Frederick Gowland Hopkins also discovered that certain food factors were important to health. In 1912, Polish scientist Casimir Funk named the special nutritional parts of food as "vitamine" after "vita" meaning life and "amine" from compounds found in the thiamine he isolated from rice husks. Vitamine was later shortened to vitamin when it was discovered that not all of the vitamins contain nitrogen, and, therefore, not all are amines. Together, Hopkins and Funk formulated the vitamin hypothesis of deficiency disease - that a lack of vitamins could make people sick.

1.2 Vitamin E

Vitamin E, in the form of alpha tocopherol (α-T) was discovered in 1922 by Evans and Bishop, as a factor essential for reproduction (Evans and Bishop 1922). Lack of this factor in rats caused 'infertility' in male rats and increased risk of miscarriage / abortion in female rats. The name tocopherol then came from the Latin word 'tokos' (childbirth) and 'phorein' (to bring forth). The suffix 'ol' was added at the end to indicate the phenolic nature of the substance (Kamal-Eldin and Appelqvist 1996).

Nowadays, the term vitamin E is used as the collective name for eight naturally occurring molecules, four tocopherols and four tocotrienols, which qualitatively exhibit the biological activity of α-T.

Vitamin E occurs in nature in at least eight different isoforms: alpha, beta, gamma, and delta tocopherols and alpha, beta, gamma, and deltatocotrienols (Packer, Weber et al. 2001). The eight isomers of vitamin E share some important traits:

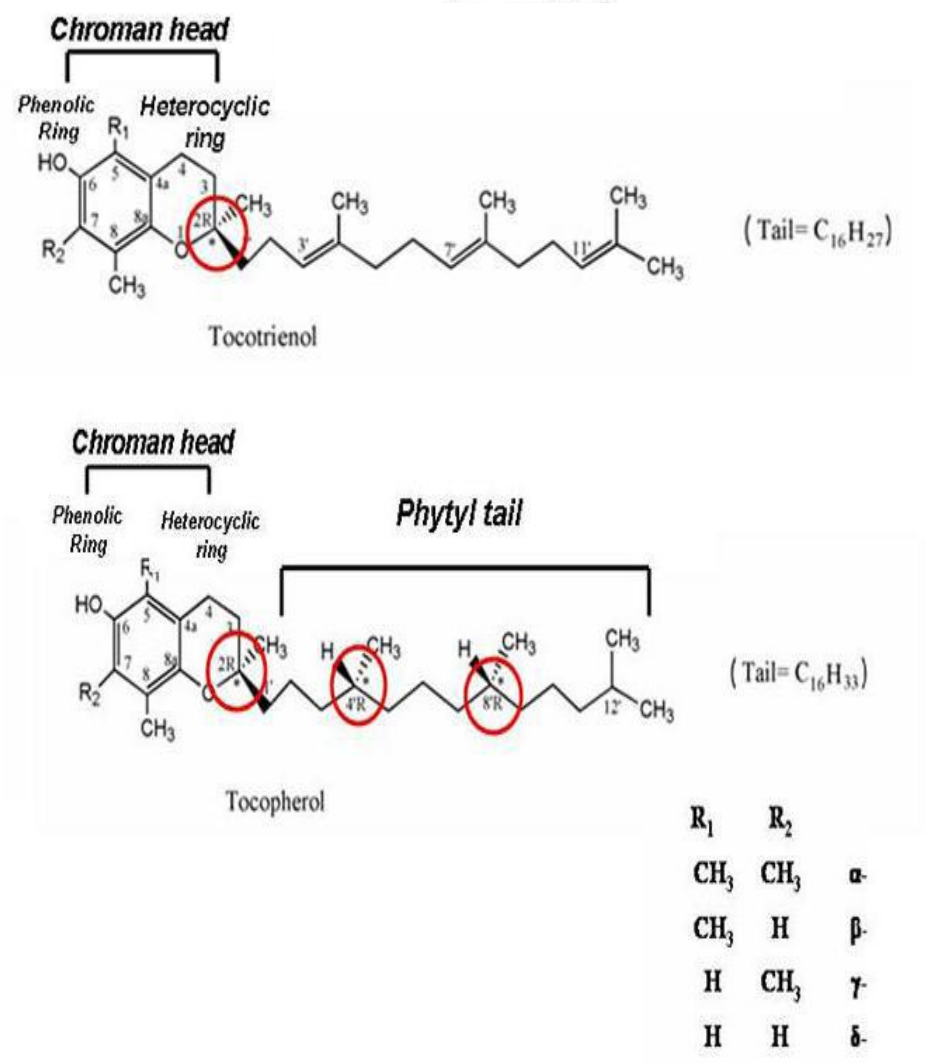
Firstly, all have a head or chroman ring in technical term. Secondly, all have a 'tail', which is called the phytyl tail for tocopherols.

Thirdly, all have the hydroxyl group, which is the active antioxidant group on the head of the molecule.

Tocotrienols differ from the corresponding tocopherols only in their aliphatic tail. Tocopherols have a phytyl side chain attached to their chromanol nucleus, whereas the tail of tocotrienol is unsaturated and forms an isoprenoid chain.

The tocotrienol tail has three double bonds while the tocopherol tail has none. The alpha, beta, gamma, and delta isoforms of tocotrienols and tocopherol differ by the number [alpha has 3, beta and gamma have 2, while delta has 1] and position of their methyl groups on the chromanol nucleus (Traber 1997) (Packer, Weber et al. 2001). The structures of tocopherol and tocotrienols are illustrated in figure 1.1

Figure 1.1 The molecular structures of tocopherol and tocotrienol.

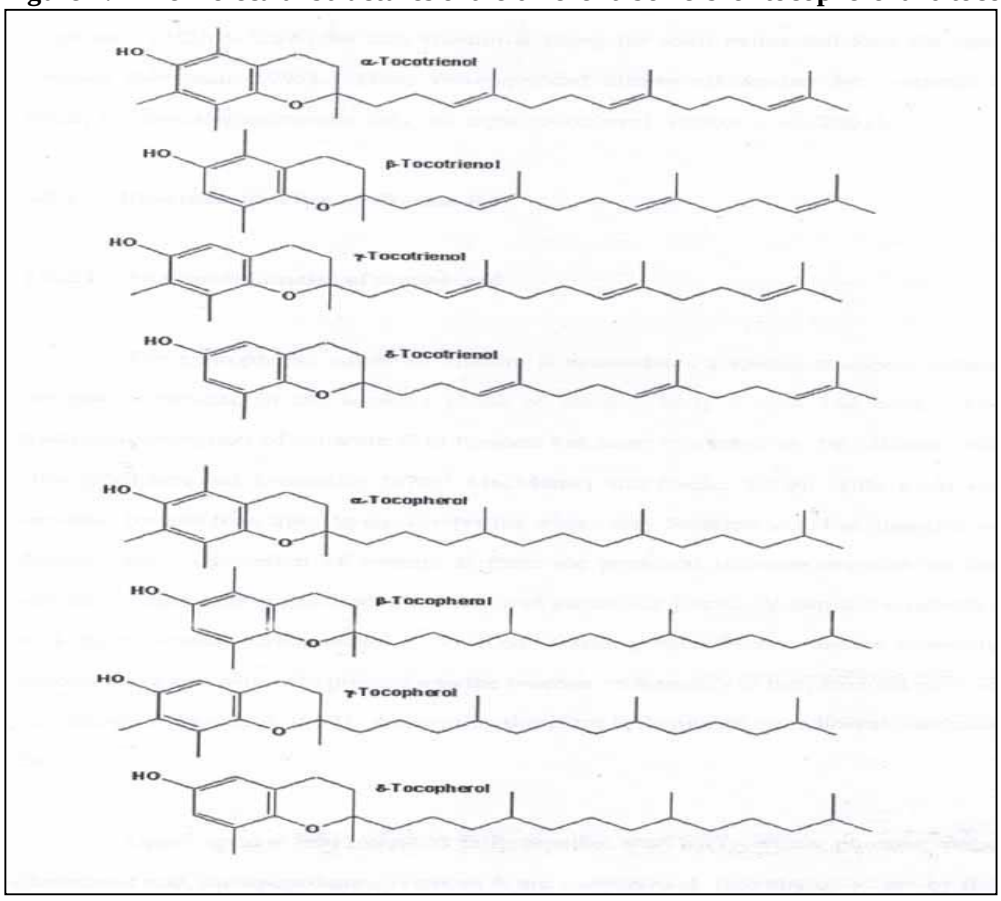


1.3 Source of Vitamin E

Lipid rich plant products and vegetable oils are the main natural sources of vitamin E. These include nuts, wheat germ, sunflower, safflower, palm oils, rice bran and others. Tocopherols are present in polyunsaturated vegetable oils and in the germ of cereal seeds. Tocotrienols are found in high concentrations and is the predominant vitamin E in palm oil, barley and rice bran (Theriault, Chao et al. 1999). Approximately 70%-75% percent of the vitamin E content in palm oil consists of tocotrienol isomers, while the rest are alpha tocopherol. Palm oil was reported to be the richest source of tocotrienols in nature (Tan and Brzuskiewicz 1989). Other cooking oils such as corn oil, soy and sunflower oils are good sources of tocopherol but contain no tocotrienols.

Vitamin E is included as one of the body's essential nutrient which must be obtained either from dietary intake or as a supplement. The recommended daily dietary allowance [RDA – USA] for this vitamin is 15 mg for adult human (Zingg 2007). These recommended dietary allowances for vitamin E define the human requirement only for α-T (Packer, Weber et al. 2001).

Figure 1.2 The molecular structures of the different isomers for tocopherol and tocotrienol



1.4 Absorption, Transport and Distribution to tissue

The hydrophobic nature of vitamin E necessitates a special transport system for this substance in the aqueous phase of plasma, body liquids and cells. The fractional absorption of vitamin E in humans has been estimated to be between 60-70% (Kelleher and Losowsky 1970) (MacMahon and Neale 1970). Bile acids are secreted by the liver into the small intestine where they function to aid in digestion of dietary fat. Absorption of vitamin E from the proximal intestine depends on the amount of lipid / fat in the food, bile acids and pancreatic juices. Vitamin E emulsifies with lipid soluble components in the food forming micelles that can be passively absorbed in a non saturable process into the mucosa cells mainly at the proximal parts of the intestine (Meydani and Hayek 1995). Absorption therefore is facilitated by a liberal intake of fat.

1.4.1 α-Tocopherol absorption

Upon uptake into intestinal cells, together with triglycerides, phospholipids, cholesterol and apolipoproteins, the isoform of vitamin E are reassembled into chylomicrons via the Golgi apparatus in the mucosal cells (Traber, Kayden et al. 1986). Chylomicrons are excreted by exocytosis into the lymphatic system to reach the blood (Kayden and Traber 1993).

Chylomicron lipolysis, facilitated by the enzyme lipoprotein lipase, allows part of vitamin E to be distributed to tissues. The remaining chylomicron remnants deliver the remaining α-T to the liver. Inside the liver, vitamin E is incorporated into lyosomes. Uptake of vitamin E into lysosomesis non specific, but there is a specific transport protein to α-T, that is the α-tocopherol transfer protein [α–TTP] (see chapter 1.6) which is sized at 32 kDa. α–TTP transports α-T from lysosomes into lipoproteins, especially Very Low Density Lipoprotein [VLDL], before the liver cells secrete VLDL into the blood stream for distribution to other organs (Stocker and Azzi 2000). VLDLs provide transport and delivery of alpha-tocopherol to peripheral cells (Bjorneboe, Bjorneboe et al. 1990).

Secretion of α-T into VLDL can lead to the enrichment of all circulating lipoproteins with α-T (Kayden and Traber 1993) (Bjorneboe, Bjorneboe et al. 1990). Upon secretion into the plasma, the VLDL is catabolised by the lipoprotein lipase and returned to the liver after conversion in the circulation to low density lipoprotein (LDL).

α-T that is secreted from the liver in VLDL can have alternative fates. Some of the alpha tocopherol can be transferred to High Density Lipoprotein [HDL] during

lipolysis, some can travel with the VLDL core during conversion to LDL, and some can return to the liver as VLDL remnants, the intermediate density lipoprotein (IDL). Thus, transport of tocopherol in the plasma is mainly via by plasma lipoproteins, especially LDL and HDL in association with plasma surface components. However, plasma vitamin E is in a constant state of flux between the lipoproteins.

α-TTP gene mutation results in low serum and cell α-T. Thus, the two factors needed for realising an adequate level of α-T in the body are dietary availability and the expression of liver α-TTP. α-TTP is also expressed in tissues including the trophoblast region of placenta. The α-TTP probably plays an important role in supplying the vitamin to the foetus, and explains the foetal resorption occurring in rats fed a vitamin E deficient diet. Plasma phospholipid transfer protein facilitates the exchange of tocopherol between LDL and HDL. α-T that is bound to lipoprotein is transported from the blood into cells in peripheral tissues with the help of the scavenger receptor B1 [SR-B1] (Kayden and Traber 1993). In the cells, α-T is placed into cell membrane components including mitochondria and the endoplasmic reticulum (Machlin, Harris et al. 1991).

Dimitrov reported that the plasma elevation of α-T was affected by dietary fat intake. Individuals consuming a high fat diet showed significantly greater plasma α-T concentrations compared with those fed a low fat diet. This is explained by α-T being a fat soluble vitamin, thus its absorption will be affected by dietary lipids and bile in the gastrointestinal tract (Dimitrov, Meyer et al. 1991).

1.5 Tocotrienol absorption

The fate of supplemented tocotrienols and the relationship between its intestinal absorption, blood levels and tissue distribution is still not well studied (Watkins, Cockrill et al. 1999). Some investigators even had difficulties in detecting plasma tocotrienols before and after supplementation; a study reported that in fasting humans the plasma tocotrienol concentration was not increased significantly after tocotrienol supplementation (Hayes, Pronczuk et al. 1993).

Studies using thoracic duct cannulated rats showed that, similarly to α-T, alpha, delta and gamma tocotrienols were also transported via the lymphatic system after oral absorption (Ikeda, Imasato et al. 1996). This is because they are too lipophilic to be absorbed via the hepatic portal vein. In the same work is also reported that alpha tocotrienol was preferentially absorbed compared with delta and gamma tocotrienols. A

report suggested that tocotrienols are transported non-specifically, like any lipid-soluble compound, most likely being incorporated with triglyceride in the core of the triglyceride rich chylomicrons (Hayes, Pronczuk et al. 1993).

Once transported to the adipose tissue, a modest level of tocotrienols especially gamma tocotrienols appears to be deposited and stored with the triglycerides, presumably to be released during lipolysis. Unlike α-T, which has a cytosolic binding protein in the liver to sequester and enhance their conservation and re-secretion into lipoproteins, no similar transport / binding protein have been found as yet for tocotrienols.

The bioavailability of alpha, gamma and delta tocotrienols, under fed and fasted conditions, were studied in eight healthy volunteers after a single dose of 300mg mixed tocotrienols composed of approximately 56.1%, 29.4%, 14.4% and 31.5% respectively of gamma, alpha and delta tocotrienol and α-T (Yap, Yuen et al. 2001). Absorption of tocotrienols was markedly increased and also more consistent when taken with food. Peak plasma concentrations for alpha, gamma and delta tocotrienol were reported to be achieved at 4.3 hrs after dosing for alpha and gamma tocotrienol and at 3.9 hours after dosing for delta tocotrienol (Yap, Yuen et al. 2001). Plasma concentrations of all tocotrienols increased markedly when dosed with food; mean maximum concentration [Cmax] and area under the curve [AUC] values for all tocotrienol isomers for the fed state were higher compared with the values of the fasted state. This is because absorption of fat soluble vitamins in general, requires emulsification by bile salts before transportation across the gut mucosa. A high fat diet causes stimulation of bile secretion, which may thus explain the increased absorption of tocotrienol in the fed state. There were no significant differences in the plasma half life of the tocotrienol isomers when supplementation was given in the fed or fasted state. The volume of distribution of tocotrienol appeared to be relatively big, which the authors reported may be indicative of either incomplete absorption or extensive redistribution from the blood or both. Whereas plasma transport of tocopherol is mainly via plasma lipoproteins, especially LDL and HDL, the exact plasma transport of tocotrienol is not well elucidated and may differ from tocopherol. Hayes and colleagues reported that there appears to be a lack of tocotrienols in LDL or HDL (Hayes, Pronczuk et al. 1993). They also reported that the concentration of tocotrienols in platelets was only about 3-5% that of the alpha tocopherol, although the platelet concentration of tocotrienols doubled after supplementation with "palm vitee" [tocotrienol enriched palm oil capsules] in humans. The increase in tocotrienols in platelets was greater than the percentage of increase

observed for tocopherols (Hayes, Pronczuk et al 1993). A study published in the same year however contradicted Hayes study (Suarna, Hood et al. 1993). Suarna and colleagues reported that after dietary supplementation with mixed tocotrienols, alpha and gamma tocotrienols were actually detected in circulating lipoproteins. Suarna's study had used a sensitive method of detecting tocotrienols that was electrochemical detection following chromatographic separation. There is currently no data in humans on tissue accumulation of tocotrienols (Suarna, Wu et al. 2006).

In hamsters, tissue concentrations of tocotrienols were typically much lower than tocopherol (1:180 on average) following palm vitee supplementation. The exception is in the adipose tissue where the tocotrienol to tocopherol ratio in supplemented hamsters was greater than 1 (Hayes, Pronczuk et al. 1993). The adipose tissue was the only tissue where the tocotrienol level exceeds that of tocopherol concentration.

1.6 α-Tocopherol Transfer Protein (α-TTP)

The major regulatory mechanism for controlling plasma α-T concentrations is the α-T transfer protein (α-TTP). α-TTP has been isolated and its cDNA sequences reported from a variety of species including human, mouse, rat, dog, and cow (see Entrez retrieval system, National Center for Biotechnology Information). α-TTP has been crystallized and the α-T-binding pocket identified (Hosomi, Arita et al. 1997; Meier, Tomizaki et al. 2003; Min, Kovall et al. 2003).

Interestingly, the structure has a hinge and a cover that entraps α-T in the binding pocket. α-TTP has differing affinities for various forms of vitamin E with ;

RRR-α-tocopherol=100%, β-tocopherol = 38%, γ-tocopherol = 9%, δ-tocopherol = 2%, α-tocotrienol = 12% (Arita, Nomura et al. 1997)

Thus, the affinity of α-TTP for vitamin E forms is one of the critical determinants for their plasma concentrations (Hosomi, Arita et al. 1997). The human α-TTP gene is located at the 8q13.1–13.3 region of chromosome 8 (Arita, Sato et al. 1995; Doerflinger, Linder et al. 1995). α-TTP expression was first reported in hepatocytes (Yoshida, Yusin et al. 1992). α-TTP mRNA has also been detected in rat brain, spleen, lung, and kidney (Hosomi, Goto et al. 1998) and in mouse liver and adrenals, but is low or undetectable in mouse cerebral cortex, lungs, heart, and spleen (Gohil, Godzdanker et al. 2004). α-TTP protein has been detected in human brain (Copp, Wisniewski et al. 1999). Furthermore, α-TTP is present in pregnant mouse uterus and human placenta (Kaempf-Rotzoll, Igarashi et al. 2002; Kaempf-Rotzoll, Horiguchi et al. 2003). Muller-Schmehl

et al. (Muller-Schmehl, Beninde et al. 2004) reported that concentrations of placental α-TTP mRNA were second only to those in the liver. The reports of uterine and placental α-TTP emphasize the importance of vitamin E during pregnancy and emphasize that the fetal resorption test (Machlin, Gabriel et al. 1982) is not likely to be reliable for assessing bioavailability for various forms of vitamin E because any trace amounts of α-T present in the test with vitamin E would be preferentially taken up by α-TTP to protect the uterus and placenta.

1.7 Causes of human Vitamin E Deficiency

The importance of α-TTP function in determining human vitamin E status was elucidated when patients with vitamin E deficiency caused by defects in the α-TTP were discovered. Overt vitamin E deficiency occurs only rarely in humans. Most often, vitamin E deficiency had been described as a symptom secondary to fat malabsorption. α-T deficiency causes both peripheral neuropathy (Traber, Sokol et al. 1987) and increased erythrocyte hemolysis (Kayden, Silber et al. 1965).

1.7.1 Ataxia

Genetic defects in α-TTP are associated with a characteristic syndrome, ataxia with vitamin E deficiency [AVED, previously called familial isolated vitamin E (FIVE) deficiency]. AVED patients have neurologic abnormalities, which are similar to those of patients with Friedreich's ataxia (Ben Hamida, Doerflinger et al. 1993; Ben Hamida, Belal et al. 1993). The symptoms are characterized by a progressive peripheral neuropathy and ataxia (Sokol, Kayden et al. 1988). Retinitis pigmentosa is also a symptom associated with vitamin E deficiency, and several mutations in the α-TTP gene in patients with AVED has been described (Matsuya, Matsumoto et al. 1994; Yokota, Shiojiri et al. 1996). Importantly, vitamin E supplementation stops or slows the progression of retinitis pigmentosa in these patients (Yokota, Shiojiri et al. 1996).

1.7.2 Fat Malabsorption Syndromes

Vitamin E deficiency secondary to fat malabsorption occurs because vitamin E absorption requires biliary and pancreatic secretions. Children with cholestatic liver disease, who have impaired secretion of bile into the small intestine, have severe fat malabsorption (Traber, Sokol et al. 1993). Neurologic abnormalities, which appear as early as the second year of life, become irreversible if the vitamin E deficiency is

uncorrected (Sokol, Heubi et al. 1983; Traber, Sokol et al. 1993). Children with cystic fibrosis can also become vitamin E deficient because the impaired secretion of pancreatic digestive enzymes causes steatorrhea and vitamin E malabsorption, even when pancreatic enzyme supplements are administered orally (Kaempf-Rotzoll, Horiguchi et al. 2003). More severe vitamin E deficiency occurs if bile secretion is impaired (Stead, Muller et al. 1986; Sokol, Reardon et al. 1989). It should be emphasized that any disorder that causes chronic fat malabsorption, including chronic diarrhoea in children, can lead to vitamin E deficiency. Thus, poor intake of nutrients generally could lead to vitamin E deficiency if the fat malabsorption is sufficiently severe and the child has low body stores.

1.7.3 Genetic defects in lipoprotein synthesis

Studies of patients with hypobetalipoproteinemia or abetalipoproteinemia (low to non detectable circulating chylomicrons, VLDL, or LDL) have demonstrated that lipoproteins containing apolipoprotein B are necessary for effective absorption and plasma vitamin E transport (Rader and Brewer 1993). These patients have steatorrhea from birth because of the impaired ability to absorb dietary fat, which also contributes to their poor vitamin E status. Clinical features also include retarded growth, acanthocytosis, retinitis pigmentosa, and a chronic progressive neurological disorder with ataxia. Clinically, both hypobetalipoproteinemic or abetalipoproteinemic subjects become vitamin E deficient and develop a characteristic neurologic syndrome—a progressive peripheral neuropathy—if they are not given large vitamin E supplements (approximately 10 g per day) (Rader and Brewer 1993; Traber, Rader et al. 1994). Despite low plasma concentrations, adipose tissue α–tocopherol concentration reach normal levels in patients given large (10 g/day) vitamin E doses (Traber, Rader et al. 1994). These findings emphasize the difficulty of assessing vitamin E status in patients with abnormal plasma lipid concentrations. Tissue concentration can be altered, but the plasma vitamin E concentration reflects the abnormal circulating lipid levels (Sokol, Heubi et al. 1984).

1.7.4 Severe malnutrition

Hepatic α-TTP is required to maintain normal plasma α-T concentrations (Ouahchi, Arita et al. 1995). It is, therefore, not surprising that vitamin E–deficiency symptoms have been reported in children with severely limited food intake, which not only might

be limiting in vitamin E, but also limiting in the dietary protein necessary to synthesize α-TTP. The study reported that 100 patients with protein energy malnutrition (PEM) had low plasma α-T concentrations (8 μmol/L or less) and low α-T/lipid ratios, as well as neurologic abnormalities characteristic of vitamin E deficiency. With 6 weeks vitamin E supplementation, not only were the subjects' circulating α-T levels normalized, but there was also improvement in their neurologic abnormalities (Kalra, Grover et al. 2001). This pair of reports clearly identifies vitamin E deficiency as a cause of the PEM neurologic syndrome (Kalra, Grover et al. 1998; Kalra, Grover et al. 2001). In general, the degree to which vitamin E deficiency is associated with kwashiorkor and/or marasma is not clear because fat malabsorption has been reported as a confounding factor during recovery from extreme malnutrition (Murphy, Badaloo et al. 2002).

1.8 Non-Antioxidant effects of α-Tocopherol

Vitamin E has been considered mainly an antioxidant and has received a lot of attention as a possible preventive and/or reducing approach against cardiovascular diseases. This partly stems from the fact that it is the principal lipid soluble chain breaking antioxidant in human tissues, membrane and plasma (Machlin, Harris et al. 1991; Traber and Sies 1996). It has been described_also as a major lipid soluble chain breaking antioxidant that prevents the propagation of free radical reactions in membranes and lipoproteins (Azzi 2004). It was also reported to be the predominant antioxidant in the LDL particles (Esterbauer, Dieber-Rotheneder et al. 1991). However, recent meta-anaylsis on vitamin E supplementation did not show any benefit on cardiovascular disease (Miller, Pastor-Barriuso et al. 2005; Bjelakovic, Nikolova et al. 2007).

1.8.1 Vitamin E and cardiovascular disease

A critical reconsideration of the rationale at the basis of the effort made to identify a therapeutic role for vitamin E (mainly as α-T) in chronic and age-related diseases has occurred in the last years. Cardiovascular disease (CVD), following the original work of Fred Gey (Gey, Puska et al. 1991) showing distribution of this aliment that inversely correlated with the vitamin E intake of the studied populations, has been the object of a large series of *in vitro* and *in vivo* studies that initially suggested a role of this vitamin in the prevention of early atherosclerotic lesions (Traber and Atkinson 2007), and (Azzi

2007) and in the regulation of immuno-inflammatory, coagulative and cell protection mechanisms (Zingg 2007). Observational evidence of a role of vitamin E in the primary prevention of CVD came mostly from prospective cohorts such as the Nurses' Health Study and the Health Professionals' Follow-Up Study (Jialal and Devaraj 2002).

In the last two decades, as one of the most recent examples, in the recent evaluation of the Physicians' Health Study II (PHS-II) trial, vitamin E as well as vitamin C was not found to promote CVD prevention (Sesso, Buring et al. 2008). The Authors concluded that ''neither vitamin E nor vitamin C supplementation reduced the risk of major cardio-vascular events. These data provide no support for the use of these supplements for the prevention of cardiovascular disease. The un ambiguous conclusion from these trials appears to be that using vitamin E supplements, as well as any other antioxidant supplement, to prevent CVD progression and mortality is not recommended, and rather it should be discouraged. However, an optimal intake of natural vitamin E, introduced with a well-balanced diet, appears to be best way to obtain prevention effects, if any, on myocardial infarction, stroke and other atherothrombotic conditions and is now a well accepted health recommendation that has been extended also to all the natural antioxidants in food (Lichtenstein, Appel et al. 2006).

The evidence discussed has clearly demonstrated the absence of a relevant preventive role of vitamin E in CVD. However, a debate on the discrepancy between the expected results and the negative evidence provided by the clinical trials is still open. A further complication has come from metanalysis studies revealing possible vitamin E toxicity in patients treated with high doses of α-T (Miller, Pastor-Barriuso et al. 2005; Bjelakovic, Nikolova et al. 2007). These studies may have suffered from the study selection bias and from the used statistic methods (Berry, Wathen et al. 2009). However, as discussed in a commentary by Blumberg and Frei (Blumberg and Frei 2007) some functions of this vitamin and particularly that of an in vivo antioxidant might need very high dosages to be achieved (Roberts, Oates et al. 2007), compatible with potential damage and the supposed increase mortality (Miller, Pastor-Barriuso et al. 2005; Bjelakovic, Nikolova et al. 2007).

1.8.2 Does vitamin E act as an antioxidant in humans?

Today, the antioxidant properties of α-T are a very well established chemical paradigm.

In one study has been show that the vitamin E can act as an antioxidant in the test tube, in lipid and phospholipid suspensions (Barclay, Artz et al. 1995), in cell-free Hevea

brasiliensis latex (Whittle, Audley et al. 1967), or in plants, although in this case the alternative function of cellular signaling by modulating jasmonic acid levels has been also proposed (Munne-Bosch, Weiler et al. 2007). There is little doubt that, *in vivo*, if given in pharmacological concentrations, possibly by parenteral administration to humans or animals, α-T must act as an antioxidant; however, this situation goes obviously beyond the concept of physiological function. Such an antioxidant function, that is, intrinsic part of the chemistry of the molecule, may in fact not be always desirable, similarly to the possible negative effects of the administration of other antioxidants in large amounts. In more recent years the mechanism of action of α-T has been thoroughly reinvestigated. In light of new experimental findings, the view of Tappel (Tappel 1980) that the chain-breaking antioxidant vitamin E is the main protector against *in vivo* lipid peroxidation and of Burton and Ingold (Burton, Joyce et al. 1982) that vitamin E functions in living systems primarily as a lipid antioxidant and free radical scavenger had to be revised. Among the important discoveries that have brought to this new paradigm is the finding that the α-T has been shown not to protect *in vivo* against oxidative damage nor to prevent diseases which have at their basis an oxidative insult. (Azzi 2007)

Altogether, the_conclusion can be drawn that α-T is not physiologically_acting as an antioxidant.

Further evidence comes from the experimental observations that α-T is able to modulate a number of cell functions in a unique way, not shared by any other antioxidants (Mahoney and Azzi 1988; Boscoboinik, Szewczyk et al. 1991).

The non antioxidant properties of tocopherol were discovered when, in several experimental models, the four tocopherol analogs had different effects, although they share a similar antioxidant capacity. It can be speculated that the selective uptake and transport of α-T represents the evolutionary selection of a molecule with specific functions, different from its antioxidant properties.

1.9 Functions of α-Tocopherol

Three quarters of a century after its discovery, we are just beginning to understand vitamin E physiology and molecular action.

The role of vitamin E in cellular signaling has been shown (Azzi, Breyer et al. 2000); it includes a number of cell functions:

1. Effects of α-T at cellular level

2. Transcriptional regulation by α-T
3. Signal transduction
4. Inhibition of monocyte-endothelial adhesion (Yoshikawa, Yoshida et al. 1998)
5. Inhibition of platelet adhesion and aggregation (Mabile, Bruckdorfer et al. 1999)

1.9.1 Effects of α-T at cellular level

In 1991 inhibition of PKC activity was found to be at the basis of the vascular smooth muscle cell growth arrest induced by α-T (Boscoboinik, Szewczyk et al. 1991; Tasinato, Boscoboinik et al. 1995). Many reports have subsequently confirmed the involvement of PKC in the effect of α-T on different cell types, including monocytes, macrophages, neutrophils, fibroblasts, and mesangial cells (Devaraj, Li et al. 1996; Devaraj, Adams-Huet et al. 1997; Tada, Ishii et al. 1997; Azzi, Breyer et al. 2001). α-T, but not β-tocopherol, was found to inhibit thrombin-induced PKC activation and endothelin secretion in endothelial cells (Martin-Nizard, Boullier et al. 1998). α-T, and not β-tocopherol, inhibits the activity of PKC from monocytes, followed by inhibition of phosphorylation and translocation of the cytosolic factor p47(phox) and by an impaired assembly of the NADPH-oxidase and of superoxide production (Cachia, Benna et al. 1998). α-T has the important biological effect of inhibiting the release of the pro inflammatory cytokine, IL-1β, via inhibition of the 5-lipoxygenase pathway (Devaraj and Jialal 1999). Inhibition of PKC by α-T in vascular smooth muscle cells is observed to occur at concentrations of α-T close to those measured in healthy adults (Gey 1990). β-Tocopherol per se is not very effective but prevents the inhibitory effect of α-T. The mechanism involved is not related to the radical scavenging properties of these two molecules, which are essentially equal. *In vitro* studies with recombinant PKC have shown that inhibition by α-T is not caused by tocopherol-protein interaction (Clement, Tan et al. 2002). α-T does not inhibit PKC expression as well. Inhibition of PKC activity by α-T occurs at a cellular level by producing dephosphorylation of the enzyme, where by β-tocopherol is much less potent (Ricciarelli, Tasinato et al. 1998). Dephosphorylation of PKC occurs via protein phosphatase (PP)2A, which is activated by the treatment with α-T (Ricciarelli, Tasinato et al. 1998; Neuzil, Weber et al. 2001). One group (Koya, Lee et al. 1997) has reported that prevention of glomerular dysfunction in diabetic rats can be achieved by treatment with α-T. Such a protection occurs through inhibition of PKC. In this case, however, α-T would act on the diacylglycerol pathway by activating the enzyme diacylglycerol kinase with consequent

diminution of diacylglycerol and PKC activation. In these studies, high glucose was responsible for increased diacylglycerol synthesis, which was counteracted, in the presence of α-T, by the activation of diacylglycerol kinase (Azzi, Boscoboinik et al. 1998).

Table 1.1 Effects of α-T and their supposed molecular mechanisms.

Reaction	Reference
Inhibition of cell proliferation	(Boscoboinik, Szewczyk et al. 1991)
Inhibition of platelet adhesion and aggregation	(Saldeen, Li et al. 1999)
Inhibition of cell adhesion	(Steiner, Li et al. 1997)
Inhibition of ROS in monocytes and neutrophils	(Wu, Hayek et al. 2001)
Inhibition of PKC	(Ricciarelli, Tasinato et al. 1998)
Activation of PP_2A	(Neuzil, Weber et al. 2001)
Inhibition of 5-lipoxygenase	(Devaraj and Jialal 1999)
Activation of diacylglycerol kinase	(Lee, Koya et al. 1999)
Inhibition of α-tropomyosin expression	(Aratri, Spycher et al. 1999)
Inhibition of liver collagen α1 expression	(Chojkier, Houglum et al. 1998)
Inhibition of collagenase MMP1 expression	(Ricciarelli, Maroni et al. 1999)
Modulation of αTTP expression	(Shaw and Huang 1998)
Inhibition of scavenger receptor SR-A	(Teupser, Thiery et al. 1999)
Inhibition of scavenger receptor CD36	(Ricciarelli, Zingg et al. 2000)
Inhibition of ICAM-1 and VCAM-1 expression	(Wu, Koga et al. 1999)

(Ricciarelli, Zingg et al. 2001)

1.9.2 Transcriptional regulation by α-T

In the last 15 years, the possibility of gene regulation by α-T has been analyzed (Azzi, Gysin et al. 2004). Up-regulation of α-tropomyosin expression by α-T, and not by β-tocopherol, suggests a non antioxidant mechanism (Aratri, Spycher et al. 1999). Long- and short-term α-T supplementation inhibits liver collagen a1(I) gene expression (Chojkier, Houglum et al. 1998). Age-dependent increase of collagenase expression in human skin fibroblasts can be reduced by α-T (Ricciarelli, Maroni et al. 1999). In rats, the liver α-TTP and its mRNA are modulated by dietary vitamin E deficiency (Shaw and Huang 1998). Scavenger receptors, particularly important in the formation of atherosclerotic foam cells, are also modulated by α-T. In smooth muscle cells and monocytes/macrophages, the oxidized LDL scavenger receptors SR-A and CD36 are down regulated at transcriptional level by α-T but not by β-tocopherol (Teupser, Thiery

et al. 1999; Devaraj, Hugou et al. 2001). The relevance of CD36 expression in the onset of atherosclerosis has been clarified by Febbraio and co-workers, who have shown that disruption of the CD36 gene protects against atherosclerotic lesion development in mice (Febbraio, Podrez et al. 2000). The following questions remain open. In some cases, differential effects of α-T and β-tocopherol have been found, pointing to a non-antioxidant mechanism at the basis of gene regulation (Aratri, Spycher et al. 1999; Ricciarelli, Zingg et al. 2000). In other cases, however, only α-T has been tested, thus leaving the mechanism of α-T action un clarified. Furthermore, the involvement of PKC has not always been assessed and it remains to be established whether the transcriptional regulation of certain genes is a consequence of PKC inhibition (Azzi 2007).

1.9.3 Inhibition of monocyte-endothelial adhesion

α-T enrichment of monocytes and polymorphonuclear leukocytes decreases agonist-induced and LDL-induced adhesion to human endothelial cells both in vivo and in vitro (Islam, Devaraj et al. 1998; Yoshida, Yoshikawa et al. 1999). Monocytes as well as neutrophils diminution of adhesion induced by α-T is dependent on the inhibition of adhesion molecule expression (Steiner, Li et al. 1997; Wu, Koga et al. 1999). These events are relevant to the onset of inflammation as well as in the early stages of atherogenesis (Reiter, Jiang et al. 2007).

1.9.4 Inhibition of platelet adhesion and aggregation

α-T inhibits aggregation of human platelets by a PKC-dependent mechanism both *in vitro* and *in vivo*(Venugopal, Devaraj et al. 2002). Another study has indicated that both α- and γ-tocopherol (γ-T) decrease platelet aggregation and delay intra-arterial thrombus formation (Saldeen, Li et al. 1999; Liu, Wallmon et al. 2003). That γ-T was significantly more potent than α-T suggests that a simple antioxidant mechanism is not applicable to these effects.

The studies reported above are consistent with the conclusions of Iuliano et al. (Iuliano, Mauriello et al. 2000): that circulating LDL accumulates in human atherosclerotic plaques and that such accumulation by macrophages is prevented by α-T *in vivo*. The protection by α-T may not be due only to the prevention of LDL oxidation, but also to the down-regulation of the scavenger receptor CD36 and to the inhibition of PKC

activity. Although not all agree on the molecular details, PKC inhibition is accepted as a common denominator of cellular events regulated by α-T:

- cell proliferation,
- cell adhesion,
- enhancement of immune response,
- free radical production and
- gene expression.

However, the molecular mechanisms at the basis of these events are not yet fully elucidated. A few observations, such as diacylglycerol kinase (Lee, Koya et al. 1999) activation, 5-lipoxygenase (Jialal, Devaraj et al. 2001) and cyclooxygenase (Wu, Hayek et al. 2001) inhibition, still miss a mechanistic explanation. On the other hand, the expression of several genes such as CD36 (Ricciarelli, Zingg et al. 2000), SR class A(Teupser, Thiery et al. 1999), collagenase (Ricciarelli, Maroni et al. 1999), and ICAM-1 (Wu, Koga et al. 1999) appears to be regulated by α-T in a PKC independent way. A further understanding of the molecular events at the basis of α-T gene regulation is part of ongoing studies.

In conclusion, numerous events are related to non-antioxidant properties of α-T, both at transcriptional and posttranscriptional level.

However, whether α-T acts by a pleiotropic mechanism, or it binds to a receptor capable of regulating different reactions, still remains unknown.

1.10 Gene Regulatory Activity of Vitamin E

With the discovery that the antioxidant effects of various tocopherols and tocotrienols have little relation to their vitamin E activities *in vivo* has come the realization that they have many other functions in tissues, most of which are specific to α-tocopherol.

α-Tocopherol is a gene regulator, causing up-regulation of mRNA or protein synthesis that could be the result of effects on gene transcription, mRNA stability, protein translation, protein stability and post-translational events.

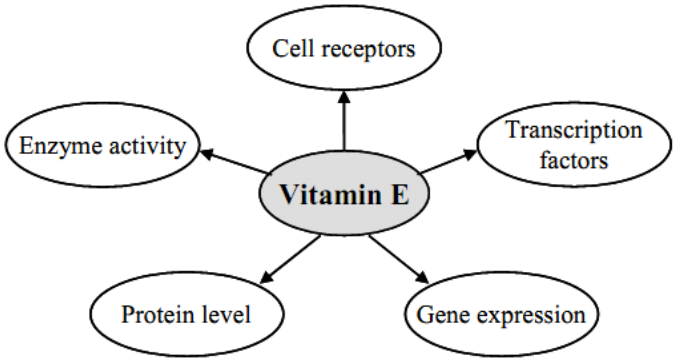
Effects have also been observed on genes connected with tocopherol catabolism, lipid uptake, collagen synthesis, cellular adhesion, inflammation and cell signalling. Vitamin E modulates the activity of several enzymes involved in signal transduction, perhaps through influencing protein-membrane interactions. However, no receptor that binds specifically to vitamin E has yet been discovered.

There are suggestions that α-tocopherol may affect the process of cellular trafficking, and especially the intracellular traffic of enzymes and vesicles, membrane fusion, and the release of the contents of vesicles.

Vitamin E is a cell signalling molecule (Azzi, Gysin et al. 2004);

- It interacts with cell receptors (e.g., LDL-receptor) and
- It interacts with transcription factors (e.g., pregnane X receptor)
- It interacts with (redox-regulated) and gene expression (e.g., scavenger receptor CD36).
- It modulates protein levels (e.g., glutathione)
- It changes enzyme activity levels (e.g., protein kinase C) both in cultured cells as well as *in vivo* (Figure 1.3).

Figure 1.3. Vitamin E is a cell signalling molecule and affects cell receptors, transcription factors, gene expression, protein levels and enzyme activities of vitamin E specific molecular targets.



(Rimbach, Moehring et al. 2010)

α-T modulates two major signal transduction pathways centered on protein kinase C and phosphatidylinositol 3-kinase. Changes in the activity of these key kinases are associated with changes in cell proliferation, platelet aggregation, and NADPH-oxidase activation. Several genes are also regulated by tocopherols partly because of the effects of tocopherol on these two kinases, but also independently of them.

These genes can be divided in five groups:

Group 1. Genes that are involved in the uptake and degradation of tocopherols:

α-TTP, cytochrome P450 (CYP3A), γ-glutamyl-cysteine synthetase heavy subunit, and glutathione-S-transferase (Masaki, Okano et al. 2002).

Group 2. Genes that are implicated with lipid uptake and atherosclerosis:

CD36 (Ricciarelli, Zingg et al. 2000), SR-BI (Kolleck, Schlame et al. 1999), and SR-AI/II (Teupser, Thiery et al. 1999).

Group 3. Genes that are involved in the modulation of extracellular proteins: tropomyosin, collagen-α-1, MMP-1, MMP-19, and connective tissue growth factor (Mauch, Kolb et al. 2002).

Group 4. Genes that are connected to adhesion and inflammation:

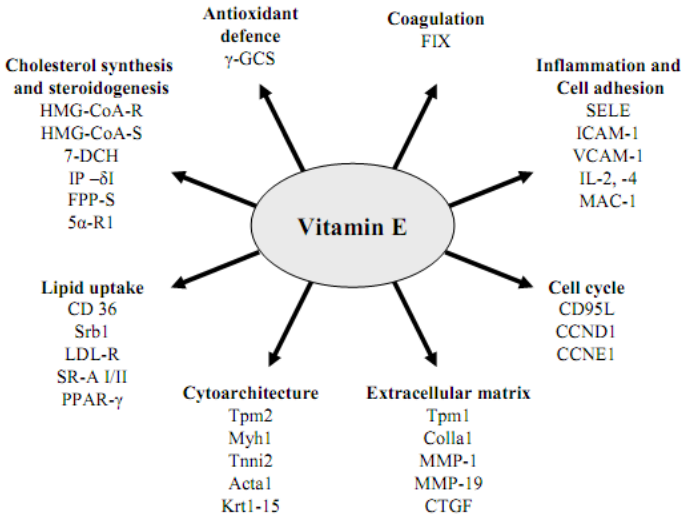
E-selectin, ICAM-1 integrins(Noguchi, Hanyu et al. 2003), glycoprotein IIb, IL-2, IL-4, IL-1b, and transforming growth factor-β (TGF-β)(Parola, Muraca et al. 1992).

Group 5. Genes implicated in cell signaling and cell cycle regulation:

PPAR-γ, cyclin D1, cyclin E, Bcl2-L1, p27, CD95 (APO-1/Fas ligand), and 5a-steroid reductase type 1.

The transcription of p27, Bcl2, α-tocopherol transfer protein, cytochrome P450 (CYP3A)(Landes, Pfluger et al. 2003), γ-glutamyl-cysteine sythetase heavy subunit, tropomyosin, IL-2, and CTGF appears to be upregulated by one or more tocopherols (Villacorta, Graca-Souza et al. 2003). All the other listed genes are downregulated. Gene regulation by tocopherols has been associated with protein kinase C because of its deactivation by α-T and its contribution in the regulation of a number of transcription factors (NF-κB, AP1)(Godbout, Berg et al. 2005). A direct participation of the pregnane X receptor (PXR) / retinoid X receptor (RXR) has been also shown. The antioxidant-responsive element (ARE) and the TGF-β-responsive element (TGF-β-RE) appear in some cases to be implicated as well (Azzi, Gysin et al. 2004).

Figure 1.4 Transcriptional regulation of vitamin E target genes.



Acta1: Actin alpha 1 skeletal muscle	**CCND1**: Cyclin D1
CCNE1: Cyclin E1 C	**CD36**: Scavenger receptor CD36(Saldeen, Li et al. 1999)
CD95L: CD95 APO-1/Fas ligand	**Colla1**: Collagen α1
CTGF: Connective tissue growth factor	**FIX:** Coagulation factor IX
FPP-S: Farnesyl pyrophosphate-synthetase	**HMG-CoA-R**:3-hydroxy-3-methylglutaryCoenzyme A reductase
HMG-CoA-S:3-hydroxy-3-methylglutaryl-coenzyme A-synthase	**ICAM-1**:Intercellular adhesion molecule-1
IL-2: Interleukin-2	**IL-4**: Interleukin-4
IP-δI: Isopentenyl diphosphate-delta isomerase	**Krt1-15**: Keratin complex 1 acidic gene15
LDL-R: Low-density lipoprotein-receptor M	**MAC-1**: Integrin, α M
MMP-1:Matrix metallopeptidase-1 (interstitial collagenase)	**MMP-19**: Matrix metallopeptidase-19
Myh1: Myosin, heavy polypeptide 1	**(Ricciarelli, Maroni et al. 1999)PPAR-γ**: Peroxisome proliferators-activate
SELE: Selectin E	**SR-A I/II**: Scavenger receptor class-A I/II
SrbI: Scavenger receptor class B type 1	**Tnni2**: Troponin I skeletal fast 2
Tpm1: α-Tropomyosin	**Tpm2**: Tropomyosin 2 beta
VCAM-1: Vascular cell adhesion molecule-1	**γ-GCS**:Gamma-glutamyl cysteinyl synthetase
5α-R1: Steroid-5-alpha-reductase 1	**7-DCH**: 7-Dehydrocholesterol reductase

(Rimbach, Moehring et al. 2010)

CHAPTER 2
Novel Tocopherol Derivates

2.1 Novel Tocopherol Derivates

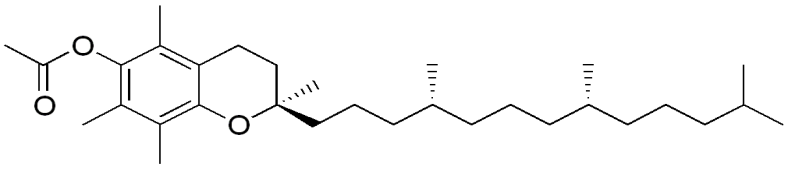
Cells incubated with different natural vitamin E analogues or synthetic derivates show striking differences in cellular response. Since the natural tocopherols and tocotrienols have essentially equal antioxidant activity, the differences seen among them cannot be the result of scavenging free radicals. Similar to that, the synthetic derivates with a modified -OH group have no chemical antioxidant activity in their non-hydrolyzed form. Thus, cellular differences seen with each vitamin E derivate can only be explained by specific non antioxidant interactions with cellular signaling and gene expression pathways.

Commercially available vitamin E consists of either a mixture of naturally occurring tocopherols and tocotrienols, αT, or synthetic racemic α-T, consisting of all possible combinations of R and S side-chain stereoisomers at equal amounts. Some of these natural and the non natural tocopherol isomers are excluded from the plasma and secreted with the bile (Traber and Kayden 1989; Brigelius-Flohe and Traber 1999).

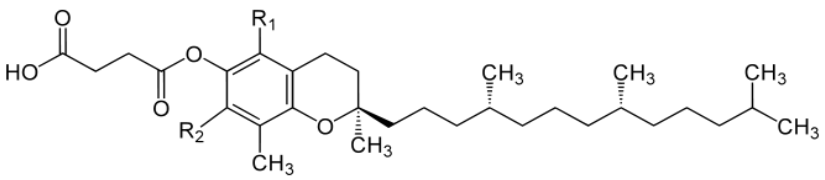
The natural vitamin E analogues chemical structures are illustrated in figure 2.1:

Figure 2.1 Vitamin E analogues chemical structures. Structure of α-tocopheryl acetate, α-tocopheryl succinate, and α-tocopheryl phosphate.

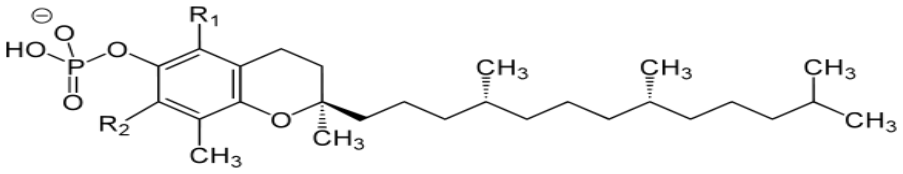
- α-tocopheryl acetate,



- α-tocopheryl succinate, and



- α-tocopheryl phosphate.



(Zingg 2007)

These tocopherol derivates have been synthesized for usage in supplements and cosmetics. In addition to stabilization, their solubility, transport and metabolism may also be different. These stabilized tocopherol derivates are modified at the –OH group of chromanol ring and thus are not susceptible to oxidation.

Some of these stabilized esters of α-T can be considered to be pro-vitamins, since they are usually converted to the natural forms by intestinal or epidermal esterases and thus ultimately perform the same function in the body as the natural vitamin E (Muller, Manning et al. 1976; Lauridsen, Hedemann et al. 2001).

Many further vitamin E derivates with modified –OH group have been synthesized and their cellular effects investigated (Zingg 2007).

These molecules often act as completely novel compounds, are transported differently and have their own effects on cellular signalling, gene expression, proliferation and apoptosis (Burton, Smart et al. 1989; Drevon 1991; Birringer, EyTina et al. 2003). The different cellular activity of these vitamin E derivatives may reflect their ability to interact with specific enzymes or proteins involved in their transport. Most of these derivatives are modified at the tocopherol-6-O position, it is often unknown to what degree these derivatives become hydrolyzed and to what degree they enhance or disrupt pathways usually targeted by the natural tocopherols.

2.2 α-Tocopheryl Phosphate (α-TP)

The phosphorylated form of α-T, α-TP (Fig. 2.2), was synthesized and tested in several experimental systems since the early 1940s (Zakharova, Shuaipov et al. 1989). After developing a novel isolation and detection method, aTP was shown only recently to occur naturally in foods and in animals as well as in human tissues. The recent discovary of aTP as a natural compound boosted new interest in the molecular actions and metabolism of this compound (Zingg, Meydani et al. 2010).

Recently, a new formulation of α-TP has been described consisting of:

- the tocopheryl phosphate ester and
- the bis-tocopheryl phosphate ester, or
- di-α-tocopheryl phosphate.

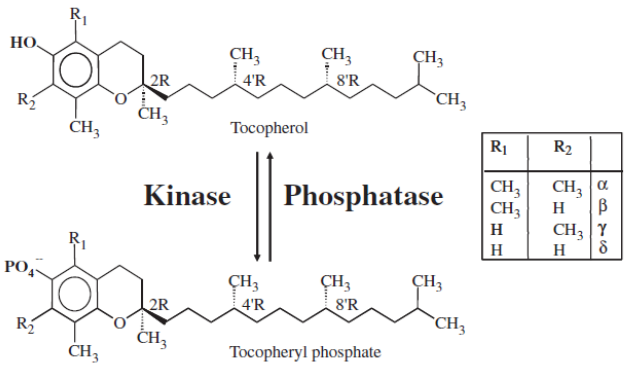
The former compound is the ester derivative of phosphate with the hydroxyl group of tocopherol, while the latter is obtained by esterification of two tocopherol moieties with one phosphate molecule (Gianello, Libinaki et al. 2005).

α-TP is an important amphipathic molecule, it is located mainly in membranes.

α-TP being a water-soluble molecule, it is resistant to both acid and alkaline hydrolysis, and undetectable using conventional assays for vitamin E. α-TP synthesis must be catalyzed by a kinase, and, to avoid excessive accumulation of the compound, a competent phosphatase must be present in cells: a postulate confirmed alkaline phosphatase is capable of slowly hydrolyzing α-TP (Rezk, Haenen et al. 2004).

It was shown that low amounts of tocopherol can become phosphorylated and dephosphorylated, suggesting that the inter conversion may serve some cellular functions (Negis, Meydani et al. 2007).

Figure 2.2: Chemical structures of α-T and α-TP and their interconversion by kinases and phosphatase



Reviewed in (Zingg, Meydani et al. 2010)

The phosphatase(s) and kinase(s), involved in interconverting α-T and α-TP may be tightly regulated, which may explain possible differences in tissue, organ, and human responses to α-T intake. The level of α-TP in tissues may depend on the presence of αT and α-TP in the diet, the transport efficiency into cells, or the activity of the αT kinase and αTP phosphatase, which may be present only in certain cells or be activated by

specific conditions, and the effective local concentrations at the site of αT phosphorylation may be much higher (Zingg, Meydani et al. 2010).

2.3 α-Tocopheryl phosphate is a natural form of vitamin E

Recent experiments have indicated that α-T is the precursor of a more active form of vitamin E. Interest in tocopheryl phosphate derivatives has increased after the discovery that α-TP is present in plant and animal tissues as well as in foods (Munteanu, Zingg et al. 2004).

By using a new method, both α-TP and α-T were extracted from a single specimen and detected by Electrospray Mass Spectrometry (ESMS), High Performance Liquid Chromatography (HPLC), Liquid Chromatography–Mass Spectrometry (LCMS/MS), and Gas Chromatography–Mass Spectrometry (GC-MS) (Gianello, Libinaki et al. 2005).

Using ESMS has been identified a molecular mass identical to that of TP in samples of biological tissue (Gianello, Libinaki et al. 2005). α-TP was detected in biological tissues including liver and adipose tissue. α-TP is therefore a natural form of vitamin E. In animal tissues (including humans) the amount of α-TP is of the same order of magnitude as that of α-T and in a few case cases it can be decisively higher (rat and pig liver) (Libinaki, Ogru et al. 2006). In animal tissues (including humans), the amount of α-TP (0,1 μg/g) is about 100 times lower than that of α-T (10 μg/g) (Gianello, Libinaki et al. 2005). This low amount suggests that it is not a storage form of α-T.

Table 2.1 α-T and α-TP levels detected in biological tissues.

TISSUE		α-Tocopherol phosphate (μg/g)	α-Tocopherol (μg/g)
LIVER	Rat	0,1	10
ADIPOSE	Rat	26-30	115
TISSUE	Human	22-28	25

(Ogru, Gianello et al. 2003)

Tocopheryl phosphate levels detected in foods sometimes the amounts of α-TP, such as in chocolate and cheese, are 10- to 30-fold higher than free αT (see Table 2.2)(Ogru, Gianello et al. 2003)

Table 2.2 α-T and α-TP levels detected in foods.

Sample	α-tocopheryl phosphate (μg/g)	α-tocopherol (μg/g)
Wheat germ	**22**	**1190**
Sun flower seeds	**9**	**500**
Almonds	**5**	**270**
Wheat germ	**10**	**110**
Chocolate	**237**	**27**
Cheddar	**337**	**17**
Brie	**437**	**14**

(Ogru, Gianello et al. 2003)

Having confirmed the existence of TP in biological tissue, the question arised where it is generated. Two main possibilities exist;

- First, TP is ingested as part of the diet, as is α-T.
- Second, it could be possible for TP to be produced in vivo by phosphorylation of α-T, which, intriguingly, implies the existence of a kinase.

To investigate the possible conversion of α-T to α-TP, radiolabeled α-[14C] tocopherol was incubated with pre adipocyte cells (3T3-L1) fat cells and was also given orally to a rats. Twenty-four hours later, the fat cells and rat livers were extracted to search for the presence of [^{14}C] TP produced in vitro and in vivo, respectively.

The results have shown that α-TP is a natural form of vitamin E and is synthesized *in viv*, as demonstrated by the fact that the cells are able to synthesize radiolabeled α-TP from radiolabeled α-TP and α-T (Gianello, Libinaki et al. 2005).

2.4 α-TP Prompts of number of questions

As discussed in the following, the natural presence of α-TP in the human body prompts a number of questions:

1. Is α-TP, when added to cells, toxic?
2. Is it going to produce, when added to cells, the same effects as α-T?

3. If α-TP is active, does it need to enter into cells in order to exhibit its activity?
4. Is the activity of α-TP due to its hydrolysis to α-T ?

2.4.1 Is α-TP, when added to cells, toxic?

Several studies have shown that at cellular level, α-TP is not toxic. Depending on the cell line no α-TP cytotoxicity has been observed up to a concentration of 100 µM. The conclusion can be drawn that, at least at cellular level, α-TP below or up to 100 µM can be used without causing cell damage (Negis, Meydani et al. 2007).

Recently, Munteanu et al. (Munteanu, Zingg et al. 2004) reported that a mixture of α-TP modulated cell proliferation and gene expression in rat aortic smooth muscle cells (RASMC) and human THP-1 monocytic leukaemia cells at a relatively low concentration. At higher concentrations, the mixture of α-TP induced apoptosis in human THP-1 monocytic leukaemia cells. Munteanu et al. (Munteanu, Zingg et al. 2004) focused on a beneficial role of the mixture of α-TP in atherosclerosis and inflammation. In our opinion, the apoptotic effect of α-TP on monocytic leukaemia cells might be of interest too. In fact Bashir et al (Rezk, van der Vijgh et al. 2007) has shown that α-TP inhibits osteosarcoma cell proliferation and induces cell death and apoptosis.

Due to its potent biological activities and the presence of amounts in the diet and our body, α-TP might strengthen our therapeutic arsenal.

Clinical studies are, however, needed to substantiate this hypothesis.

2.4.2 Is it going to produce, when added to cells, the same effects as α-T?

Only recently, health benefits of α-TP have become apparent.

A number of cellular reactions (cell proliferation and gene expression) has been previously shown to be more sensitive to α-TP than to α-T. It emerges from a study that α-TP inhibits cell growth in human THP-1 cells while α-T does not produce any significant effect (Negis, Meydani et al. 2007). α-TP modulates the activity of several enzymes; in cell culture, it affects proliferation, signal transduction, and gene expression at concentrations lower than tocopherol (Munteanu, Zingg et al. 2004). In high cholesterol fed rabbits and LDL knock-out mice α-TP prevents atherosclerosis at much lower concentrations than tocopherol (Negis, Aytan et al. 2006). However, an study demonstrated the ability of α-TP to reduce myocardial ischemia reperfusion injury by enhancing ventricular performance and reducing myocardial infarct size and

cardiomyocyte apoptosis (Mukherjee, Lekli et al. 2008). Being α-TP more active than α-T and given the molecular structure of the two compounds it appears possible that the conversion of α-T to α-TP produces a more active compound. α-TP, added to cell media is capable of producing effects, such as modulation of proliferation and gene expression (Munteanu, Zingg et al. 2004).

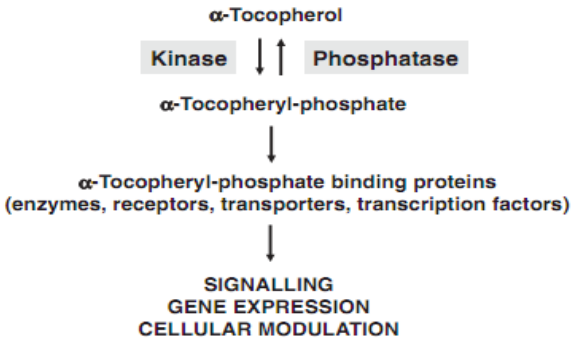
2.4.3 If α-TP is active, does it need to penetrate into cells in order to exhibit its activity?

It could be assumed that α-TP acts at the cell membrane, possibly modulating surface receptors; alternatively one can suppose that α-TP is internalized by the cell and that its action occur within the cytosol. It appears also possible that α-TP acts specifically in organelles such as mitochondria, Golgi apparatus or nucleus. Such an action would possibly require intracellular transport proteins such as tocopherol associated protein (TAP), which in fact is increasing transport of the synthetic vitamin E derivative, a-tocopheryl succinate, into cells (Ni, Wen et al. 2005; Neuzil, Dong et al. 2006). The experimental data has produced evidence that α-TP penetrates across the cell membrane and that this event is mediated by a carrier (Negis et al), possibly a member of the family of the organic anion transporters (OAT) (Kim 2003; Sekine, Miyazaki et al. 2006) that are inhibited by glibenclamide and probenecid, two inhibitors of a number of OAT. Glibenclamide prevented α-TP induced cell growth inhibition, indicating that α-TP acts after its uptake inside cells. The possibility that α-TP is more efficient than α-T due to its better penetration through the plasma membrane and its easier hydrolysis by cellular esterases can be excluded, sincein THP-1 cells, only a minimal amount (<10%) of α-TP was hydrolyzed to α-T, and amounts of α-T 10-fold higher than those liberated from α-TP were not able to inhibit cell proliferation. An additional possibility, that the α-TP found in tissues may represent a storage form of α-T appears to be unlikely, given the small amounts recovered from tissues of the order of 150 to 250 ng/g tissue, thus in the submicromolar range. These very low amounts found in tissues (of the same order of magnitude of the well-known signaling molecule phosphatidylinositol phosphate (IP) are compatible with the proposal that α-TP is a signaling molecule (Zingg, Meydani et al. 2010).

2.5 α-TP Modulation of Proliferation

In order to exhibit its cell modulatory effects, αTP must be recognized by a protein (possibly a receptor), capable of transducing the ligand information into cellar responses. This possibility is open to experimental analysis and is an obvious alternative to the elusive search for α-T receptors. In conclusion, the discovery of the synthesis and hydrolysis of α-TP in tissues and cells is not only an interesting observation but it has now been rationalized in an experimentally testable hypothesis.

Figure 2.3 Hypothetical scheme of α-T phosphorylation and biological activity of α-TP



(reviewed in (Zingg, Meydani et al. 2010))

2.6 Function non antioxidant of α-TP

αTP has per se no chemical antioxidant activity since it is phosphorylated at the chromanol -OH group, which in α-T is essential for the scavenging of free radicals. Despite that, it has been suggested to reduce oxidative stress by preventing the propagation of free radicals in membranes from one polyunsaturated fatty acid to another or possibly by interfering with their enzymatic generation by specific interaction with enzyme(s) and/or receptor(s) (Rezk, Haenen et al. 2004; Tanaka, Moritoh et al. 2007). An ''indirect'' antioxidant function of α-TP via induction or regulation of enzymes involved in scavenging ROS and RNS was so far not observed. In contrast to αT (Masaki, Okano et al. 2002), α-TP was ineffective in elevating the intracellular level of the redox-active antioxidant glutathione (GSH). The α-TP plays a role in modulating the antinflammatory effects of α-T (Sampayo-Reyes and Zakharyan 2006).

Recently, Libinaki et al (Libinaki, Tesanovic et al. 2010) reported that a mixture of α-TP compared with that of α-T on key pro-inflammatory markers involved in

atherogenesis, including interleukin (IL)-1β, IL-6, IL-8, plasminogen activator inhibitor-1, tumour necrosis factor-α and C-reactive protein (CRP). This study reported that after treatment with α-TP, incorporated into the rabbit food resulted in a significant reduction in plasma levels of all pro-inflammatory cytokines and biomarkers that appeared to be somewhat dose dependent.

The reduction in these key pro-inflammatory markers appears to follow the improvement in the atherogenic state of the animals, indicating that the anti-inflammatory properties of α-TP may be potentially beneficial in inflammatory disease states (Libinaki, Tesanovic et al. 2010).

2.7 Biological function

α-TP may be ultimately the molecule which specifically interacts with a receptor or transcription factor and in this way modulates cellular functions. αTP when tested in vitro modulated many enzymes possibly acting a cofactor, or as a''second messenger'', or as ligand of a receptor or transcription factor in the membrane capable of exerting regulatory effects (Negis, Zingg et al. 2005).

Several cellular functions and activities have been suggested for α-TP:

- Induction of hippocampal long term potentiation (Xie and Sastry 1993)
- Protection of mouse skin against ultraviolet-induced damage (Nakayama, Katoh et al. 2003)
- Activation of cAMP phosphodiesterase (Sakai, Okano et al. 1976)
- Activation of rat liver phenylalanine hydroxylase (Abita, Parniak et al. 1984).

At the molecular level αTP (and possibly also αT with a different activity of specificity) may act as a ''second messenger'' may bind to membrane receptors and activate or inhibit signal transduction via G-proteins or receptor tyrosine kinases similar to other phosphorylated lipids, such as ceramide-1-phosphate, sphingosine-1-phosphate (Benaud, Oberst et al. 2002), or others.

It is plausible that αTP acts as an intracellular signaling molecule mediating some of the effects seen with αT on gene expression and cellular signaling (Fig. 2.4) (Zingg 2007).

2.8 αTP modulates gene expression

In recent years vitamin E (αT) has been shown to modulate the activity of several pathways involved in signal transduction and gene expression (Zingg 2007).

When tested with cells *in vitro*, α-TP has been shown to have more potent cellular effects than α-T itself in terms of inhibition of cell proliferation and regulation of gene expression.

Zingg et al. compared the cellular effects of αT with those of the novel phosphorylated analog, αTP (Zingg, Libinaki et al. 2010).

Gene expression microarrays showed that α-TP was able to regulate more genes than α-T, and most of the α-TP -regulated genes were up-regulated (Zingg, Libinaki et al. 2010). The study by Zingg et al., carried out *in vitro*, suggests an activation of signaling pathways initiated by αTP.

Specifically α-TP;

- Regulated genes with a possible role in the neurological symptoms of vitamin E deficiency
- Regulated genes with a possible role in the anti-cancer effects of vitamin E
- Regulated genes with a regulatory role in nutrient metabolism and glucose and lipid homeostasis (Zingg, Libinaki et al. 2010).

2.8.1 Regulation of genes relevant for neurological disorders and α-TP

It should also be noted that vitamin E deficiency in humans with full neurological symptoms is rare and usually is the consequence of mutations of the α-T transfer protein leading to AVED.

α-TP may play an essential role in preventing the predominant neurological symptoms of severe vitamin E deficiency and of other neurodegenerative disorders as well as the cellular damage particularly after ischemia/reperfusion injury (Zingg, Meydani et al. 2010).

Moreover, it can be speculated that the main neurological symptoms of severe vitamin E deficiency are in part the result of the lack of conversion of αT into the more potent α-TP, which may activate phosphatidyl inositol 3-kinases (PI3K).

PI3K belongs to a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking, which are, among other functions, also involved in cancer development.

The serine/threonine protein kinase Akt, also known as protein kinase B (PKB), was initially identified as one of the downstream targets of PI3K.

Akt is a serine/threonine protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration.

αTP ⟹ PI3K ⟹ Akt

α-TP activating PI3K/Akt could be essential for the survival of specific neurons or muscle cells (de Jesus Ferreira, Crouzin et al. 2005).

It is noteworthy that in amyotrophic lateral sclerosis (ALS) (Lambrechts and Carmeliet 2006) and in Alzheimer disease (Lee, Kumar et al. 2009) patients, Akt signaling is decreased and the activation of PI3K/Akt by vascular endothelial growth factor A (VEGF) decreases neuron cell death (Li, Xu et al. 2003). It thus seems possible that induction of PI3K/Akt/VEGF by α-TP could be the basis for some of the beneficial effects seen after vitamin E supplementation in patients with ALS but also with other neurodegenerative disorders (Lambrechts and Carmeliet 2006), and treatment directly with α-TP may be more potent than with α-T in preventing these diseases (Zingg, Meydani et al. 2010).

αTP ⟹ PI3K ⟹ Akt ⟹ VEGF

The up-regulation seen with the dystrophin-associate sarcoglycan-γ protein may be relevant for the preventive effects of vitamin E against muscular dystrophies (Kefi, Amouri et al. 2003), and the reported higher preventive activity of α-TP may be in part the result of the stronger up-regulation of that gene (Hove and Harris 1947).

2.8.2 Regulation of genes relevant for cancer, αT and α-TP

DNA mutation is a critical step in carcinogenesis, and elevated levels of oxidative DNA lesions (8-OH-G) have been noted in various tumors, strongly implicating the involvement of such damage in the etiology of cancer. However, reactive oxygen species within cells may act as secondary messengers in intracellular signaling cascades, which induce and maintain the oncogenic phenotypes, although they can also induce cellular senescence and apoptosis and can therefore function as anti tumorigenic species. A diminution of cell proliferation by vitamin E has been described (Boscoboinik, Szewczyk et al. 1991) to be associated with protein kinase C (PKC) inhibition (Ricciarelli, Zingg et al. 2001). Especially, γ-tocopherol (γ-T) has been shown to be effective in decreasing cell cycle activity by inhibiting cyclins expression and activity (Giovannucci 2000; Gysin, Azzi et al. 2002). Additionally, a retardation of cell growth may be attributed to the anti-inflammatory property of γ-T (Jiang, Christen et al.

2001). Inhibition of cell proliferation by vitamin E has also been correlated to the inhibition of PKB, (Kempna, Reiter et al. 2004; Reiter, Azzi et al. 2007). Vitamin E may be effective against tumor progression by reducing the release of the angiogenic peptide, VEGF from human tumor cells (Schindler and Mentlein 2006). In prostate cancer cells, growth inhibition efficacy of α-T is correlated with α-T retention and its transporter gene expression (Ni, Pang et al. 2007). Furthermore, α-T associated protein (TAP) and its role in suppressing prostate cancer cell growth by inhibition of the PI3K pathway have been described by Kempna et al. (Kempna, Reiter et al. 2004) and Neuzil et al. (Neuzil, Dong et al. 2006). In advanced colorectal cancer patients, vitamin E was shown to increase cytolytic activity of natural killer cells (Hanson, Ozenci et al. 2007). A word of caution is needed when considering the positive roles of vitamin E in diminishing tumor progression. Since removal of oxidized bases is performed predominantly by the base excision repair (BER) pathway, and it has been shown that induction of DNA repair genes occurs in response to oxidative stress (Powell, Swenberg et al. 2005), antioxidants may exert a negative role on this repair mechanism. In conclusion, protection by vitamin against free radical induced DNA mutations is improbable.

On the other hand, in vitro evidence that different tocopherols slow down cell proliferation is generally accepted. However, such an event may not be sufficient to result in beneficial clinical outcomes.

The possibility that α-T can be used in the general prevention of cancer appears to be invalidated by a number of clinical studies. However, the effectiveness of α-T in some particular types of cancer or in particular subpopulations (Zingg, Azzi et al. 2008) may not be excluded at this time. In vitro, it appears improbable that α-T can act as a free radical scavenger under physiological conditions and protect against radical induced DNA mutations and cancer. On the other hand, a number of *in vitro* and *in vivo* studies suggest a role of α-T and other tocopherols in inhibiting cancer cell proliferation.

Recently, the α-TP has been discovered as a natural and more active form of α-T, capable of affecting gene expression and tumor cell proliferation more strongly than α-T. In a particular tumor, ovarian cancer cells, α-TP suppresses telomerase activity and may be an important protective agent against ovarian cancer cell growth as well as a potentially effective therapeutic agent for this for cancer (Bermudez, Ahmadi et al. 2007). The discovery of α-TP has opened a new field, not only in the understanding of the molecular mechanism of action of this activated α-T form, but also in the possible

role of the activation process, potentially needed for the expression of the antiproliferative effects of α-T.

2.8.3 Regulation of genes relevant for metabolism and α-TP

The activation of genes with a regulatory role in nutrient metabolism and glucose and lipid homeostasis (e.g., growth factor receptor-bound protein 19 (GRB10), UL16-binding protein 1 (ULBP1), tribbles homolog 3 (TRIB3/NIPK/SKIP3), a family of stress-inducible proteins (SESN2), phosphoenolpyruvate carboxykinase (PEPCK), TSC22D3/GILZ, and insulin-induced gene 1 (INSIG1) suggests some regulatory role of αTP important for diabetes, obesity, and the metabolic syndrome (Song and DeBose-Boyd 2006).

In line with a possible regulatory role of α-TP on glucose and lipid homeostasis, a dose-dependent decrease in serum glucose level was measured in male rats after treatment with α-TP, and decreased cholesterol and lipid concentrations were observed in hypercholesterolemic rabbits (Libinaki, Tesanovic et al. 2010). The vitamin E phosphate wasreported to have atherosclerotic-preventing effects in rabbits fed with a high-cholesterol diet (Libinaki, Tesanovic et al. 2010).

CHAPTER 3
LIPID METABOLISM

3.1 Obesity

A mounting concern throughout the world is the increasing prevalence of obesity and there is growing evidence that obesity is associated with a chronic system low-grade inflammatory state and that inflammation is one of the potential mechanisms of obesity-related morbidity (Clement and Langin 2007). The anatomical distribution of adipose tissue is a key indicator of metabolic alterations in cardiovascular diseases. Obesity has emerged as an independent risk factor and the excess of fat mass in the upper part of the body constitutes a classical risk factor for diabetes and cardiovascular diseases (Clement and Langin 2007). It has also been confirmed to correlate with an increased risk for:

1) insulin resistance,
2) hypertension,
3) hypertryglyceridemia,
4) prothrombotic state and
5) proinflammatory state (Smith 2007).

Obesity arises when energy intake, principally stored as triglycerides, exceeds energy expenditure (Flier, Kulkarni et al. 2001; Flier 2004). Obesity is a complex trait influenced by diet, developmental stage, age, physical activity and genes (Brockmann and Bevova 2002; Friedman 2003). There is convincing experimental evidence showing that the balance between energy intake (food consumption) and energy expenditure (basal metabolic rate, i.e. biochemical processes required to maintain cellular viability, physical activity and adaptive thermogenesis) is tightly regulated. A homeostatic network maintains energy stores through a complex interplay, regulating storage and mobilization of fat stores (Friedman 2000; Flier, Kulkarni et al. 2001; Sainsbury, Cooney et al. 2002; Flier 2004; Cone 2005).

There are genes that encode the molecular components of this system may underlie obesity and related disorders. In the past decade, identification of myriad lipid and protein signals secreted from this tissue has led to its recognition as a major endocrine organ (Rondinone 2006; Trayhurn and Bing 2006).

Abnormally and/or excessively differentiated adipocytes may help induce the metabolic syndrome by producing insulin-resistant hormones and cytokines (e.g., tumor necrosis

factor-α). Insulin is a key afferent signal that controls energy balance. Insulin is secreted from the endocrine pancreas in proportion to fat mass and exerts potent effects on peripheral nutrient storage. Insulin causes long-term inhibitory effects on energy intake. Together, these responses maintain energy balance via both intake and expenditure pathways (Porte, Baskin et al. 2002; Cone 2005).

Intricate metabolic networks tightly coordinate the flow of sugars and fats through synthesis, storage, and breakdown pathways. The differentiation of pre-adipocytes into adipocytes is regulated by an elaborate network of transcription factors that control the expression of hundreds of genes responsible for establishing the mature adipocyte phenotype.

Adipogenesis is a developmental process by which cells differentiate to fulfill adipose tissue specific functions, among which are fat storage and production of adipose hormones (also called adipokines). Lipogenesis results in cellular lipid accumulation, via the uptake of lipogenic substrate from the diet, endogenous fatty acid synthesis, and fatty acid storage as triglycerides. It should be noted that lipolysis, which releases fatty acids into the blood, is the reciprocal important function of the white adipose tissue (WAT). However, little is known on the transcriptional regulation of lipolysis. Transcriptional regulation of the genes involved in fatty acid metabolism is presently considered as the major long-term regulatory mechanism controlling lipid homeostasis (Rosen, Wing et al. 2005).

3.2 Energy resource of cells

In general, cells break down carbohydrates, amino acids and fats to generate ATP, the universal energy resource of cells (Rudin-Brown, Greenley et al. 2004). Carbohydrates are broken down via glycolytic enzymes to pyruvate and further to acetyl-CoA which powers generation of NADH and $FADH_2$ through the tricarboxylic acid cycle (TCA) cycle. In turn, NADH and $FADH_2$ are used to generate ATP via oxidative phosphorylation and ATP synthesis. Mobilization of stored triacylglycerides is initiated by lipolytic enzymes such as hormone-sensitive lipase. Liberated fatty acids are then activated to their respective acyl-CoA derivates by acyl-CoA synthases/ligases. Breakdown of fatty acyl-CoAs to acetyl-CoA occurs in peroxisomes or mitochondria via β-oxidation enzymes (Rudin-Brown, Greenley et al. 2004).

3.3 Mitochondrial Fatty Acid β-Oxidation

3.3.1 The fatty acid β-oxidation pathway:

Fatty acid β-oxidation is the process of breaking down a long chain acyl-CoA molecule to acetyl-CoA molecules. The number of acetyl-CoA produced depends upon the carbon length of the fatty acid being oxidized. This process involves a variety of enzymes, with the four main enzymes involved in fatty acid β-oxidation being, in order;

1. acyl-CoA dehydrogenase,
2. enoyl-CoA hydratase,
3. hydroxyacyl-CoA dehydrogenase, and
4. ketoacyl-CoA thiolase (Boengler, Buechert et al. 2008).

At the end of each β-oxidation cycle, two new molecules are formed, an acetyl-CoA and an acyl-CoA that is two carbons shorter.

Additionally, during β oxidation NADH and $FADH_2$ are formed. One $FADH_2$ is produced during the reaction catalyzed by acyl-CoA dehydrogenase. A NADH is produced during the reaction catalyzed by hydroxyacyl-CoA dehydrogenase. The $FADH_2$ and NADH produced during the process of fatty acid β-oxidation is used by the electron transport chain to produce ATP.

There are different isoforms of these enzymes of β-oxidation, which have different affinities for different fatty acid chain lengths.

Interestingly, the enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, and ketoacyl-CoA isoforms specific for long chain fatty acids form an enzyme complex on the inner mitochondrial membrane.

Auxiliary enzymes are required for the β-oxidation of unsaturated fatty acids and odd chain fatty acids. Odd numbered fatty acids are broken down by β-oxidation to acetyl-CoA molecules and propionyl-CoA.

While propionyl-CoA could be metabolized through alternative pathways, it is primarily metabolized in the cell to succinyl-CoA by three enzymes (propionyl-CoA carboxylase, methylmalonyl-CoA epimerase, and methylmalonyl-CoA mutase) (Becker, Goodman et al. 1957; Boengler, Buechert et al. 2008).

This succinyl-CoA can then enter the TCA cycle. Compared to even-numbered fatty acids, odd-numbered fatty acids occur infrequently in nature (Henschel, Wenzel et al. 1997).

The two auxiliary enzymes, enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase are necessary for the complete oxidation of unsaturated fatty acids.

During the β-oxidation cycle in which the cis-double bond begins on the third carbon of the acyl-CoA, the first step involves enoyl-CoA isomerase isomerizing it before enoyl-CoA hydratase, and the other two enzymes, can act on the acyl-CoA. A double bond on an even numbered carbon requires both the auxiliary enzymes. Once the double bond is on the fourth carbon of the acyl-CoA at the beginning of a β-oxidation cycle it begins to be oxidized. Following action of acyl-CoA dehydrogenase, 2,4-dienoyl CoA reductase acts on the acyl-CoA followed by enoyl-CoA isomerase. Enoyl-CoA hydratase then acts on the acyl-CoA and the process resumes its normal order.

3.3.2 Allosteric control of fatty acid β-oxidation:

The activity of the enzymes of fatty acid β-oxidation is affected by the level of the products of their reactions (Schulz 1994). Each of the β-oxidation enzymes are inhibited by the specific fatty acyl-CoA intermediate it produces (Eaton 2002).

Interestingly, 3-ketoacyl-CoA can also inhibit enoyl-CoA hydratase and acyl-CoA dehydrogenase (Eaton 2002).

β-oxidation can also be allosterically regulated by the ratio of NADH/NAD+ and acetyl-CoA/CoA level. A rise in the NADH/NAD+ or acetyl-CoA/CoA ratios results in inhibition of fatty acid β-oxidation. Increases in the acetyl-CoA/CoA ratio have specifically been shown to lead to feedback inhibition of ketoacyl-CoA thiolase (Schulz 1994).

3.4 Fatty acid transporters

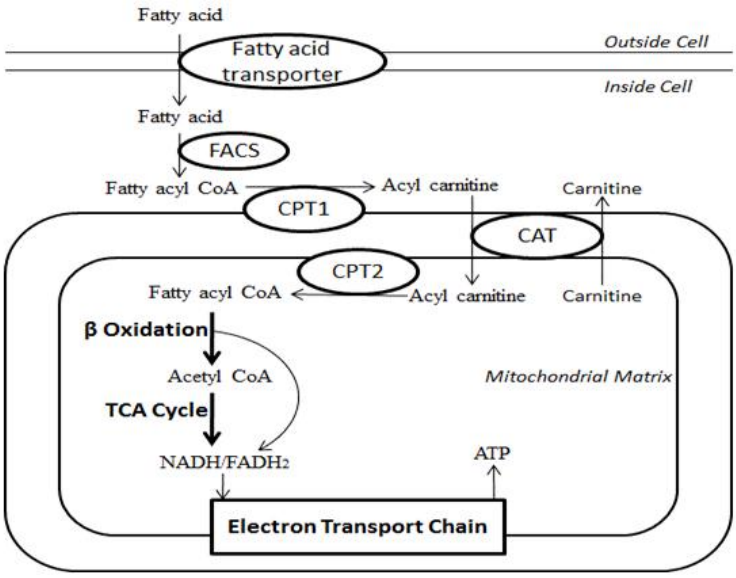
Fatty acid β-oxidation is a multi step process by which fatty acids are broken down by various tissues to produce energy. Fatty acids primarily enter a cell via fatty acid protein transporters on the cell surface (Lopaschuk, Ussher et al. 2010). Fatty acid transporters include fatty acid translocase (FAT/CD36), tissue specific fatty acid transport proteins (FATP), and plasma membrane bound fatty acid binding protein ($FABP_{pm}$) (Lopaschuk, Ussher et al. 2010). Once inside the cell, a CoA group is added to the fatty acid by fatty acyl-CoA synthase (FACS), forming long chain acyl-CoA.

Carnitine palmitoyl-transferase 1 (CPT1) conversion of the long chain acyl-CoA to long chain acylcarnitine allows the fatty acid moiety to be transported across the inner mitochondrial membrane via carnitine translocase (CAT), which exchanges long chain acylcarnitines for carnitine. An inner mitochondrial membrane CPT2 then converts the long chain acyl-carnitine back to long chain acyl-CoA. The long chain acyl-CoA enters the fatty acid β-oxidation pathway, which results in the production of one acetyl-CoA from each cycle of fatty acid β-oxidation.

This acetyl-CoA then enters the mitochondrial tricarboxylic acid (TCA) cycle.

The NADH and FADH2 produced by both fatty acid β-oxidation and the TCA cycle are used by the electron transport chain to produce ATP.

Figure 3.1: An overview of fatty acid oxidation.



Fatty acid β-oxidation is the process by which fatty acids are broken down to produce energy. Fatty acids primarily enter a cell via fatty acid protein transporters on the cell surface. Once inside, FACS adds a CoA group to the fatty acid. CPT1 then converts the long chain acyl-CoA to long chain acylcarnitine. The fatty acid moiety in transported by CAT across the inner mitochondrial membrane. CPT2 then converts the long chain acylcarnitine back to long chain acyl-CoA. The long chain acyl-CoA can then enter the fatty acid β-oxidation pathway, resulting in the production of one acetyl-CoA from each cycle of β-oxidation. This acetyl-CoA then enters the TCA cycle. The NADH and FADH2 produced by both β-oxidation and the TCA cycle are used by the electron transport chain to produce ATP.

3.5 Cellular fatty acid transport:

There has been considerable effort in recent years to elucidate the mechanisms by which the fatty acids are taken up by cells, particularly determining whether fatty acids are transported across the cellular membrane by simple diffusion or whether this transport is facilitated by membrane associated proteins. While various results support both methods of transport, transport by membrane associated proteins is believed to be the predominant means of fatty acid uptake into cells (Su and Abumrad 2009). Various membrane proteins that facilitate cellular fatty acid uptake have been identified. The membrane associated fatty acid transporters CD36/FAT, FABPpm, and FATPs, differ in their molecular weight and degree of post-translational modification (Glatz, Luiken et al. 2010).

Identification of this first FATP led to the discovery of several other isoforms of FATP (FATP1-6) (Folmes and Lopaschuk 2007). FATP1 is predominantly expressed in heart and skeletal muscles (Nickerson, Momken et al. 2007).

FATP2 and FATP5 are expressed mainly in liver, where they are involved in hepatic lipid metabolism in association with acyl-CoA synthetase.

FATP4 is essential for absorption of dietary lipids and has a critical role in normal skin structure and function.

To date, FATP3 and FATP6 have been shown to have little or no fatty acid transport function (Nickerson, Momken et al. 2007).

The role of the membrane protein CD36/FAT in fatty acid uptake and β-oxidation in mammals has been studied extensively. CD36/FAT is an 88 kDa fatty acid translocase membrane protein expressed in proportion to the rate of fatty acid oxidation in muscle tissue (for example, it is expressed more in the heart than in skeletal muscle) (Su and Abumrad 2009). CD36/FAT is involved in angiogenesis, inflammation, as well as lipid metabolism. Unlike FATP, CD36/FAT has the ability to translocate between intracellular endosomes and the plasma membrane of cells, which enables CD36/FAT to play a critical role in fatty acid uptake regulation. Insulin and muscular contraction can stimulate CD36/FAT translocation from intracellular stores to the plasma membrane, which leads to an enhanced uptake and β-oxidation of fatty acids.

Is assumed that AMP-activated protein kinase (AMPK) activation and the energy status of muscle cells may participate in the CD36/FAT translocation response (Holloway, Luiken et al. 2008). Post-translational modification of CD36/FAT through ubiquitination can also regulate the intracellular protein levels of CD36/FAT by

targeting the protein for degradation. Therefore, insulin increases the availability of CD36/FAT for translocation by inhibition of ubiquitination. However, fatty acids promote ubiquitination, which leads to CD36/FAT degradation (Lopaschuk, Ussher et al. 2010).

3.6 Fatty acid esterification to Acyl-CoA:

A fatty acid must be converted to fatty acyl-CoA in order for it to enter the mitochondria and be oxidized (Lopaschuk, Ussher et al. 2010). The enzyme responsible for esterification of fatty acids to long chain fatty acyl-CoA is FACS. For this reaction, FACS consumes the equivalent of two ATP. Another enzyme, cytosolic thioesterase (CTE), can remove the CoA converting the fatty acyl-CoA back to a fatty acid. Fatty acyl-CoA can either be converted to acyl carnitine, allowing it to be transported into the mitochondria and enter fatty acid β-oxidation or be converted to lipid metabolites (triacylglycerol, diacylglycerol, ceramide, etc.).

3.7 The acetyl-CoA carboxylase, malonyl-CoA decarboxylase:

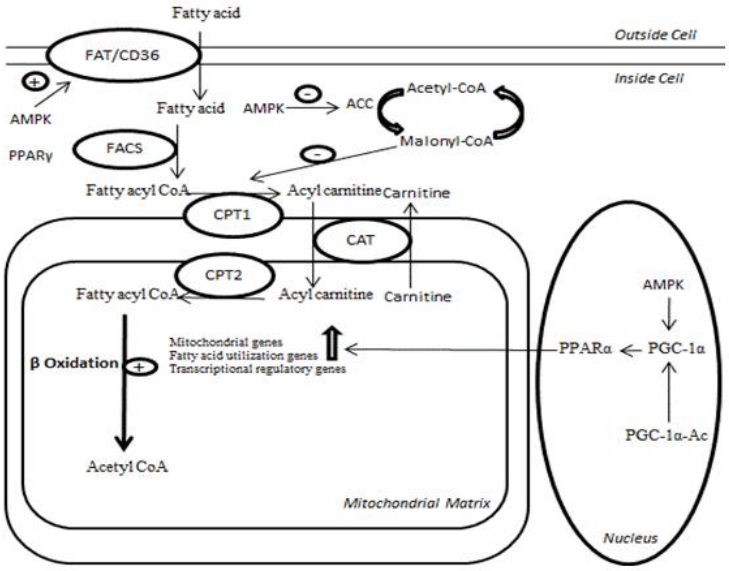
3.7.1 Acetyl-CoA carboxylase (ACC)

Acetyl-CoA carboxylase (ACC) is a central enzyme involved in fatty acid β-oxidation and fatty acid biosynthesis. ACC catalyzes the carboxylation of acetyl-CoA producing malonyl-CoA, which can be used by fatty acid synthase for fatty acid biosynthesis (Lopaschuk, Ussher et al. 2010). While malonyl-CoA is used as a substrate for fatty acid biosynthesis, malonyl-CoA is also a potent inhibitor of mitochondrial fatty acid uptake secondary to inhibition of CPT1 (Lopaschuk, Ussher et al. 2010).

There are two forms of ACC, a 265 kDa ACC1 isoform, which is highly expressed in the liver and adipose tissue, and a 280 kDa ACC2 isoform which is more specific to highly metabolic organs such as skeletal muscle and the heart (Lopaschuk, Ussher et al. 2010). AMPK plays a major role in ACC1 and ACC2 regulation by phosphorylating and inhibiting ACC activity. In situations of increased energy demand, AMPK is activated, where it then phosphorylates and inactivates both isoforms of ACC (Figure 3.2**)**. ACC2 inhibition can lead to an increase in fatty acid β-oxidation, while fatty acid biosynthesis decreases when ACC1 is inhibited (Lopaschuk, Ussher et al. 2010) Long term regulation of ACC depends on regulation of its gene expression. Several transcriptional factors can regulate ACC gene expression, including sterol regulatory element binding protein (SREBP) and carbohydrate response element binding protein

(ChREBP) (Tong 2005). The family of SREBPs governs transcriptional activation of a large number of genes involved in regulation of lipid metabolism, including lipogenesis, cholesterol transport and synthesis (Adam, Opie et al. 2010).

Figure 3.2 The fatty acid β-oxidation pathway



The four main enzymes involved in β-oxidation are: acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxy acyl-CoA dehydrogenase, and ketoacyl-CoA thiolase. Acyl-CoA dehydrogenase creates a double bond between the second and third carbons down from the CoA group on acyl-CoA and in the process produces a FADH2. Next, enoyl-CoA hydratase removes the double bond just formed, in the process of adding a hydroxyl group to the third carbon down from the CoA group and a hydrogen on the second carbon down from the CoA group. Hydroxyacyl-CoA dehydrogenase removes the hydrogen in the hydroxyl group just attached and in the process produces a NADH. In the final step, ketoacyl-CoA thiolase attaches a CoA group on to the third carbon down from the CoA group resulting in the formation of two molecules, an acetyl-CoA and an acyl-CoA that is two carbons shorter.

3.7.2 Malonyl-CoA decarboxylase (MCD)

Malonyl-CoA decarboxylase (MCD) is the enzyme responsible for decarboxylation of malonyl-CoA to acetyl-CoA (Lopaschuk, Ussher et al. 2010). Generally, the level of malonyl-CoA is decreased when MCD activity is increased, resulting in an elevated rate of fatty acid oxidation. It has been reported that protein kinases that phosphorylate and inhibit ACC might activate MCD. However, MCD appears to be primarily regulated by

transcriptional means. Therefore, MCD and ACC appear to work in harmony to regulate the pool of malonyl-CoA that can inhibit CPT1 (Lopaschuk, Ussher et al. 2010).

3.8 Mitochondrial carnitine palmitoyl transferase (CPT):

The CPT isoform, CPT1, resides on the inner surface of the outer mitochondrial membrane, and is a major site of regulation of mitochondrial fatty acid uptake (Lopaschuk, Ussher et al. 2010). As mentioned, CPT1 is potently inhibited by malonyl-CoA, the product of ACC that binds to the cytosolic side of CPT1.

Mammals express three isoforms of CPT1, which are encoded by different genes.

The liver isoform (CPT1α), the muscle isoform (CPT1β), and a third isoform of CPT1 (CPT1c), which is primarily expressed in the brain and testis (Schreurs, Kuipers et al. 2010).

More specifically, the heart expresses two isoforms of CPT1, an 82 KDa (CPT1α) isoform and the predominant 88 KDa (CPT1β) isoform (that has the highest sensitivity to malonyl-CoA inhibition). Insulin and thyroid hormone can regulate the sensitivity of CPT1α in the liver; however, the CPT1β isoform is not affected (Schreurs, Kuipers et al. 2010). Previous studies have reported that the levels of malonyl-CoA are inversely correlated with fatty acid β-oxidation rates (Lopaschuk, Ussher et al. 2010). Furthermore, studies on ACC2 knockout mice suggest two separate cellular malonyl-CoA pools, malonyl-CoA produced by ACC1 (used mainly for lipogenesis), and a cytosolic pool of malonyl-CoA produced by ACC2 involved in the regulation of CPT1 and fatty acid β-oxidation (Schreurs, Kuipers et al. 2010).

3.9 The regulation of gene expression

The regulation of gene expression is carried out in many ways, with interactions between ligands, transcription factors and the promoters of target genes. In addition to these transcriptional mechanisms; translation and post-translational modification also play an important role. In reality, the concept of pathways – which have been very successful used in the study of enzyme-metabolite-enzyme interactions – cannot be readily applied to gene expression. However, studies have consistently shown a set of transcription factors to be involved in the control of metabolic gene expression. The list below is not exhaustive, but provides and introduction to the major regulators of metabolic gene expression, particularly in the context of carbohydrate and fat oxidation. The proteins involved in fatty acid β-oxidation are regulated by both transcriptional and

post-transcriptional mechanisms. There are a number of transcription factors that regulate the expression of these proteins.

3.9.1 Sterol Response Element Binding Protein (SREBP)

Sterol regulatory element binding proteins (SREBP) are transcription factors belonging to the class containing basic-helix-loop-helix-leucine-zipper (bHLH/LZ) domains (Kim, Takao et al. 2005).

To date, three variants of SREBP have been described:

- SREBP-1a and SREBP-1c are splice variants of the same gene (SREBF1), transcribed via alternate promoters.
- SREBP-2 is transcribed from a separate locus (SREBF2) and only shares 50% homology with SREBP1, but is expressed ubiquitously (Hua, Yokoyama et al. 1993).

SREBP-1a is expressed in most tissues at a low level, but at more significant levels in cell lines and the intestine.

SREBP-1c is expressed at higher levels in skeletal muscle, adipose tissue, liver, the adrenal medulla and the brain (Shimomura, Shimano et al. 1997).

The SREBP-1 isoforms are primarily responsible for regulating fatty acid biosynthetic genes, whereas SREBP-2 controls cholesterol biosynthesis (Horton 2002).

SREBP-1c has a slightly shorter transactivation domain than SREBP-1a, which gives it a lower affinity for the promoters of target genes (Shimano, Shimomura et al. 1997).

The mechanism which regulates the activity of SREBPs is known as 'Regulated intramembrane proteolysis (Rip) (Luong, Hannah et al. 2000).

In this system, SREBPs are translated from mRNA into 125 kDa precursor proteins which are then localized to the endoplasmic reticulum (ER). In the ER SREBPs associate with two proteins; SREBP-cleavage activating protein (SCAP), and insulin-induced gene (INSIG) (Nohturfft, Brown et al. 1998). SREBPs are retained in the ER, and only translocate to the Golgi apparatus for activation after being released through one of two mechanisms.

The mature SREBP is then free to bind with SREs in the nucleus, where it can initiate transcription of target genes (Wang and Howells 1994).

Each of the SREBPs can activate all of their target genes, but with varying affinities and differing effects on transcription.

SREBP1 is involved in the lipogenic program, whereas SREBP2 targets genes involved in cholesterol biosynthetic pathways.

Unlike SREBP-2 and 1-a, the expression of SREBP-1c is not regulated by cholesterol. SREBP-1c is expression is induced by insulin (Shimomura, Bashmakov et al. 1999). This effect on SREBP-1c expression appears to be mediated via the PI3K-signalling pathway. Akt and PKC have both been shown to play a role in this action (Matsumoto, Ogawa et al. 2003). SREBP-1c expression is also induced by Liver X Receptor-α (LXRα) (Yoshikawa, Shimano et al. 2001). LXR is activated by cholesterol derivatives, and the function of its binding with SREBP-1c may be to induce lipogenesis to create cholesterol esters, which would be capable of buffering high cholesterol concentrations (Tontonoz and Mangelsdorf 2003).

3.9.2 Carbohydrate Response Element Binding Protein (ChREBP)

ChREBP is another transcription factor belonging to the basic-helix-loop-helix-leucine zipper (bHLH/LZ) family. It binds to carbohydrate response element (ChRE) sequences, which are comprised of two, 5′-CACGTG type E-box motifs separated by 5 base pairs (Yamashita, Takenoshita et al. 2001).

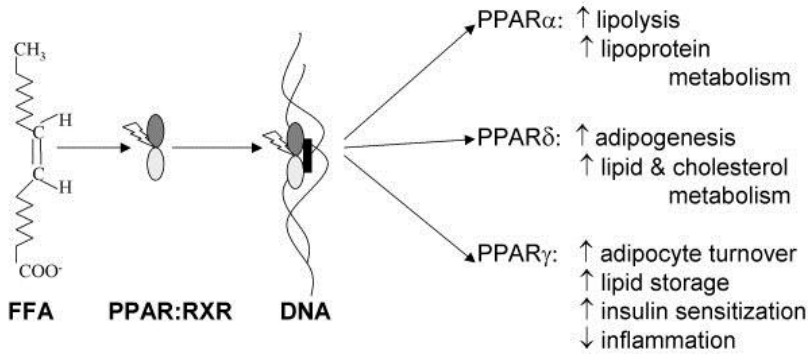
As its name indicates ChREBP is responsible for affecting glucose induced changes in gene expression. ChREBP itself is expressed in many tissues, but most highly in metabolically active tissues such as; liver, white and brown adipose and skeletal muscle (Yamashita, Takenoshita et al. 2001; Iizuka, Oka et al. 2004). ChREBP is positively regulated by glucose, independently of insulin, in a process whereby, increased glucose is metabolised via the pentose phosphate pathway (PPP). One product of the PPP is xylulose 5-phosphate (Xu 5-P), a metabolite which increases the activity of a protein phosphatase (PP2A) (Doiron, Cuif et al. 1996). PP2A activation allows ChREBP to translocate to the nucleus where it can induce lipogenic gene expression (Kabashima, Kawaguchi et al. 2003). When blood glucose concentrations are low, ChREBP is localised in the cytosol, but it rapidly translocates to the nucleus when blood glucose concentrations rise. This is the result of dephosphorylation of serine and threonine residues. The target for PKA, Ser196, is dephosphorylated, which permits entry of ChREBP into the nucleus. Ser565 and Thr666 are then dephosphorylated by PP2A, which allows it to bind with DNA and initiate transcription of target genes. ChREBP is negatively regulated by glucagon via cAMP and PKA (Burke, Collier et al. 2009), which through phosphorylation prevent DNA binding (Uyeda, Yamashita et al. 2002).

ChREBP works in partnership with SREBP to regulate lipogenesis; with some targets having binding sites for both TFs (Rufo, Teran-Garcia et al. 2001). This relationship allows a lipid storage program to be initiated only when both insulin and glucose signaling is coordinated, providing an integrated response (Towle 2001). ChREBP does not work alone, instead it forms a heterodimer with Max-like protein X (Mlx) to mediate glucose induced gene expression in liver (Ma, Trivinos-Lagos et al. 1999) Mlx acts to distinguish between E-boxes which are glucose responsive and those which are not (Dentin, Girard et al. 2005). L-type pyruvate kinase (L-PK) and lipogenic genes such as, ACC and FASN are targeted by ChREBP, to promote fat storage through de novo lipogenesis and glycolysis (Dentin, Girard et al. 2005). High fat diets are also capable of suppressing the expression of L-PK and FAS, by expediting the degradation of ChREBP, possibly by the action of PUFAs (Dentin, Girard et al. 2005).

3.9.3 The Peroxisome-Proliferator-Activated-Receptors (PPARs)

The Peroxisome-Proliferator-Activated-Receptors are members of the nuclear receptor superfamily. They have a wide range of targets involved in metabolic regulation. At present three PPAR isotypes (α,γ,δ) have been discovered, and in common with all ligand activated receptors, they have a ligand binding domain (LBD), activating-function domains (AF) and a DNA binding domain (DBD). After ligand binding occurs, the PPARs form obligate heterodimers with RXR, without which they are unable to bind to DNA.

Figure 3.3: Overview of PPAR activation and effects.



FFA interact with PPAR, which dimerize with retinoid X receptor (RXR) and translocate to the nucleus where the complex interacts with PPRE to activate gene transcription. The general effects of transcriptional activation of PPARα, PPARδ and PPARγ are shown on the right of the figure. **(Fatehi-Hassanabad and Chan 2005)**.

The endogenous ligands for the PPARs are fatty acids and their derivatives; eicosanoids and prostaglandins (Kliewer, Sundseth et al. 1997). Elevated levels of FFAs activate PPARs and depending on the isoform, the response is different. In PPARs, the DBD binds to a PPAR-response-element (PPRE) in the promoter of the target gene, and transcriptional activation is initiated. The activity of PPARs can also be increased through insulin and AMPK induced phosphorylation (Diradourian, Girard et al. 2005).

3.9.3.1 PPARα

PPARα is expressed in liver, heart, kidney, intestine, pancreas, skeletal muscle and brown adipose tissue where it induces the expression of genes involved in the transport and β-oxidation of fatty acids and glucose oxidation. PPARα target genes constitute a comprehensive set of genes that participate in many if not all aspects of lipid catabolism. This includes fatty acid transport across the cell membrane (fatty acid transporter protein genes), intracellular binding (liver fatty acid binding protein gene), activation via the formation of acyl-CoA (long chain fatty acid acyl CoA synthase gene), catabolism by β-oxidation in peroxisomes and mitochondria, and catabolism by β-oxidation in microsomes (acyl-CoA oxidase gene, medium-chain acyl-CoA dehydrogenase, and 3-hydroxy 3-methylglutaryl-CoA synthase genes).

3.9.3.2 PPARδ

PPARδ is expressed ubiquitously, it induces the expression of genes which regulate fatty acid β-oxidation, mitochondrial respiration and oxidative metabolism. It also plays a role in the regulation of HDL concentrations. PPARδ is present at higher levels in glycolytic myofibers. Post-translational modifications resulting from signalling proteins such as protein kinase A and MAPK, may phosphorylate PPARδ to increase its activity (Barish 2006).

3.9.3.3. PPARγ

PPARγ has three main splice variants – PPAR-γ1b expressed ubiquitously at low levels, PPAR-γ2, which is expressed uniquely in adipose tissue and PPAR-γ3 found in macrophages, the small intestine and adipose tissue. PPARγ is most highly expressed as the PPARγ2 isoform in white adipose tissue, where it exerts greatest influence. Its main roles are in adipocyte differentiation, fatty acid transport and storage, and glucose metabolism. It induces the expression of genes including UCP2 &-3, FATP, FABP, and GLUT4. Variants in the sequence of the PPARγ gene are not common, but in a few

studies SNPs have been linked to obesity, diabetes and hypertension (Meirhaeghe and Amouyel 2004). A recent study has shown that obesity induced by high fat feeding in mice, activates cyclin dependent kinase 5 (CDK5), which phosphorylates PPARγ, and dysregulates the expression of target genes including the insulin sensitising adipokine, adiponectin (Choi, Banks et al. 2010).

3.9.3.4 Peroxisome-proliferator-activated-receptor-γ Co-activator-1α (PGC1α)

Peroxisome-proliferator-activated-receptor-γ co-activator-1α (PGC1α), is a transcriptional coactivator which, despite its name, is a coactivator of all three PPARs.
Its main function is to regulate mitochondrial biogenesis, and hence it is more abundant in the metabolically active tissues, such as skeletal and cardiac muscle and brown adipose tissue, with lower levels found in liver and white adipose tissue. It is induced by cold exposure via the β-adrenergic/cAMP pathway.
PGC-1α is part of a family of transcriptional co-activators which induce the expression of genes involved in a number of metabolic processes.
These include adaptive thermogenesis, carbohydrate and fatty acid oxidation and mitochondrial biogenesis.
Roles have also been suggested for PGC-1α involvement in hepatic gluconeogenesis and glucose uptake in skeletal muscle (Oberkofler, Holzl et al. 2003; Puigserver and Spiegelman 2003). Coactivators such as PGC-1α do not bind to DNA but affect their targets via interactions with nuclear receptors such as PPARS α, δ and γ, by increasing their ability to initiate transcription.
PGCs can act by docking to specific receptors alone, but can also recruit more co-activators such as CBP/p300 and p160/SRC-1 (Puigserver and Spiegelman 2003).

3.9.4 AMP-activated protein kinase (AMPK)

AMPK is a heterotrimeric enzyme complex, is the key regulator of energy metabolism in cells. In the liver, activation of AMPK phosphorylates and inactivates the rate-limiting enzymes of lipogenesis, such as acetyl-CoA carboxylase (ACC) (Hardie, Corton et al. 1997). AMPK is classically regulated by various metabolic stresses causing an increase in the AMP:ATP ratio or other cellular energy and metabolic states, such as glycogen, lipid, and NAD/NADH redox potential (Kawanaka, Nolte et al. 2000; Taylor, Ellingson et al. 2005). However, recent studies have suggested that other unidentified pathways can regulate AMPK, regardless of cellular energy status. Thus,

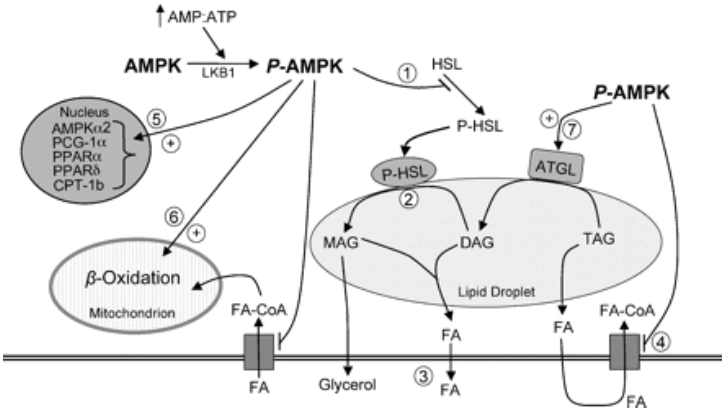
inhibition of AMPK by chemicals, or physiological state, may contribute to the intracellular accumulation of lipids.

It is well documented that AMPK phosphorylation inhibits the key transcription factor responsible for fatty acid SREBP-1 through mammalian target of rapamycin (mTOR) and liver-X receptor-α (LXRα) (Porstmann, Santos et al. 2008). Conversely, inhibition of AMPK has been suggested as a central event causing the development of chemical induced fatty liver. Repressed AMPK activates anabolic pathways, such as fatty acid synthesis, and inhibits catabolic pathways, such as fatty acid oxidation. In studies performed with hepatocytes and in the livers of mice was demonstrated that inhibition of AMPK leads to the activation of lipogenesis, mediated by SREBP-1 (You, Matsumoto et al. 2004). AMPK positively regulated fatty acid oxidation by activating PPAR α and PPARγ coactivator (PGC)-1 (Lee, Kim et al. 2006). Thus, inhibition of AMPK by chemicals, or physiological state, may contribute to the intracellular accumulation of lipids.

The activation of AMPK downregulates the expression of SREBP1c, a transcription factor that regulates cholesterol and lipid synthesis. Reduction of SREBP1c results in downregulation of genes involved in lipogenesis, including ACC, fatty acid synthase (FAS), and glycerol-3-phosphate acyltransferase (GPAT).

Conversely, inhibition of AMPK has been suggested as a central event causing the development of chemical induced fatty liver (Gaidhu, Fediuc et al. 2009).

Figure 3.4: The role of AMPK activation in lipid metabolism in white adipocytes.



Acute activation of AMPK inhibits HSL activity (1), which prevents hydrolysis of DAG (2) and subsequent liberation of glycerol and FAs (3). Acute and chronic pharmacological AMPK activation powerfully suppresses FA uptake (4), conferringan anti-lipogenic role for AMPK in white adipose tissue. Furthermore, chronic AMPK activation upregulates the expression of genes (PGC-1 ,PPAR ,PPARy, CPT-1b) (5) that increase the ability of the cell to dispose of FAs intracellularly through -oxidation (6). ATGL content is also increased with AMPK activation (7) and catalyzes the hydrolysis of one FA from TAG. ⊕ and → denote stimulation; ----] denotes inhibition. MAG, monoacylglycerol; LKB1, AMPK kinase (Gaidhu, Fediuc et al. 2009).

3.10 The Retinoid X Receptors (RXR)

The RXR also forms part of the nuclear receptor superfamily of transcription-factors which are ligand-activated.

The three receptor subtypes are known as, α, β, & γ. The isotypes are encoded by different genes, which through alternative splicing produce a two isoforms each (α1,α2 etc). As the names suggests, of 9-cis retinoic acid (RA) coactivates RXR. They were first discovered to bind heterodimerically with the thyroid receptor (TR) and later discovered to dimerize with pregnane X receptor, farnesoid X receptor, liver X receptor, constitutively activated receptors (CAR) and PPARs (Szanto, Narkar et al. 2004).

Evidence exists that shows RXR to be capable of activating PPAR targets as a homodimer binding to PPRE sites in gene promoters (A, Tan et al. 2004). RXRα is found in liver, kidney, epidermis and intestine, RXRβ is found ubiquitously, but RXRγ is restricted to skeletal muscle, and the pituitary gland (Mangelsdorf, Borgmeyer et al. 1992). As Germain et al. have suggested, the fact that RXR is an obligate heterodimer for many nuclear receptors, the number of targets which they regulate at the transcriptional level is vast (Germain, Chambon et al. 2006). As a result of the myriad binding targets, most research has focussed on the binding partners of RXR.

3.11 Liver X Receptors (LXR)

LXRs are another class of nuclear receptors related to RXRs and PPARs. There are two LXR genes, α and β, transcribed from different loci.

LXRα is found in liver, adipose tissue, macrophages, bone, intestine and spleen, whilst LXRβ is expressed ubiquitously.

LXRs bind to the LXR response element (LXRE) which has the consensus sequence TGGTCACTCAAGTTCA. LXRs are activated by oxysterols, and are involved in

cholesterol homeostasis, in conjunction with SREBP1. It regulates a range of metabolic genes, due in part to its promiscuous nature and ability to dimerize with other receptors including RXR (Zhang, Repa et al. 2001; Joseph, McKilligin et al. 2002). PPARs suppress SREBP-1c expression by inhibiting the binding of RXR:LXR to the SREBP-1c promoter (Yoshikawa, Ide et al. 2003). It may be that the ratio of available RXR binding with either PPARs or LXR determines the program of lipogenesis or β-oxidation, however these findings are yet to be replicated *in vivo*.

Aims of the thesis

On the basis of the preliminary results indicated above, this work aims to validate the hypothesis that **alpha-TP regulates lipid metabolism through the induction of a number of genes**.
On the basis of the hypothesis made above, the following aims will be pursued:

1. To show that α-TP treatment is not toxic for cells *in vitro*

2. To measure the deposition of lipids in the NIH3T3-L1 cells in control conditions and after treatment with α-TP.

3. To study the transcription of genes regulating lipid metabolism, by quantitative RT-PCR and by western blot, in 3T3/adipocyte cells, challenged with progressive amounts of α-TP to simulate physiological conditions.

The present study preliminary data carried out in pre-adipocytes and differentiated adipocytes will be validated and extended, through the coordinated induction of a number of target genes.

CHAPTER 4
Materials and Methods

4.1 Reagents

The stock solutions (50 mM) of αT (Cognis, Cincinnati, OH, USA), αTP (by Phospha-genics Ltd, Melbourne, Australia) and γTP (by Phospha-genics Ltd, Melbourne, Australia) were prepared in ethanol. For cell proliferation assays in 96-well microtiter plates, working stock dilutions were prepared in 1% ethanol to keep total ethanol concentrations in the cell culture medium below 0.1%.

4.2 Cell culture

The NIH3T3-L1 cell line were purchased from the American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine and 5% CO_2 at 37°C.

4.2.1 Differentiation of NIH3T3-L1 cells into adipocytes

The cells were cultured to confluence in DMEM supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 5µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone with medium change every 2 days as previously described (Ejaz, Wu et al. 2009). Two days after cell confluence, differentiation was initiated by adding differentiation medium 0.5 mM isobutylmethylxanthine (IBMX), 0.25 µM dexamethasone (DEX), 1 µg/ml insulin (INS) in the above medium. After 48h, the medium was replaced with fresh DMEM plus 10% fetal bovine serum supplemented with 5µg/ml insulin. Five days later, the fully differentiated adipocytes were used in the experiments.

4.3 Proliferation assay

Cell proliferation measured using the mitochondrial metabolic rate was determined with the 3-[4,4-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Undifferentiated cells 3T3 L1 were grown in 96-well microtiter plates (3000 cells in 200 µl medium well) and treated in quadruplicate with solvent control ethanol or αT, αTP and γTP of different concentrations (10, 20, 30, 40 and 50 µM) for 24, 48 and 72 h. For assays, cell proliferation was measured at 490 nm using the Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) with an assay time of 4 h.

Table 4.1 Cell proliferation Assay Adipocytes

Sample	Treatment αT αTP	Ethanol	Time of addition of 20 µl Cell titer reagent
1	Control	5%	24 48 72
2	αT 10µM	4%	
3	αT 20 µM	3%	
4	αT 30	2%	
5	αT 40	1%	
6	αT 50	0%	
7	Control	5%	24 48 72
8	αT P 10	4%	
9	αT P20	3%	
10	αT P 30	2%	
11	αT P 40	1%	
12	αT P 50	0%	
13	Control	5%	24 48 72
14	γTP 10	4%	
15	γTP20	3%	
16	γTP 30	2%	
17	γTP 40	1%	
18	γTP 50	0%	

4.4 Treatment with αT, αTP, γTP

NIH3T3-L1 cells were counted and grown in 6-well plates at a density of 15000 cells/mL and treated in duplicate.

- The cells were cultured to confluence in DMEM supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 5µg/ml insulin, 0.5 mM 3-isobutyl-1-

methylxanthine (IBMX) and 1 µM dexamethasone differentiated and incubated with αT, αTP, and γTP over a period of 5 days

- The cells were cultured to confluence with a modified protocol using DMEM containing 10% modified FBS without lipid, 2 mmol/L L-glutamine, 5µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone and differentiated and incubated with αT, αTP, and γTP over a period of 5 days
- The cells were cultured to confluence in DMEM supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, the cells were incubated only with αT, αTP, and γTP for a period of 24 hours.
- The cells were cultured to confluence in DMEM and differentiated with 5µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone and cells were incubated with αT, αTP, and γTP for a period of 24 hours.

Table 4.2 Treatment with α-T and α-TP with different protocols.

Cells	Incubation Time	Treatment
Proliferation	24h	No differentiated incubated with αT, αTP, and γTP
Differentiation	24h	Differentiated incubated with αT, αTP, and γTP
Differentiation	5 Days	Differentiated incubated only with αT, αTP, and γTP
Differentiation	5 Days	Differentiated incubated with αT, αTP, and γTP and Modified DMEM 10% FBS without lipid

The cells were plated with α-T, α-TP and γ-TP 1, 10, 20 and 40 µM concentrations with vehicle control (ethanol, 0.1%).

4.5 Oil Red O (ORO) Staining

Lipid accumulation was measured with the oil red O, ORO/isopropanol method (Lillie and Ashburn 1943; Ramı `rez-Zacarı `as et al. 1992; Fukumoto and Fumimoto 2002) ORO (1.4 g; Sigma, O-0625) was dissolved in 400 ml of 99% 2-propanol and left over night at room temperature (22 °C). The solution was next filtered using Whatman #4 filter paper (Whatman International, 1004 185), mixed with 144 ml glass-distilled H_2O and left over night at 4 °C. The solution was then filtered again using Whatman #4 filter paper, left to stand for 30 min, and filtered (Whatman #4) again before use. Before staining, the media was removed from the wells with a Pasteur pipette or by gently inverting the plate over a waste container, then gently rinsing with PBS. One milliliter of 10% formalin was added to each well for 10 min to fix the cells. Each well was then rinsed with 60% isopropanol for 30 s. ORO stain was applied 1 ml per well for 10 min and removed, and then the wells were rinsed four times with glass-distilled H2O. Next, the wells were rinsed with 100% isopropanol for approximately 10 min, the solution was transfer to 1.5 ml tubes and the absorbance of 200 microL was measured in microtiter plates at OD 500 nm (Ramirez-Zacarias, Castro-Munozledo et al. 1992), against a blank containing 100% isopropanol.

4.6 Lipid analysis

3T3-L1 cells were washed with PBS, and lipids were extracted by hexane-2-propanol (3:2, v/v). The amounts of intracellular triglyceride were determined by the Triglyceride E-test from Wako and normalized to the amounts of total cellular protein determined by BCA protein assay (Pierce) according to each manufacturer's instructions.

4.7 Extraction RNA

Total RNA from proliferating and differentiated NIH3T3-L1 cells was isolated treated with control ethanol (0.1%) as indicated in the figure.

The RNA extraction protocol;

500 µl TRIZOL was added and incubated at room temperature for 5 min. Then 200 µl chloroform was added and the samples vortexed thoroughly each sample for 3 min. Each sample was centrifuged at 13000 x g for 15 min at 4°C. The upper aqueous phase RNA was collected and then 500 µl isopropanol was added, and then samples were vortexed for 10 min, then centrifuged at 13000 x g for 20 min at 4°C. Supernatant was discharged and pellet washed with 1 ml 75% ethanol. Then the supernatant was spun at

7500 x g for 5 min at 4 °C. Again, the supernatant was discharged and the RNA pellet was dried for 10 min. Then 30 ul of RNase-free water was used to dissolve the RNA pellet and the pellet was stored at -80°C.

For RNA quantification, 1 ul of sample RNA was used to measure the optical density (OD) 260 and (OD) 280 nm using Nanodrop via UV spectrophotometry.

4.8 Quantitative RT-PCR

A selected subset of genes was assessed by quantitative real time RT-PCR using the primers and conditions of the TaqMan two-step RT-PCR protocol (Applied Biosystems, Foster City, CA, USA).

Basically, total RNA was reverse transcribed and quantitative real-time TaqMan PCR was performed with 2x with Fast Universal PCR Master mix employing pre-designed primers and MGBprobe (Applied Biosystems, Foster City, CA) for either

Sestrin 2, TRB3, Insig, CD36, and AP2 (Applied Biosystems, Foster City, CA).

The analysis was run on a 7700 Fast real-time PCR system (Applied Biosystems). Each TaqMan reaction contained 10 ng of sample cDNA in a total reaction volume of 20 µl. As endogenous control human GAPDH was used, which is stably expressed in preadipocytes, and throughout the differentiation to adipocytes. The usability of GAPDH as reference gene was evaluated by comparing the different threshold values (ct values) treated versus untreated cells. The relative expression was determined by the comparative threshold method as described in the ABI PRISM 7700 sequence detection system and an ABI StepOne Plus (Applied Biosystems, UK). 5µL of RNase-free water was used as a negative/No Template Control (NTC).

The threshold cycle (Ct Value) is the point at which fluorescence rises to a point considered statistically significant above the baseline values. All standards, samples and negative controls were assayed in quadruplicate to ensure accurate results and the data were analyzed using the ΔΔCt method (Schefe, Lehmann et al. 2006).

The Thermo-cycling parameters for amplification were:

- 2 minutes at 50°C for incubation,
- 0:20 sec at 95°C for polymerase activation
- 0:01 sec at 95°C to denature
- with a final extension step of 0:20 sec at 60°C.

Each reaction was in a final volume of 20uL.

4.9 Protein Extraction and Western Blot

Cell monolayers (10^6 cells in 10 ml medium per dish) were washed twice with ice-cold phosphate-buffered saline and lysed at 4 °C for 30 minutes with a buffer containing 50 mM Tris•HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate and 1% protease inhibitor cocktail solution (Sigma). The cell lysates were clarified by centrifugation at 10,000 × g for 10 minutes at 4 °C prior to separation by SDS-PAGE. Protein content was measured using the Pierce BCA Protein Assay Reagent kit (Pierce, Rockford, IL), following the manufacturer's procedure with minor modifications. 2 mg/ml bovine serum albumin solution in water was used as standard. The reaction was started by adding 200 µl of the reaction mixture provided in the kit, to 10 µl of sample in 96 multi-well plates. The plate was incubated 15 minutes at 37 °C and the extinction was measured at 562 nm in a microplate multireader.

4.10 SDS PAGE and immunoblotting

The resolved proteins were transferred to PVDF membrane and the membranes were blocked with powdered not-fat milk solution (5% (w/v). Individual proteins were detected with specific antibodies and visualized by blotting with horseradish peroxidase-conjugated secondary antibodies.

Immunoblots were done according to standard methods using 30 µg extract per lane and separated by 10% SDS– PAGE. The level of AMPK phosphorylation was determined using primary anti-phospho-AMPK antibody, the level of AMPK was determined using primary anti-AMPK antibody, the level of phosphorylation ACC was determined using primary anti-phospho-ACC antibody. Monoclonal mouse anti-human β-actin (1:10,000 diluted; Sigma–Aldrich) were used as primary antibodies. Anti-mouse secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology) were used for detection. Proteins were visualized with an enzyme-linked chemiluminescence detection kit (Immun-Star HRP) according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Chemiluminescence was monitored by exposure to film (Kodak BioMax), and the signals were analyzed using a Fluorchem 8900 workstation and the AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA).

CHAPTER 5
Results

Zingg et al. compared the cellular effects of α-T with those of the novel phosphorylated analog, α-TP (Zingg, Libinaki et al. 2010).

Using gene expression microarrays, more genes were regulated by αTP than by aT, and most of the αTP -regulated genes were up-regulated. Zingg et al., has published results carried out *in vitro* in THP-1 cells indicated that the molecule is capable of significantly up-regulating 165 known genes and down-regulating 14 known genes.

Of the 22 genes mostly up-regulated by α-TP (5 to 12 fold more than by the non-phosphorylated parent compound), eight of them resulted in being associated with the expression of enzymes of lipid metabolism regulation (see Table 5.1).

Table 5.1 Genes Up-Regulated More Than 4-Fold By Alpha-TP
T= tocopherol; TP=alpha-TP; C= control- The genes involved in lipid metabolism are underlined

Genes	T/C	TP/C	TP/T
CHAC1, Ca/H-Transporter regulator	0.9	13.1	14.7
Hypothetical protein RTP801	1.1	14.7	13.5
Inhibin beta E	0.8	9.4	11.1
Microfibrillar-associated protein 4	0.4	4.4	11.1
Microtubule-associated protein 1B	1	10.5	10.9
SKIP3, Stress induced NIPK kinase	1.2	11.3	9.3
Prostate differentiation factor mRNA	0.8	6.9	9.1
Sarcoglycan, gamma (35kD dystrophin-associated glycoprotein) (SGCG)	1.9	15	7.9
Neuroendocrine secretory protein 55	1	8	7.8
Stanniocalcin 2	1.7	11.7	7
cDNA FLJ36044 similar to COP1	0.6	4	6.6
Vascular endothelial growth factor	1.1	6.8	6.5
Protein kinase domains containing protein similar to Phosphoprotein C8FW	1.3	8.1	6.3
Asparagine synthetase	1.1	6.8	6.2
cDNA DKFZp761M02121, sestrin 2	0.9	5.6	6.1
VEGF Vascular endothelial growth factor	0.8	5.1	6
Phosphoserine aminotransferase (PSA)	1	5.7	5.9
Cystathionase and a CHORD containing protein 1 (CHP1) pseudogene	1	5.6	5.6
p8 protein homolog (COM1)	0.8	4.2	5.5
Vascular endothelial growth factor isoform 121	1	5.6	5.4
Transmembrane 6 superfamily member 1 (TM6SF1)	0.8	4.4	5.3
Insulin-like growth factor binding protein 3	1.6	7.9	5

On the basis of the results indicated above, we make the hypothesis that α-TP regulates lipid metabolism through the induction of a number of genes in NIH3T3-L1 pre-adipocytes, and we asseess the efficacy of this natural phosphorylated vitamin E derivative on the prevention and treatment of obesity.

5.1 α-Tocopheryl Phosphate inhibits the proliferation of NIH3T3-L1

In the course of this study, NIH3T3-L1 pre-adipocytes have been employed, cells that have the ability to differentiate from fibroblasts to adipocytes.

In these experiments the cellular activity of the pure phosphorylated α-tocopherol, the effect of αT and phosphorylated γ-tocopherol on NIH3T3-L1 cell proliferation was measured. The cytotoxicity is measured through metabolic rate of the mitochondria in NIH3T3-L1 cells treated with increasing concentrations (0–50 μM) of αT αTP and γTP for 4 (date not shown) 24 48 and 72h, by using the colorimetric MTT assay.

The conclusion can be drawn that, at least at cellular level αT, αTP, and γTP below or up to 40 μM can be used without causing cell damage. Other cell lines such as Hep-G2 and CaCo2 cells (up to 72h incubation) are not damaged by αTP concentrations up to 100 μM (Negis, Meydani et al. 2007).

In this study α-TP has been shown (Fig. 5.1 A) to be capable, in isolated NIH3T3-L1 cells, to inhibit their proliferation.

Cell proliferation is measured spectrophotometrically as absorbance increase consequent to the metabolic reduction of the yellow tetrazolium salt (MTS) to form purple formazan crystals by living cells.

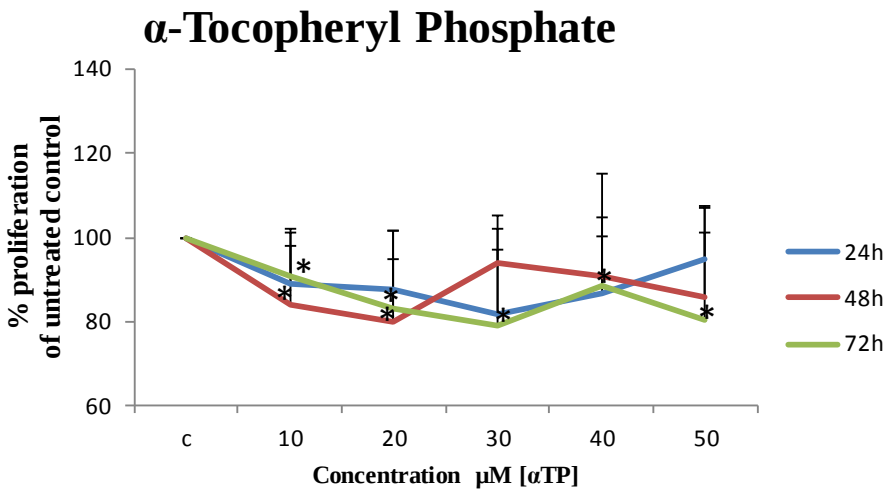
We found that the NIH3T3-L1 cell proliferation was significantly inhibited by αTP (30 μM) after 24 and 72 h treatment and to 20 μM after 48h treatment. Increasing the concentration did not result in stronger inhibition, excluding non specific cell toxicity (Fig.5.1 A) which is consistent with previous experiments carried out in THP-1 cells (Zingg, Libinaki et al. 2010).

Concentrations of αT above 10 μM significantly increased NIH3T3-L1 proliferation, (10 μM after 24 h) (Fig.5.1 B). When NIH3T3-L1 cells were treated with γT (0–50 μM), inhibition of cell proliferation was only observed at the concentration of 30 μM after 48 h treatment and of 40 μM after 72 h treatment (Fig.5.1 C),

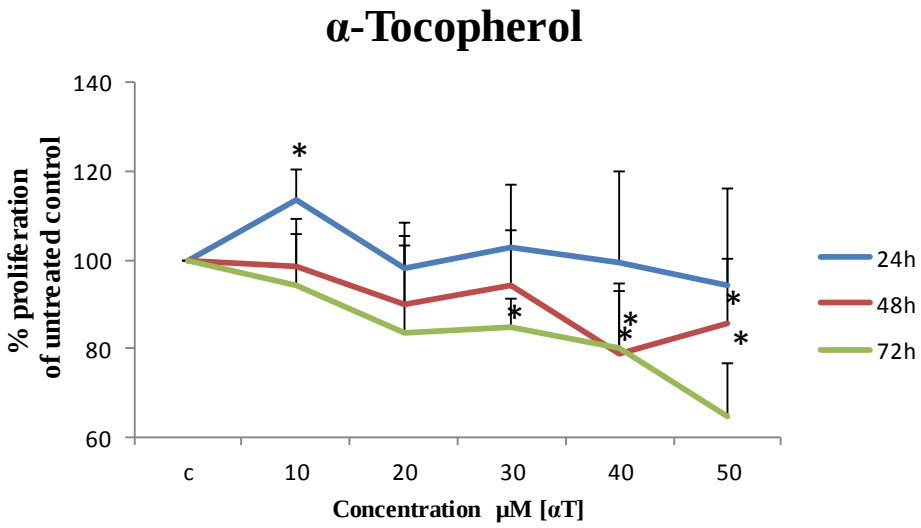
It is evident that α-TP inhibits cell growth and that, under these conditions, α-T and γ-TP do not produce any significant effect.

Figure 5.1. Effect of α-T,α-TP and γTP on the proliferation of NIH3T3-L1 pre-adipocyte proliferation, MTT assay.

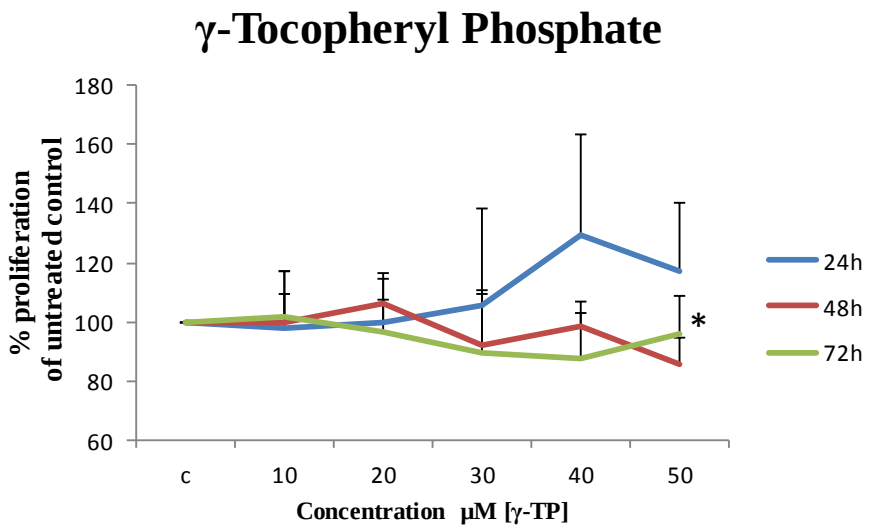
A)



B)



C)



Cells were treated with increasing concentrations of α-T, α-TP and γTP for 24, 48, and 72 h and cell proliferation was measured with an MTS assay kit (see Materials and methods). The experiment was performed in quadruplicate and the mean and standard deviation were calculated for each treatment. A statistically significant difference of $p < 0.05$ (two-tailed unpaired Student's t-test) is marked by *. **(A)** Inhibition of NIH3T3-L1 pre-adipocyte proliferation with various concentrations (0–50 µM) of αTP treated for either 24, 48 72 h (n=4, ±SEM, *<0.05, vs control (c) set to 100%). **(B)** Inhibition of NIH3T3-L1 pre-adipocyte proliferation with various concentrations (0–50 µM) of α-T, treated for either 24, 48 72 h (n=4, ±SEM, *<0.05, vs control (c) set to 100%). **(C)** Inhibition of NIH3T3-L1 pre-adipocyte proliferation with various concentrations (0–50 µM) of γTP, treated for either 24, 48 72 h (n=4, ±SEM, *<0.05, vs control (c) set to 100%).

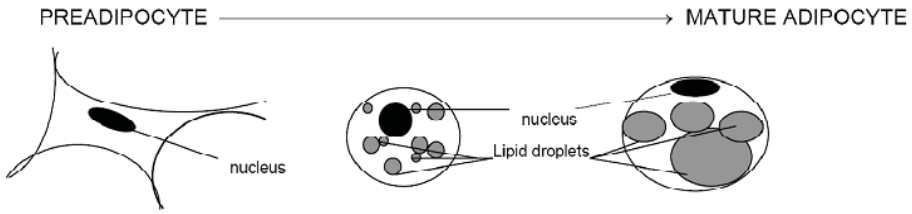
5.2 Lipid accumulation both in NIH3T3-L1 and in adipocytes.

NIH3T3-L1 pre-adipocytes have been employed, cells that have the ability to differentiate from fibroblasts to adipocytes by addition of IBMX, INS and DEX.

Adipogenesis is the development of fat cells (adipocytes) from pre-adipocytes. More recently interest in adipogenesis has increased, markedly due to its association with health hazards like obesity and a number of other pathological disorders, including diabetes, hypertension, cancer, gall bladder disease, and atherosclerosis.

The process of adipogenesis consists of three stages: growth arrest, clonal expansion and differentiation (See Figure 4.2). These stages are orchestrated by a cascade of gene expression involving different transcription factors.

Figure 5.2 Differentiation of NIH3T3-L1 preadipocytes to adipocytes.



5.2.1 α-Tocopherol and α-Tocopheryl phosphate modulate fat levels increase during adipocyte differentiation in NIH3T3-L1 cells

In preliminary experiments carried out in parallel, α-TP appeared to be more effective than α-T in inhibiting the fat accumulation of NIH3T3-L1 pre adipocyte than NIH3T3-L1 adipocyte. To verify that α-T and α-TP are involved in the adipocyte differentiation, we first examined daily changes in the levels of fat and protein during the differentiation of NIH3T3-L1 cells. Spectrophotometric analisys revealed that after treatment with α-TP, the fat levels decreased in NIH3T3-L1 pre adipocyte and then increased NIH3T3-L1.

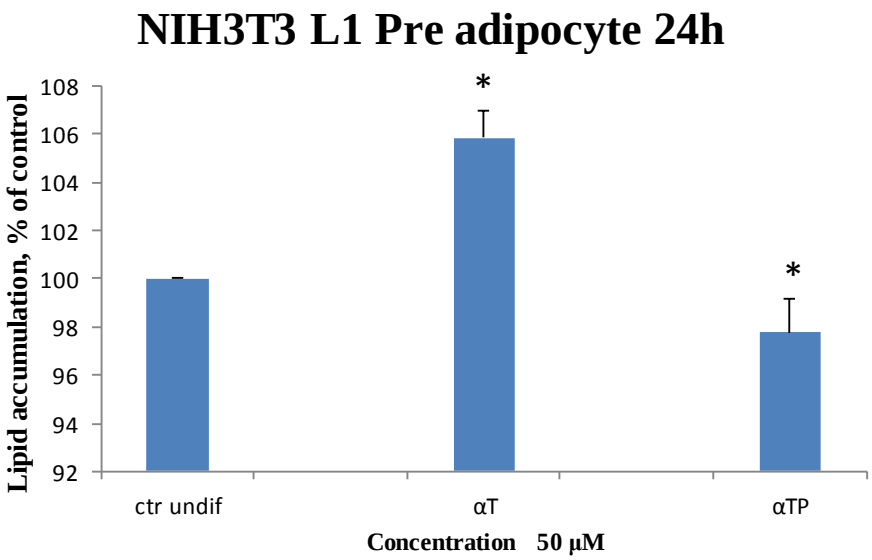
5.2.2 Induction of lipid accumulation in undifferentiated NIH3T3-L1 adipocytes

In this experiment of lipid accumulation in undifferentiated NIH3T3-L1 cells by αTP, but not by αT, was determined with oil red o staining.

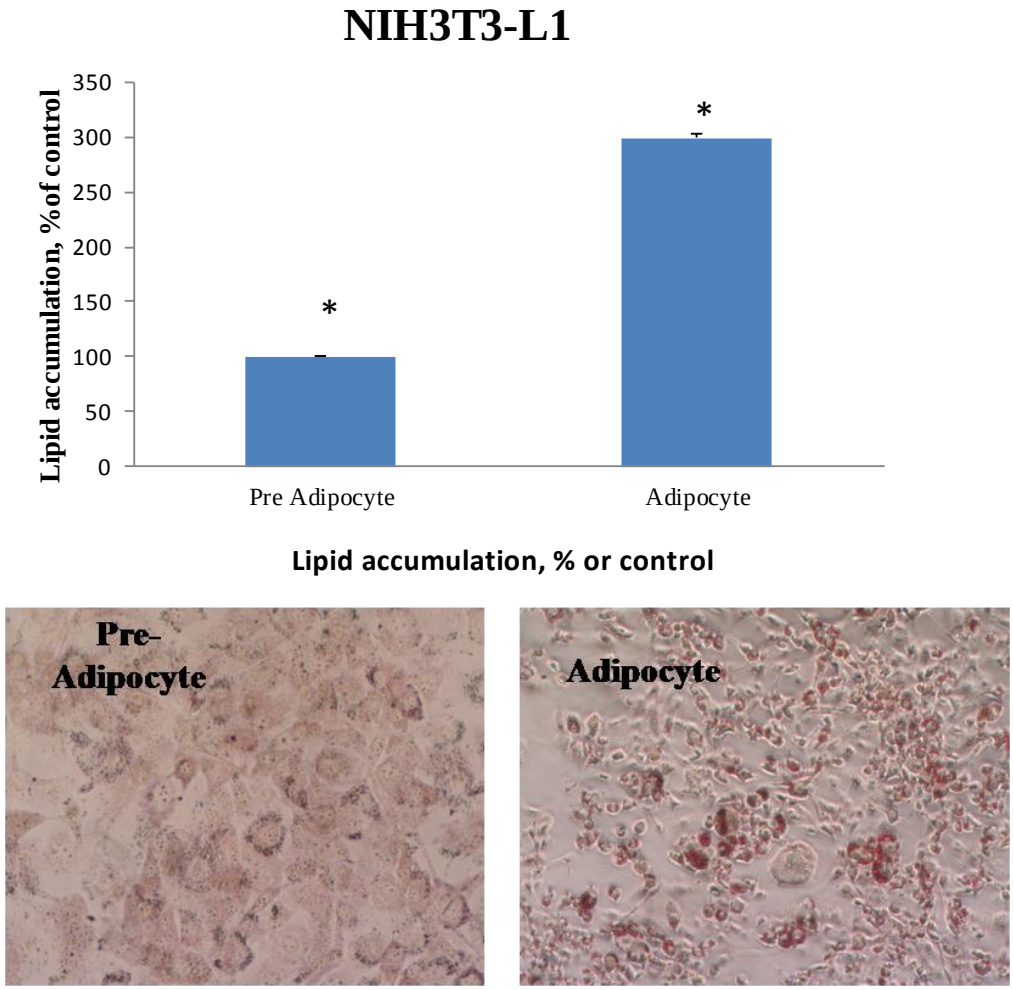
In figure 5.3 is shown that α-TP is not able to induce lipid accumulation in both NIH3T3-L1 pre-adipocytes. On the other hand, α-T was capable of inducing a significant lipid accumulation in NIH3T3-L1 pre-adipocytes. This experiment confirms that α-TP acts as such under these conditions, and not as a precursor of free α-T; in fact the parent compound is much less active, excluding the possibility that the effects of α-TP could be caused by its conversion to α-T, whereas γ-TP is less active in inhibiting the proliferation of NIH3T3-L1 (data not shown).

Figure 5.3 Induction of lipid accumulation in un-differentiated NIH3T3-L1.

A)



B)

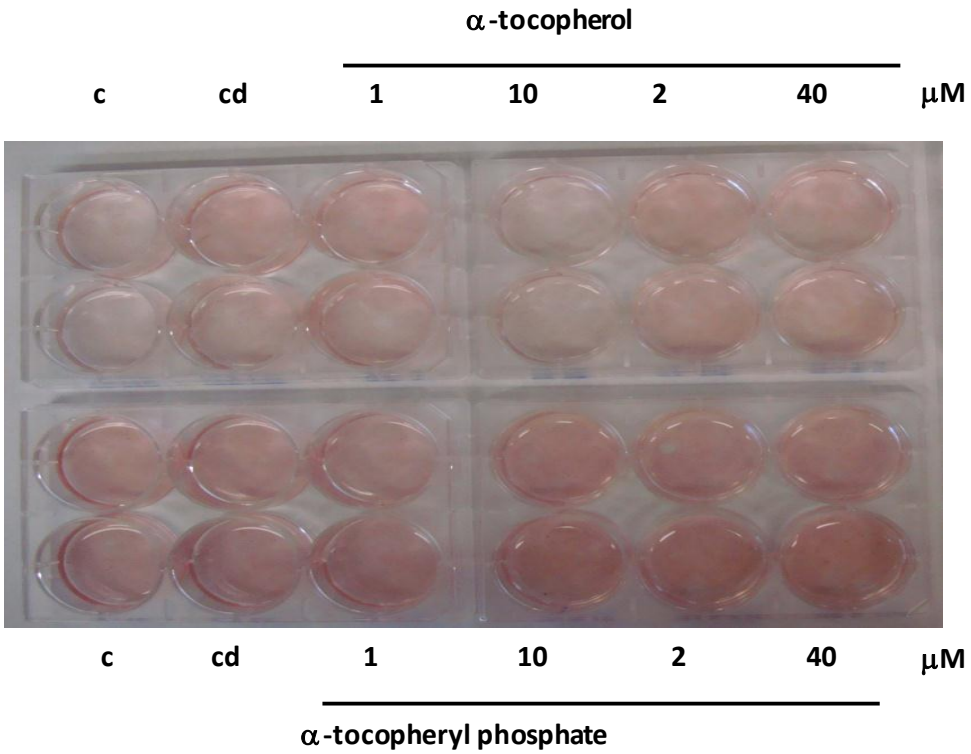


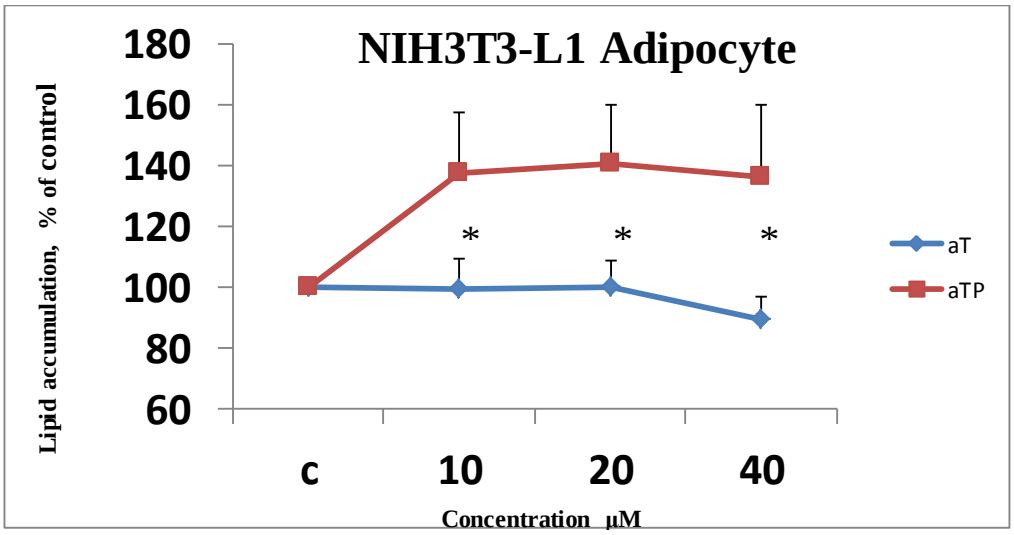
(**A**) Induction of lipid accumulation in NIH3T3-L1 un-differentiated adipocytes by αT, but not by αTP, as determined with oil red o staining. α-TP is not able to induce lipid accumulation both in NIH3T3-L1 pre adipocyte at 50 µM to 24h incubation. n=4, repeated two times with similar results, SEM. **(B)** The NIH3T3-L1 pre-adipoytes not treated with INS DEX and IBMX and the NIH3T3-L1 adipocytes treated with INS DEX IBMX.

5.2.3 Induction of lipid accumulation in differentiated NIH3T3-L1 adipocytes

In the experiment of figure 5.4 is shown that α-T is not able to induce lipid accumulation both in NIH3T3-L1 adipocytes. On the other hand, α-TP was capable of inducing a significant lipid accumulation in NIH3T3-L1 adipocytes. These result suggest that with NIH3T3-L1 adipocytes α-TP increased the fat in cells whereas when NIH3T3-L1 pre adipocyte were treated with α-TP it did not. fig 5.4.

Figure 5.4 Induction of lipid accumulation in differentiated NIH3T3-L1.





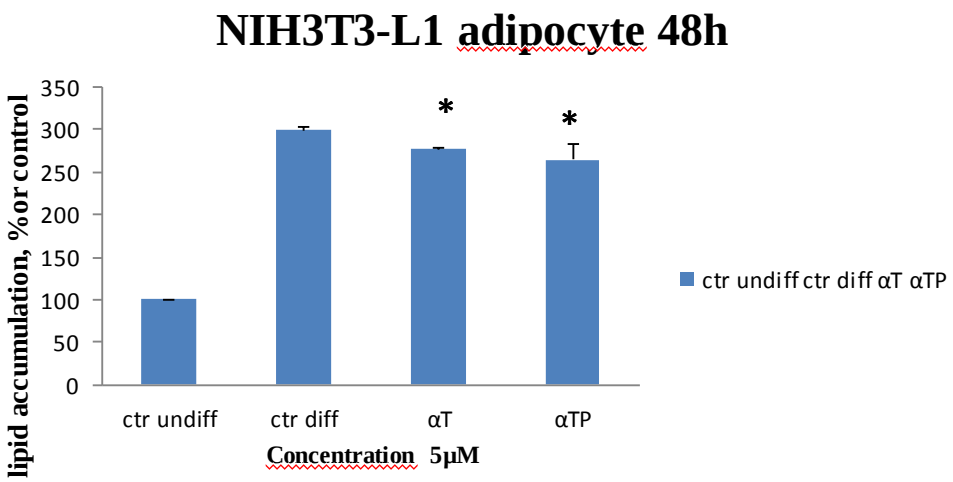
Induction of lipid accumulation in NIH3T3-L1 differentiated adipocytes by αTP, but not by αT, as determined with oil red o staining. α-T is not able to induce lipid accumulation both in NIH3T3-L1 adipocyte at 40 μM. n=4, repeated two times with similar results, SEM.

5.2.4 Induction of lipid accumulation in differentiated NIH3T3-L1 adipocytes 48h.

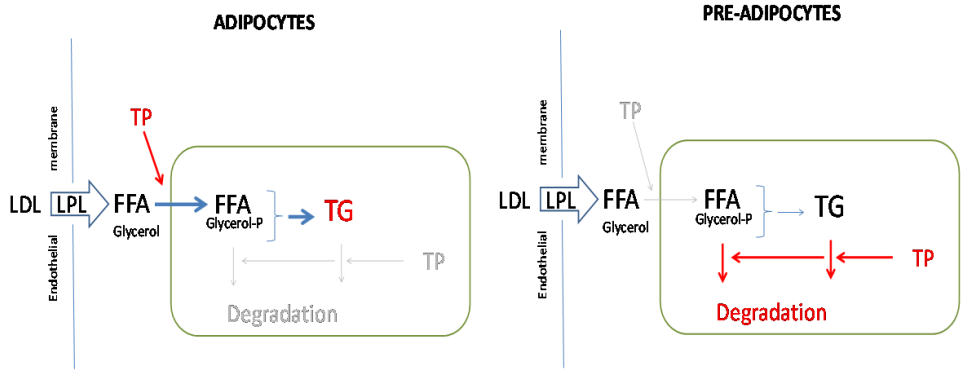
By comparing differentiated and undifferentiated NIH3T3-L1 we found that there isn't of lipid accumulation in differentiated NIH3T3-L1 cells at 48h of incubation after treatment with α-TP and α-T, as determined with oil red o staining.

In the experiment of figure 5.5 is shown that α-TP and α-T are not able to induce lipid accumulation both in NIH3T3-L1 adipocytes. These results suggest that with NIH3T3-L1 adipocytes α-TP and α-T increased the fat in cells fig 5.5.

Figure 5.5 Induction of lipid accumulation in differentiated NIH3T3-L1 at 48 h.
A)



B)



(A) Induction of lipid accumulation in differentiated NIH3T3-L1 adipocytes to 48 h incubation by αTP, and αT, as determined with oil red o staining. n=4, repeated two times with similar results, SEM **(B)** Possible mechanism of action of αTP (TP) on lipid accumulation in NIH3T3-L1 cells.

5.3 α-Tocopherol α-Tocopheryl phosphate induces gene expression in NIH3T3-L1 cells

NIH3T3-L1 cells were treated with αT, αTP and γ-TP) (datas not shown) (both at 40 μM), at different time and concentration that showed clear cellular effects but did not result in cytotoxicity or apoptosis.

The genes regulated by αT or αTP were analyzed for possible candidate genes known to mediate essential biological effects attributed to vitamin E, such as the regulation of lipid metabolism relevant for the prevention of obesity.

In line with a possible regulatory role of αTP on glucose and lipid homeostasis, a dose-dependent decrease in serum glucose level was measured in male rats after treatment with αTP (Libinaki, Ogru et al. 2006), and decreased cholesterol and lipid concentrations were observed in hypercholesterolemic rabbits (Libinaki, Tesanovic et al. 2010).

The regulation of these genes was further assessed by TaqMan RT-PCR.

In this study we compared the cellular effects of αT with those of the novel phosphorylated analog, α-TP, and γ-TP. The first which can be isolated from food and animal tissues in amounts of nmol/g of extracted material and which is formed in small amounts from αT in cultured cells and animal tissues (Nakayama, Katoh et al. 2003; Gianello, Libinaki et al. 2005; Negis, Zingg et al. 2005). The second is synthesized in the laboratory. Some of the cellular effects seen with α-T may be the result of scavenging free radicals, those seen with α-TP, which cannot act as a free radical scavenger, most probably result from specific interactions with enzyme(s) and/or receptor(s).

In line with previous results using α-TP, we find that α-TP is more potent in reducing NIH3T3-L1 cell proliferation and the expression of the genes than the un phosphorylated α-T.

As shown in the previous section, aT and aTP had regulatory effects on lipid accumulation in NIH3T3-L1 pre-adipocytes and adipocytes.

The following experiments, the genes which are most relevant in the process of fat accumulation in cells, namely Sestrin, TRB3,INSIG, CD36 and aP2 have been analyzed.

The effects of α-T and α-TP on the transcription of those genes in NIH3T3-L1 and adipocytes are presented.

The effects of α-TP, α-T and γ-TP on the transcription of those genes in NIH3T3-L1 and adipocytes are different at different condition of growth.

5.4 Sestrin

The Sesn family is composed of three members, Sesn1, Sesn2, and Sesn3 (Budanov, Shoshani et al. 2002) (Peeters, Debeer et al. 2003), (Velasco-Miguel, Buckbinder et al. 1999).

The first member of the family, Sesn1 or p53-activated gene 26 (PA26), was identified through screening of novel genes activated by p53 in tetracycline-regulated system Sesn1 is activated by genotoxic stress in a p53-dependent manner (Buckbinder, Talbott et al. 1994; Velasco-Miguel, Buckbinder et al. 1999).

Tthe second member of the family Sesn2 or hypoxia-inducible gene 95 (Hi95) was isolated by microarray-based analysis of novel genes activated by prolonged hypoxia.

Sesns areregulated positively by p53 and negatively by insulin-AKT pathway (Nogueira, Park et al. 2008). Accordingly, deletion the only Sesn gene (dSesn) in Drosophila accelerates age-related pathologies such as triglyceride accumulation, mitochondrial dysfunction, muscle degeneration, and cardiac malfunction.

Sestrins are conserved proteins that accumulate in cells exposed to stress and potentiate AMPK and inhibit activation of target of rapamycin (TOR). TOR is a key protein kinase that regulates cell growth and metabolism to maintain cellular and organismal homeostasis (Oldham 2003; Wullschleger, Loewith et al. 2006). Insulin (Ins) and insulin-like growth factors (IGF) are major TOR activators that operate through phosphoinositide 3-kinase (PI3K) and the protein kinase AKT (Hay and Sonenberg 2004). Conversely, AMPK, which is activated upon energy depletion, caloric restriction (CR), or genotoxic damage, is a stress-responsive inhibitor of TOR activation (Hay and Sonenberg 2004).

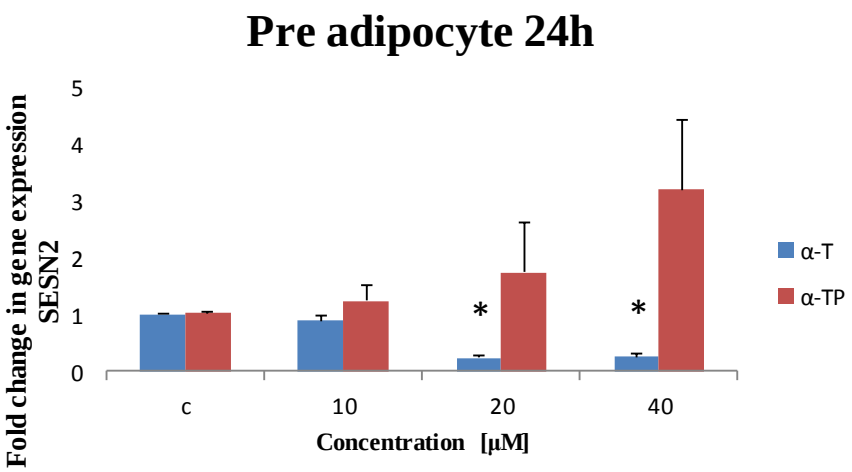
Expression of the gene encoding transcription factor dSREBP and the PPAR-gamma coactivator 1 (dPGC-1) gene are diametrically regulated by TOR and AMPK, respectively, to properly control lipid metabolism (Wullschleger, Loewith et al. 2006;

Towler and Hardie 2007; Porstmann, Santos et al. 2008). Persistent TOR activation is associated with diverse pathologies such as cancer, diminished cardiac performance, and obesity-associated metabolic diseases (Wullschleger, Loewith et al. 2006). Conversely, inhibition of TOR prolongs life span and increases quality-of-life by reducing the incidence of age-related pathologies (Harrison, Strong et al. 2009).

5.4.1 α-Tocopheryl phosphate induces Sestrin 2 gene expression in NIH3T3-L1 pre-adipocytes.

Interpretation of both, the effects α-T and effects of α-TP on the Sestrin 2 expression. The Figure 5.6 shows that incubation of NIH3T3-L1 undifferentiated cells with α-TP (40 µmol/L) induced the expression of Sestrin. This induction was observed after 24 hours of incubation (compared to the control cells) and was also noted after 24 in pre adipocytes (data shown).

Figure 5.6 Interpretation of both, effects of α-T and effects of α-TP on induction of Sestrin 2 gene expression, after 24 h incubation in NIH3T3-L1 pre adipocytes.

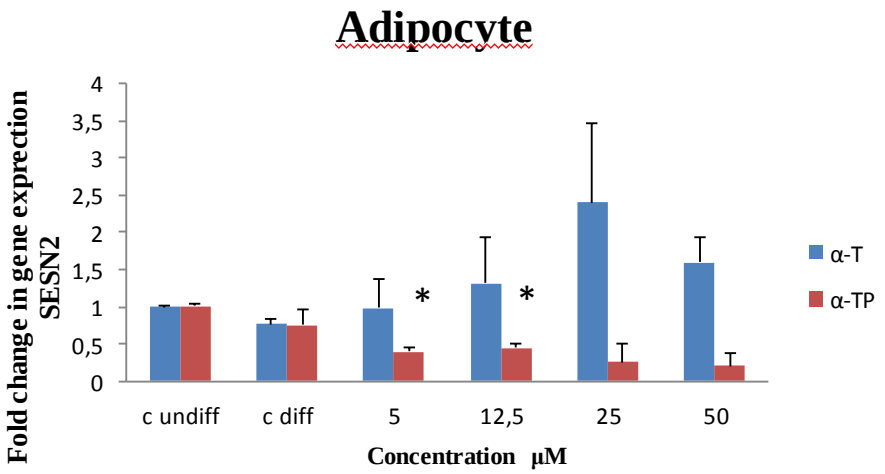


Interpretation of both, effects of α-T and effects of α-TP on induction of Sestrin gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells wereas treated with 1, 10, 20and 40 µM of α-T or α-TP. The graph corresponds to NIH3T3-L1 cells treated with normal DMEM medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, the cells were incubated only with αT and αTP for a period of 24 hours. SESTRIN expression was analyzed by RT-PCR as described in materials and methods. graphData are presented as mean ± SD after normalization of the SESTRIN to the GAPDH expression data.

5.4.2 α-Tocopherol induces Sestrin 2 gene expression in NIH3T3-L1 adipocytes.

Interpretation of both, the effects α-T and effects of α-TP on Sestrin 2 expression, we performed RT-PCR using specific primers for this gene. Figure 5.7 shows that incubation of NIH3T3-L1 cells with α-T (50 µmol/L) induced the expression of Sestrin 2. This induction was observed after 5 days of incubation (compared to the control cells) in adipocytes (data shown).

Figure 5.7 Interpretation of both, effects of α-T and effects of α-TP on induction of Sestrin gene expression in NIH3T3-L1 adipocytes

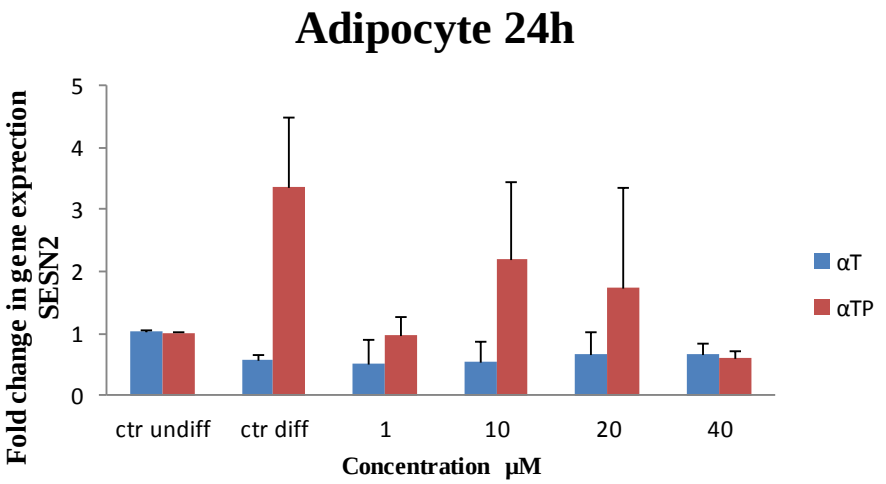


Interpretation of both, effects of α-T and effects of α-TP on induction of Sestrin gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells was treated with 1, 12,5 25 and 50 µM of α-T or α-TP. The NIH3T3-L1 cells treated with DMEM medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 5µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone differentiated and incubated with αT or αTP over a period of 5 days. SESTRIN expression was analyzed by RT-PCR as described in materials and methods. The graph shows a representative experiment using Taq Man primers specific.Data are presented as mean ± SD after normalization of the SESTRIN to the GAPDH expression data.

5.4.3 α-Tocopheryl phosphate induces Sestrin 2 gene expression in 24 h differentiated NIH3T3-L1 adipocytes.

Interpretation of both, the effects α-T and effects of α-TP on Sestrin 2 expression. Figure 5.8 shows that incubation of adipocytes cells with α-TP (40 µmol/L) induced the expression of Sestrin 2. The incubation with γ-tocopheryl phosphate does not induce the expression of sestrin 2. This induction was observed after 24 hours of incubation (compared to the control cells) on adipocytes (data shown).

Figure 5.8 . Interpretation of both, effects of α-T and effects of α-TP on induction of Sestrin 2 gene expression for 24h incubation in NIH3T3-L1 adipocytes.

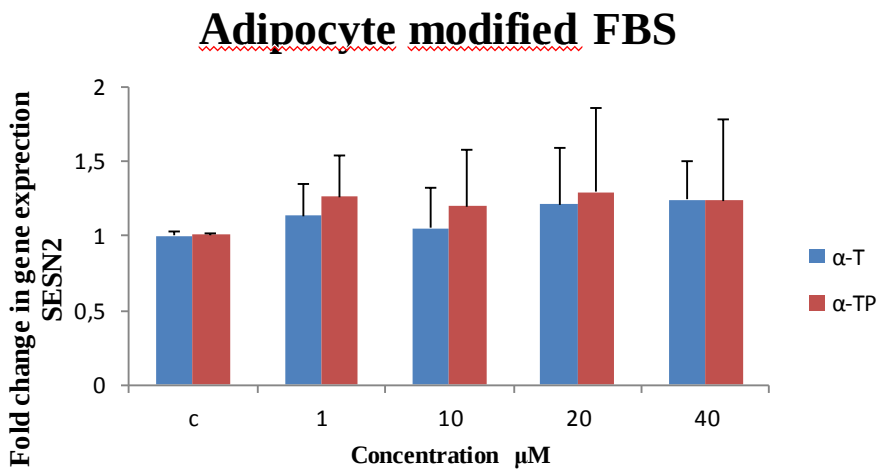


Interpretation of both, effects of α-T and effects of α-TP on induction of Sestrin gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells was treated with 1, 10 20 and 40 µM of α-T α-TP for 24 h incubation. The NIH3T3-L1 cells were treated with DMEM and differentiated with 5µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone and cells were incubated with αT orαTPfor a period of 24 hours. Sestern 2 expression was analyzed by RT-PCR as described in materials and methods. The graph shows a representative experiment using Taq Man primers specific. Data are presented as mean ± SD after normalization of sestrin 2 to the GAPDH expression data.

5.4.4 α-Tocopheryl phosphate induces Sestrin gene expression in NIH3T3-L1 adipocytes with FBS without fat.

Interpretation of both, the effects α-T and effects of α-TP on sestrin 2 expression, we performed RT-PCR using specific primers for this gene. The Figure 5.9 shows that incubation of NIH3T3-L1 cells with α-T and α-TP (50 µmol/L) induced the expression of Sestrin 2 when incubated with modified medium without fat in the serum. This induction was observed after 5 days of incubation (compared to the control cells) on adipocytes (data shown).

Figure 5.9 Interpretation of both, effects of α-T and effects of α-TP on induction of Sestrin 2 gene expression in NIH3T3-L1 adipocytes treated with modified protocol.



Interpretation of both, effects of α-T and effects of α-TP on induction of Sestrin gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells was treated with 1, 10 20 and 40 µM of α-T or α-TP. The NIH3T3-L1 cells treated with a modified medium using DMEM containing 10% modified FBS without lipid, 2 mmol/L L-glutamine, 5µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone and differentiated and incubated with αT or αTP over a period of 5 days.Sestrin 2 expression was analyzed by RT-PCR as described in materials and methods. The graph shows a representative experiment using Taq Man primers specific. Data are presented as mean ± SD after normalization of the SESTRIN to the GAPDH expression data.

5.5Tribbles

Mammalian tribbles homologs (TRB) are a newly recognized protein family including three isoforms: TRB1,TRB2, and TRB3. Members of TRB protein family are characterized by the presence of a domain homologous to a protein kinase in the middle of molecule [kinase homology (KH) domain] (Hegedus, Czibula et al. 2007). The KH domain of TRB lacks a functional ATP binding site and contains the variant amino acid residues that are essential for kinase catalytic activity. Thus, members of the TRB family are referred to as pseudokinase to reflect the view that they are not functional kinases (Mata, Curado et al. 2000). Despite lacking kinase activity, KH domain of TRBs still contain substrate-binding domain through which TRBs interact with different factors. By interacting with various factors, TRBs have been implicated in the regulation of numerous biological processes including cell growth, cell differentiation,

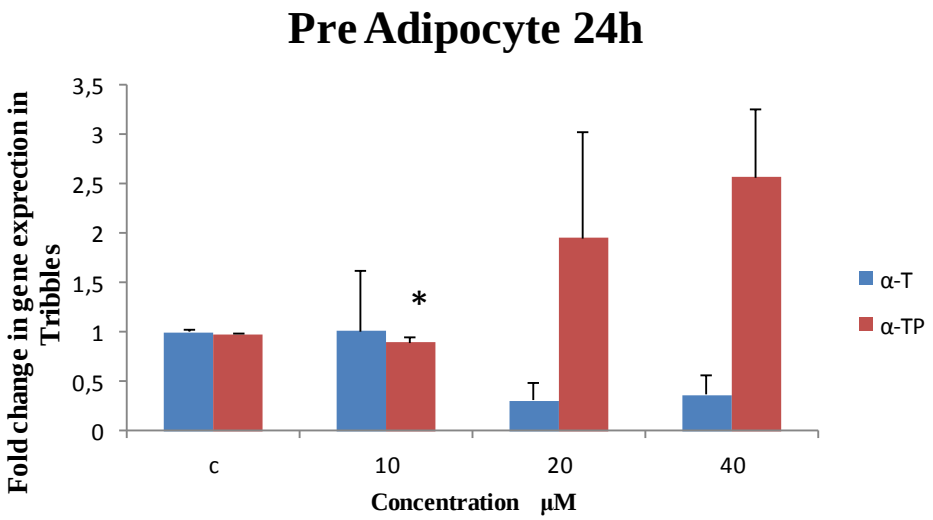
and cellular metabolism. In mammals, TRBs interact with protein kinases Akt and MAPK, transcription factor CCAAT/enhancer-binding protein beta (C/EBPβ), E3 ligase constitutive photomorphogenic 1 (COP1). By interacting with Akt, TRB3 negatively regulates insulin signaling and, thereby, liver and muscle glucose metabolism (Koh, Arnolds et al. 2006) and muscle differentiation (Kato and Du 2007). By interacting with C/EBPβ, both TRB2 and TRB3 are found to modulate adipocyte differentiation (Naiki, Saijou et al. 2007), and TRB1 is found to modulate gene expression induced by cytokines in macrophages (Yamamoto, Uematsu et al. 2007). In addition, elevated TRB3 expression is associated with insulin resistance (Du, Herzig et al. 2003) and metabolic syndromes (Bi, Tan et al. 2008), implying that regulation of TRB3 gene expression constitutes an important mechanism to modulate TRB3 function. Induction of TRB3 expression by insulin depends on phosphatidylinositol 3-kinase (PI3K) because inhibition (Ding, Kato et al. 2008) or inactivation (Taniguchi, Aleman et al. 2007) of PI3K in hepatic cells suppresses TRB3 expression. Strikingly, inactivation of Akt enhances TRB3 expression, suggesting that insulin transmits both positive and negative signals to regulate TRB3 expression (Ding, Kato et al. 2008). Currently, the mechanism under which insulin promotes TRB3 expression is not well understood. However, negative and positive regulation of TRB3 expression by insulin implies that TRB3 expression induced by insulin may represent a balanced insulin signaling. Thus, further studying of the regulation of TRB3 expression by insulin is warranted.

Since TRB3 has been shown to suppress adipocyte differentiation by negative regulation of PPAR alpha and to facilitate the proteasome degradationof acetyl-CoA-carboxylase, we analyzed in the following the regulation of TRIB3 in response to α−T and α−TP.

5.5.1 α-Tocopheryl phosphate induces Tribbles gene expression in NIH3T3-L1 pre adipocyte.

Interpretation of both, the effects α-T and effects of α-TP on the Tribbles expression, we performed RT-PCR using specific primers for this gene. Figure 5.10 shows that incubation of NIH3T3-L1 cells with α-TP (40 µmol/L) induced the expression of Tribbles. This induction was observed after 24 hours of incubation (compared to the control cells) on pre-adipocytes (data shown).

Figure 5.10 Interpretation of both, effects of α-T and effects of α-TP on induction of Tribbles gene expression for 24 h incubation in NIH3T3-L1 pre adipocytes.

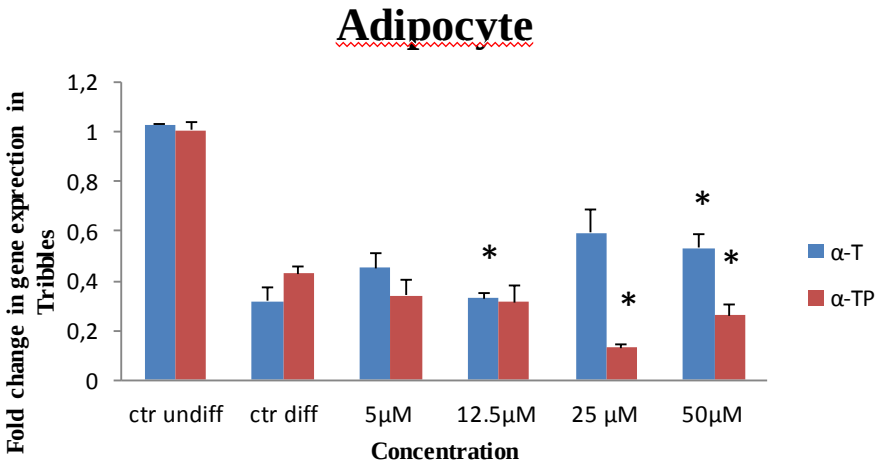


Interpretation of both, effects of α-T and effects of α-TP on induction of Tribbles gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells was treated with 10, 20 and 40 µM of α-T or α-TP. graph NIH3T3-L1 cells treated with DMEM medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, the cells were incubated only with αT or αTP for a period of 24 hours. Normal DMEM 10%FBS. Tribbles expression was analyzed by RT-PCR as described in materials and methods. graphData are presented as mean ± SD after normalization of the Tribbles to the GAPDH expression data.

5.5.2 α-Tocopherol induces Tribbles gene expression in NIH3T3-L1 adipocytes.

Interpretation of both, the effects α-T and effects of α-TP on the Tribbles expression, we performed RT-PCR using specific primers for this gene. Figure 5.11 shows that incubation of NIH3T3-L1 cells with α-T (50 µmol/L) induced the expression of Tribbles, whereas αTP reduced it. This induction was observed after 5 days of incubation (compared to the control cells) and was also noted after incubation on adipocytes (data shown).

Figure 5.11 Interpretation of both, effects of α-T and effects of α-TP on induction of Tribbles gene expression in NIH3T3-L1 adipocytes.

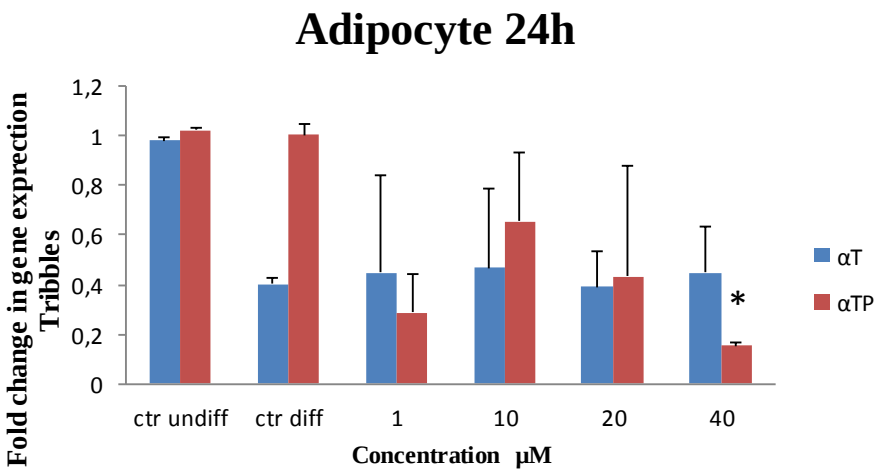


Interpretation of both, effects of α-T and effects of α-TP on induction of Tribbles gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells treated with 5, 12.5, 25 and 50 µM of α-T α-TP. The NIH3T3-L1 cells in DMEM medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 5µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone differentiated and incubated with αT or αTP over a period of 5 days. Tribbles expression was analyzed by RT-PCR as described in materials and methods. The graph shows a representative experiment using Taq Man primers specific. Data are presented as mean ± SD after normalization of the Tribbles to the GAPDH expression data.

5.5.3 α-Tocopheryl phosphate induces Tribbles gene expression in NIH3T3-L1 adipocyte differenetiated for24 h

Interpretation of both, the effects α-T and effects of α-TP on Tribbles expression, we performed RT-PCR using specific primers for this gene. Figure 5.12 shows that incubation of NIH3T3-L1 adipocytes with α-TP and α-T (40 µmol/L) reduced the expression of Tribbles. This induction was observed after 24 hours of incubation (compared to the control cells) in adipocytes (data shown).

Figure 5.12 Interpretation of both, effects of α-T and effects of α-TP on induction of Tribbles gene expression in NIH3T3-L1 adipocytes.

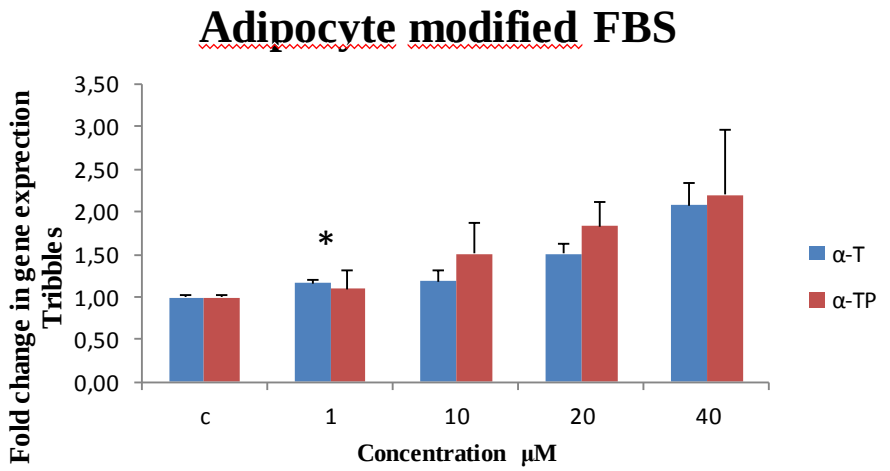


Interpretation of both, effects of α-T and effects of α-TP on induction of Tribbles gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells treated with 1, 10, 20 and 40 µM of α-T α-TP. The cells in DMEM and differentiated with 5µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone and cells were incubated with αT and αTP for a period of 24 hours. Tribbles expression was analyzed by RT-PCR as described in materials and methods. The above graph shows a representative experiment using Taq Man primers specific. Data are presented as mean ± SD after normalization of the Tribbles to the GAPDH expression data.

5.5.4 α-Tocopheryl phosphate induces Tribbles gene expression in NIH3T3-L1 adipocytes in FBS without fat.

Interpretation of both, the effects α-T and effects of α-TP on Tribbles expression,we performed RT-PCR using specific primers for this gene. Figure 5.13 shows that incubation of NIH3T3-L1 cells with α-TP and α-T (20-40 µmol/L) induced the expression of tribbles when incubated with modified medium without fat in the serum . This induction was observed after 5 days of incubation (compared to the control cells) in adipocytes .

Figure 5.13 Interpretation of both, effects of α-T and effects of α-TP on induction of Tribbles gene expression in NIH3T3-L1 adipocytes treated with modified protocol.



Interpretation of both, effects of α-T and effects of α-TP on induction of Tribbles gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells were treated with 1, 10, 20 and 40 µM of α-T α-TP. The NIH3T3-L1 cells treated with a modified protocol using DMEM containing 10% modified FBS without lipid, 2 mmol/L L-glutamine, 5µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone and differentiated and incubated with αT αTP over a period of 5 days. Tribbles expression was analyzed by RT-PCR as described in materials and methods. The graphgraph shows a representative experiment using Taq Man primers specific. Data are presented as mean ± SD after normalization of the Tribbles to the GAPDH expression data.

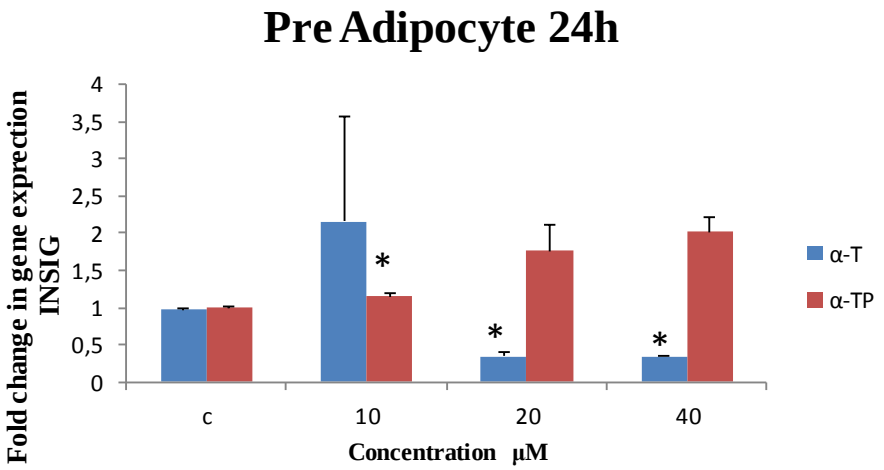
5.6 Insig

Insulin induced gene 1, also known as INSIG1, is a protein which in humans is encoded by the INSIG1 gene. INSIG plays an important role in the SREBP-mediated regulation of cholesterol biosynthesis, its action resulting in a decreased expression of HMG-CoA-reductase and in increased degradation of the enzyme (Liou, Bai et al. 2012).

5.6.1 α-Tocopheryl phosphate induces Insig gene expression in NIH3T3-L1 pre adipocytes.

Interpretation of both, the effects α-T and effects of α-TP on Insig expression, we performed RT-PCR using specific primers for this gene. Figure 5.14 shows that incubation of NIH3T3-L1 pre adipocytes with αTP (40 µmol/L) induced the expression of Insig. This induction was observed after 24 hours of incubation (compared to the control cells) in pre adipocytes.

Figure 5.14 Interpretation of both, effects of α-T and effects of α-TP on induction of INSIG gene expression for 24 h incubation in NIH3T3-L1 pre adipocytes.

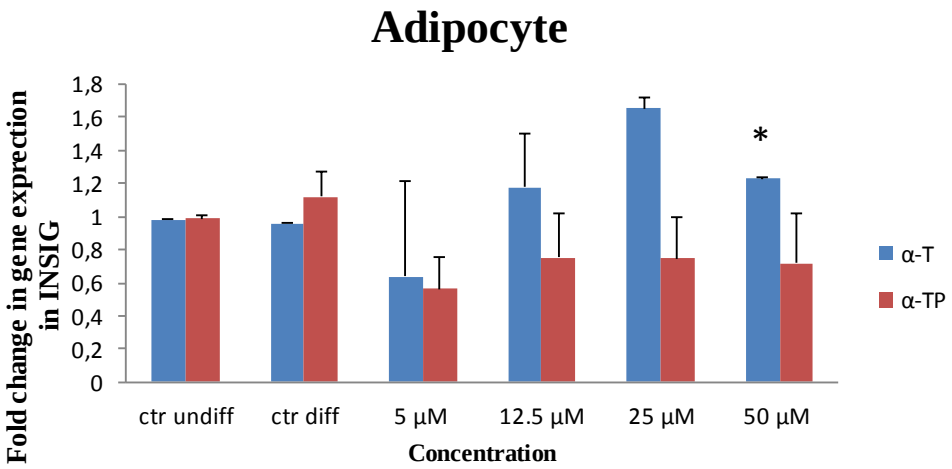


Interpretation of both, effects of α-T and effects of α-TP on induction of INSIG gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells treated with 10, 20 and 40 µM of α-T α-TP. The graph corresponds to NIH3T3-L1 cells treated with DMEM medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, the cells were incubated with αT or αTP for a period of 24 hours. Normal DMEM 10%FBS. INSIG expression was analyzed by RT-PCR as described in materials and methods. The graphshows a representative experiment using Taq Man primers specific. Data are presented as mean ± SD after normalization of the INSIG to the GAPDH expression data.

5.6.2 α-Tocopherol induces Insig gene expression in NIH3T3-L1 adipocytes.

Interpretation of both, the effects α-T and effects of α-TP on the Insig expression. We performed RT-PCR using specific primers for this gene. Figure 5.15 shows that incubation of NIH3T3-L1 cells with with α-T (25 µmol/L) induced the expression of Insig. This induction was observed after 5 days of incubation (compared to the control cells) on adipocytes (data shown).

Figure 5.15 Interpretation of both, effects of α-T and effects of α-TP on induction of INSIG gene expression in NIH3T3-L1 adipocytes.



Interpretation of both, effects of α-T and effects of α-TP on induction of INSIG gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells treated with 5, 12,5, 25 and 50 µM of α-T or α-TP. The NIH3T3-L1 cells in DMEM medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 5µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone differentiated and incubated with αT αTP over a period of 5 days. INSIG expression was analyzed by RT-PCR as described in materials and methods. The graph shows a representative experiment using Taq Man primers specific. Data are presented as mean ± SD after normalization of the INSIG to the GAPDH expression data.

5.7 CD36

CD36, a member of the class B scavenger receptor family,is an 88 kDa glycoprotein originally identified as a platelet receptor and also known as fatty acid translocase (FAT), which is expressed in numerous cell types including monocytes/macrophages, platelets, endothelial cells, and adipocytes (Nicholson and Hajjar 2004). CD36 is a multiligand receptor that is recognized by fatty acids, anionic phospholipids, thrombospondin, and oxidized lipoproteins. It is this latter property of scavenging (e.g., clearing) oxLDL which implicates CD36 in the initial steps of atherogenesis, as evidenced with studies in mice (Febbraio and Silverstein 2007) and humans.

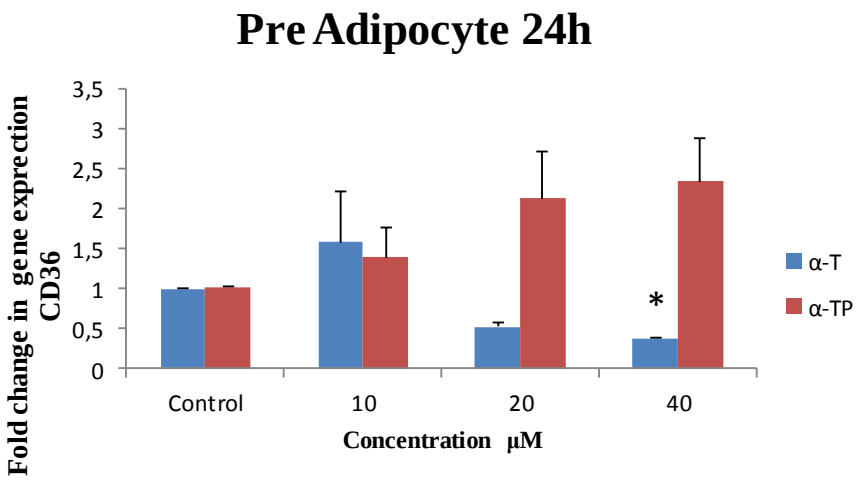
PPARγ is considered a master regulator of fatty acid metabolism through its direct role in regulating the expression of a broad range of genes involved in fatty acids and glucose metabolism. Among the genes upregulated by PPARγ are found genes related to fatty acid uptake (fatty acid transport protein FATP, CD36), glucose uptake (GLUT4), β-oxidation (acyl-CoA dehydrogenase, carnitine palmitoyltransferase CPT-1,

acyl CoA oxidase), gluconeogenesis (phosphoenolpyruvate carboxykinase PEPCK), and lipid storage (adipophilin) (Demers, Rodrigue-Way et al. 2008).

5.7.1 α-Tocopheryl phosphate induces CD36 gene expression in NIH3T3-L1 pre adipocytes.

Interpretation of both, the effects α-T and effects of α-TP on CD36 expression,we performed RT-PCR using specific primers for this gene. Figure 5.16 shows that incubation of NIH3T3-L1 adipocytes with α-TP (20-40 µmol/L) induced the expression of CD36. This induction was observed after 24 hours of incubation (compared to the control cells) and was also noted in pre adipocytes .

Figure 5.16 Interpretation of both, effects of α-T and effects of α-TP on induction of CD36 gene expression for 24 h incubation in NIH3T3-L1 pre adipocytes



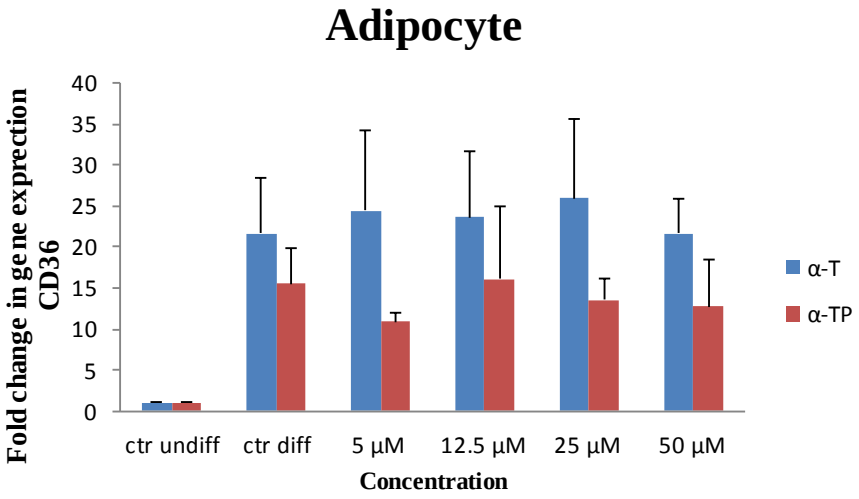
Interpretation of both, effects of α-T and effects of α-TP on induction of CD36 gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells were treated with 10, 20 and 40 µM of α-T α-TP, in DMEM medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, the cells were incubated only with αT and αTP for a period of 24 hours. CD36 expression was analyzed by RT-PCR as described in materials and methods. The graph shows a representative experiment using Taq Man primers specific. Data are presented as mean ± SD after normalization of the CD36 to the GAPDH expression data.

5.7.2 α-Tocopherol induces CD36 gene expression in NIH3T3-L1 adipocytes.

Interpretation of both, the effects α-T and effects of α-TP on the CD36 expression,we performed RT-PCR using specific primers for this gene. Figure 5.17 shows that incubation of NIH3T3-L1 adipocytes with with α-T (50 µmol/L) induced the

expression of CD36. This induction was observed after 5 days of incubation (compared to the control cells) ins adipocytes.

Figure 5.17 Interpretation of both, effects of α-T and effects of α-TP on induction of CD36 gene expression in NIH3T3-L1 adipocytes.



Interpretation of both, effects of α-T and effects of α-TP on induction of CD36 gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells were treated with α-T and α-TP 5, 12.5, 25 and 50 µM of α-T α-TP. The NIH3T3-L1 were grownin DMEM medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 5µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone, differentiated and incubated with αT and αTP over a period of 5 days. CD36 expression was analyzed by RT-PCR as described in materials and methods. The graph shows a representative experiment using Taq Man primers specific. Data are presented as mean ± SD after normalization of the CD36 to the GAPDH expression data.

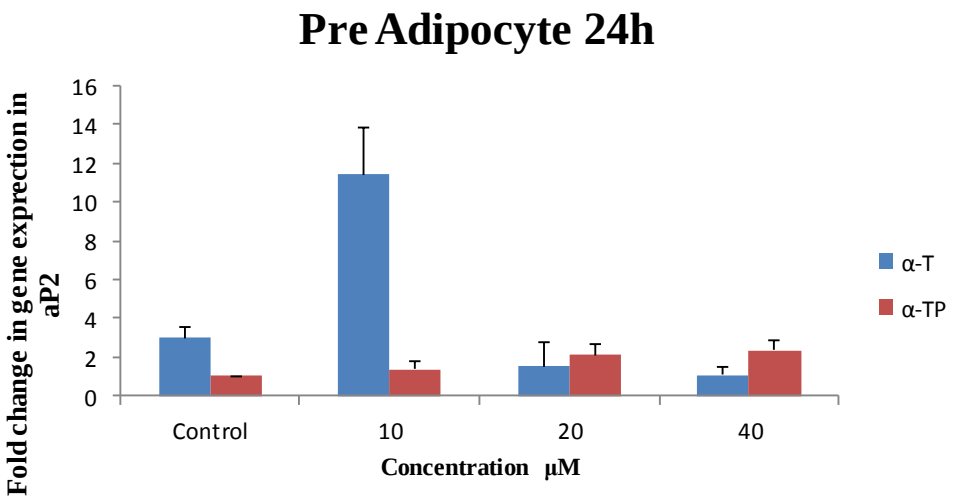
5.8 aP2

The adipocyte fatty acid-binding protein aP2 is also called fatty acid binding protein 4 (FABP4) aP2 regulates fatty acid uptake, release, and storage in adipocytes and participates in systemic glucose homeostasis and in macrophage responses in atherosclerosis. aP2, is abundantly expressed by adipocytes. Although aP2 was originally considered to be adipocyte specific, it has also recently been identified in activated macrophages, where it regulates cholesterol metabolism and cytokine production (Shum, Mackay et al. 2006). In adipocytes and macrophages, aP2 expression is regulated by PPARγ (Pelton, Zhou et al. 1999).

5.8.1 α-Tocopheryl phosphate induces aP2 gene expression in NIH3T3-L1 cells preadipocytes.

Interpretation of both, the effects α-T and effects of α-TP on the aP2 expression, we performed RT-PCR using specific primers for this gene. Figure 5.18 shows that incubation of NIH3T3-L1 pre-adipocytes with α-TP (40 µmol/L) induced the small expression of aP2. This induction was observed after 24 hours of incubation (compared to the control cells) and was also noted in pre adipocytes (data shown).

Figure 5.18 Interpretation of both, effects of α-T and effects of α-TP on induction of aP2 gene expression for 24 h incubation in NIH3T3-L1 pre adipocytes.

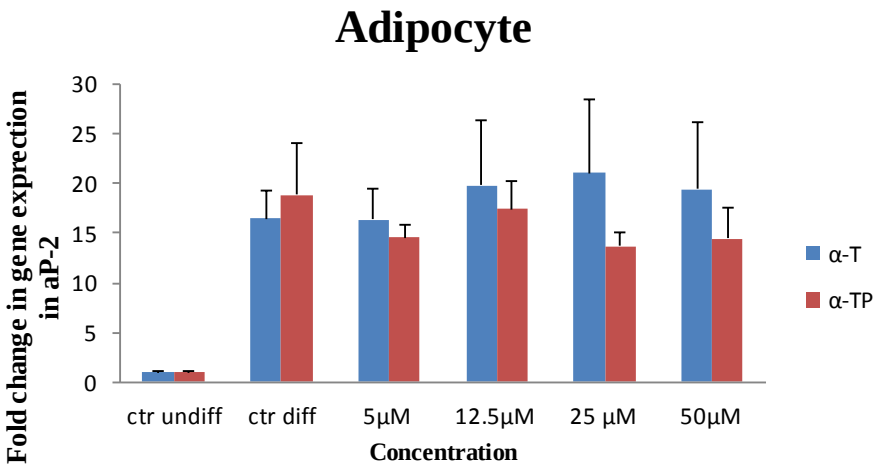


Interpretation of both, effects of α-T and effects of α-TP on induction of AP2 gene expression in NIH3T3-L1 as analyzed by RT-PCR. The NIH 3T3 L1 pre adipocytes treated with 10, 20 and 40 µM of α-T and α-TP. The graph NIH3T3-L1 cells treated with DMEM medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, the cells were incubated only with αT αTP for a period of 24 hours. Normal DMEM 10%FBS. aP2 expression was analyzed by RT-PCR as described in materials and methods. The graph shows a representative experiment using Taq Man primers specific. Data are presented as mean ± SD after normalization of the AP-2to the GAPDH expression data.

5.8.2 α-Tocopherol induces aP2 gene expression in NIH3T3-L1 adipocytes.

Interpretation of both, the effects α-T and effects of α-TP on the aP2 expression, we performed RT-PCR using specific primers for this gene. Figure 5.19 shows that incubation of NIH3T3-L1 adipcytes with α-T (50 µmol/L) induced the small expression of aP2. This induction was observed after 5 days of incubation (compared to the control cells) on adipocytes (data shown).

Figure 5.19 Interpretation of both, effects of α-T and effects of α-TP on induction of AP-2 gene expression in NIH3T3-L1 adipocytes.



Interpretation of both, effects of α-T and effects of α-TP on induction of AP2 gene expression in NIH3T3-L1 as analyzed by RT-PCR. The cells treated with 5, 12.5, 25 and 50 µM of α-T or α-TP, corresponds to NIH3T3-L1 cells treated with DMEM medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 5 µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone differentiated and incubated with αT αTP over a period of 5 days. AP-2 expression was analyzed by RT-PCR as described in materials and methods. The graph shows a representative experiment using Taq Man primers specific. Data are presented as mean ± SD after normalization of the AP-2 to the GAPDH expression data.

5.9 Mechanisms for induction of protein expression by αTP

The regulatory effects of αT and αTP on lipid metabolism, was further confirmed by Western blotting, by assessing AMPK and ACC phosphorylation. Figure 5.20 display gene expression analysis conducted from NIH3T3-L1 cells treated with various concentrations of α-T, α-TP and γ-TP for 24 hours.

In this study α-T (10 and 40 µmol/L) α-TP (10 and 40 µmol/L) and γ-TP (10 and 40 µmol/L have been shown to increase the expression of p-AMPK p-ACC by induction of a signaling cascade that ultimately leads to increased transcription.

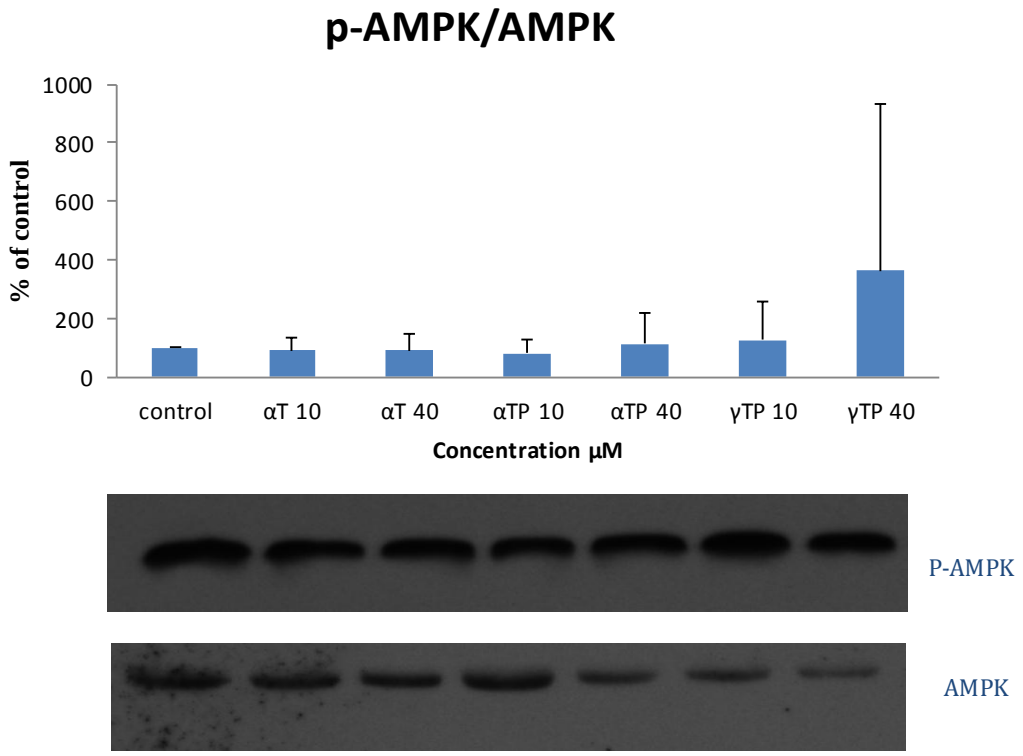
AMPK is a protein that positively regulated fatty acids oxidation by activating PPARα and PGC-1 PPARγ. Furthermore, it is well documented that AMPK phosphorylation inhibits the key transcription factor responsible for fatty acid synthesis–sterol SREBP-1. The activation of AMPK downregulates the expression of SREBP1c, a transcription factor that regulates cholesterol and lipid synthesis (Lee, Kim et al. 2006).

Conversely, inhibition of AMPK has been suggested as a central event causing the development of chemical induced fatty liver. AMPK is now also recognized as a critical modulator of aging through its interactions with mammalian target of rapamycin (mTOR), and the Sestrins. The signaling cascades initiated by the activation of AMPK exert effects on glucose and lipid metabolism, gene expression and protein synthesis. These effects are most important for regulating metabolic events in the liver, skeletal muscle, heart, adipose tissue, and pancreas.

5.9.1 α-T, α-TP and γ-TP induction of the phosphorylated form of p-AMPK expression

Treatment of NIH3T3-L1 with α-T, α-TP, and γ-TP induces the phosphorylated form of p-AMPKas assessed by the presence of the phosphorylated form of AMPK versus total AMPK protein. We observed no effect on the basal level of protein expression (Figure5.20). Although α-TP activity does not seem to have a significant effect on protein expression in our experimental conditions, we do not discard a role of α-TP in the fatty acid oxidation pathway. In fact, our results with sestrin 2 indicate that that α-TP may modulate AMPK activity *in vitro*.

Figure 5.20 p-AMPK protein expression is not stimulated by α-TP.

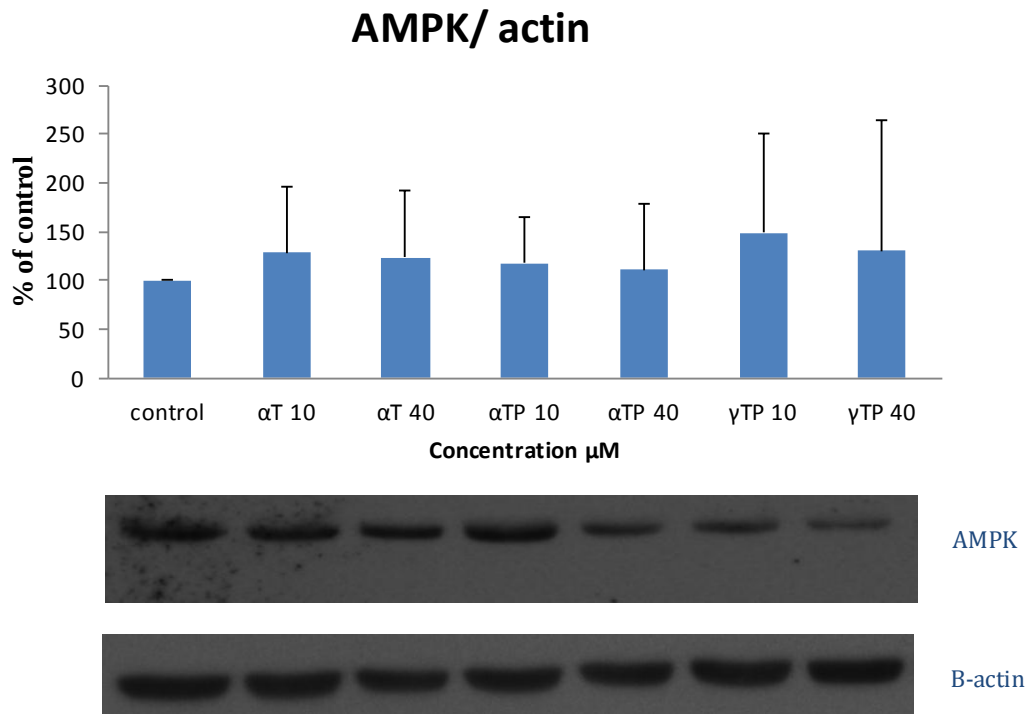


NIH3T3-L1 pre adipocytes were treated with α-T (10 and 40 µmol/L) α-TP (10 and 40 µmol/L) and γ-TP (10 and 40 µmol/L) or ethanol (0.1%) as control for 24 hours. Total protein was extracted, separated by SDS-PAGE and transferred to PVDF membrane. Blotted membrane was probed with anti-p-AMPK antibody and horseradish peroxidase coupled anti-mouse Ig antibody. Data are presented as mean ± SD quantified with scanning densitometry. α-T α-TP and γ-TP do not change total p-AMPK. (0.1 % Ethanol (EtOH) was used as control).

5.9.2 α-T α-TP and γ-TP induction of AMPK expression

Treatment of NIH3T3-L1 with α-T α-TP and γ-TP the form of AMPK (Figure 3B). We further analyzed AMPK activity of cell cultures treated with α-T α-TP and γ-TP , we observed that α-T α-TP and γ-TP stimulation of AMPK protein expression than on the basal level of protein expression (Figure 5.21).

Figure 5.21 AMPK protein expression is stimulated by α-TP.

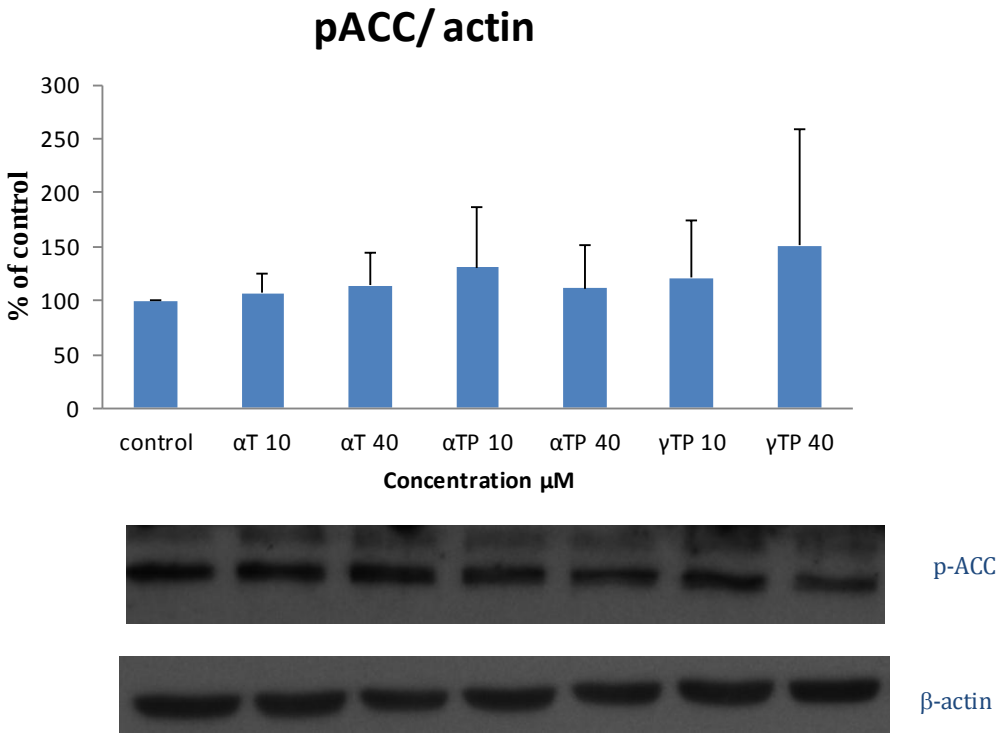


NIH3T3-L1 pre adipocytes were treated with α-T (10 and 40 µmol/L) α-TP (10 and 40 µmol/L) and γ-TP(10 and 40 µmol/L) or ethanol (0.1%) as control for 24 hours. Total protein was extracted, separated by SDS-PAGE and transferred to PVDF membrane. Blotted membrane was probed with anti-AMPK and anti-p-AMPK primary antibody and horseradish peroxidase coupled anti-mouse IgG secondary antibody. Data are presented as mean ± SD quantified with scanning densitometry. α-T α-TP and γ-TP do change total AMPK. The expression the α-TP increases the levels of AMPK (0.1 % Ethanol (EtOH) was used as control).

5.9.3 α-T, α-TP, and γ-TP induction of the phosphorylated form of p-ACC expression

Treatment of NIH3T3-L1 with α-T, α-TP, and γ-TP the phosphorylated form of ACC (Figure 3B). We further analyzed p-ACC activity in NIH3T3-L1 pre-adipocytes treated with α-T α-TP and γ-TP, we observed that α-T α-TP and γ-TP stimulation of p-ACC protein expression when compared with the basal level of protein expression (Figure 5.22).

Figure 5.22 p-ACC protein expression is stimulated by α-TP.



NIH3T3-L1 pre adipocytes were treated with α-T (10 and 40 µmol/L) α-TP (10 and 40 µmol/L) and γ-TP(10 and 40 µmol/L) or ethanol (0.1%) as control for 24 hours. Total protein was extracted, separated by SDS-PAGE and transferred to PVDF membrane. Blotted membrane was probed with anti-p-ACC primary antibody and horseradish peroxidase coupled anti-mouse IgG secondary antibody. Data are presented as mean ± SD quantified with scanning densitometry. α-T, α-TP and γ-TP do change total p-ACC. The expression the α-TP increases the levels of the phosphorylated form of ACC (0.1 % Ethanol (EtOH) was used as control).

CHAPTER 6
Conclusions

The molecular function of vitamin E has been the object of intensive investigations during at least the last 50 years (Zingg and Azzi 2004). After the original molecular understanding of vitamin E as an antioxidant at the level of cells and organs, it has become clear that, beyond and above this, vitamin E could act in *in vitro* cell systems as well as *in vivo* as a regulator of gene expression and cell signal transduction. More recent work has also shown that vitamin E can undergo phosphorylation in cells and experimental animals. The phosphorylated form of vitamin E, α-TP, was recently also found in human plasma and tissues. It has been also described that supplementation with α-TP in humans is able to increase the amounts found in plasma (Zingg, Libinaki et al. 2010).

α-TP, naturally present in the body or after supplementation, appears to be in amounts (nanomolar concentrations) not compatible with a storage form of vitamin E. The hypothesis has been then formulated that α-TP is a signaling molecule, similar in nature to other lipid-phosphate compounds such as inositol phosphates or sphingosine phosphate. Although very stable, α-TP can be somewhat hydrolyzed both in cells and in the body. However, the amounts of free α-T produced after administration to cells or to experimental animals are not sufficient to explain the superior function of α-TP relative to α-T(Zingg, Meydani et al. 2010). In fact, in a number of experiments ranging from cell signaling to protection against atherosclerosis or heart infarction, α-TP was shown, at the same concentrations, to be more effective than α-T (Libinaki, Tesanovic et al. 2010). The function assigned to α-T, alternative to the antioxidant one, has been to modulate the expression of a number of genes both in the sense of up and down regulation. In the attempt to understand the molecular function of α-TP a gene array analysis of the two molecules, α-T and α-TP has been made. Also in this case it was shown that, at the same concentration, α-TP appeared to be, in some cases, several folds more potent than the non-phosphorylated compound. The analysis of the genes over expressed in the presence of α-TP by THP-1 cells indicated that a surprising number of them belonged to a group of genes involved in cell lipid storage (See figure 6.1) and in particular, REDD-1, TRB3, C8FW [anti-G-Protein-Coupled Receptor Induced Protein

GIG2 (C8FW) (TRIB1)], Sestrin-2, and Insulin Induced Gene 1, INSIG(Zingg, Libinaki et al. 2010).

As a proof of concept, the transcription/translation of the VEGF gene was analyzed by quantitative RT-PCR and by Western blot, and the results were fully consistent with the gene array outcome.

In the course of this study, NIH3T3-L1 pre-adipocytes have been employed, cells that have the ability to differentiate from fibroblasts to adipocytes (see Figure 5.2). In parallel experiments α-TP appears to be more effective than α-T in inhibiting the proliferation of NIH3T3-L1 at the same concentrations and incubation time. This experiment confirms that α-TP acts as such under these conditions, and not as a precursor of free α-T; in fact the parent compound is much less active, excluding the possibility that the effects of α-TP be caused by its conversion to α-T.

In figure 5.5 is shown that α-T is not able to induce lipid accumulation both in NIH3T3-L1 pre-adipocytes and in adipocytes. On the other hand, α-TP was capable of inducing a significant lipid accumulation in adipocytes and it had only a small effect in NIH3T3-L1.

To evaluate the effect of α-T and of α-TP on the gene expression, we performed RT-PCR using specific primers for the genes which are most relevant in the process of fat accumulation in cells, namely Sestrin 2, TRB3 and INSIG.

These genes were shown to be strongly up-regulated in THP-1 cells by α-TP .

- Sestrins potentiate AMPK and inhibit activation of target of mTOR increased lipolysis and increased fatty acid oxidation may result. At the same time inhibition of fatty acid synthesis is also occurring.
- TRB3 has been shown to suppress adipocytes differentiation by negative regulation of PPAR alpha and to facilitate the proteasome degradation of acetyl-CoA-carboxylase.
- INSIG1 plays an important role in the SREBP-mediated regulation of cholesterol biosynthesis, its action resulting in a decreased expression of HMG-CoA-reductase and in increased degradation of the enzyme.

However, our results so far favor a model in which α-TP activates;

1. Sestrin 2 activation of AMPK and inhibition of m-TOR, with increased fatty acid oxidation and lipolysis (Budanov and Karin 2008).

2. Diminution of p53 activity via ubiquitination and degradation (Dornan, Wertz et al. 2004) balanced by an increase via Sestrin 2 ⟶ AMPK

3. Inhibition of Akt via PI3Kinase inhibition I think it stimulates Akt (mediated by Sestrin-AMPK; Sestrin ⟶ AMPK ⟶ PPAR-alpha), via TRB-3 directly overexpressed by α-TP;

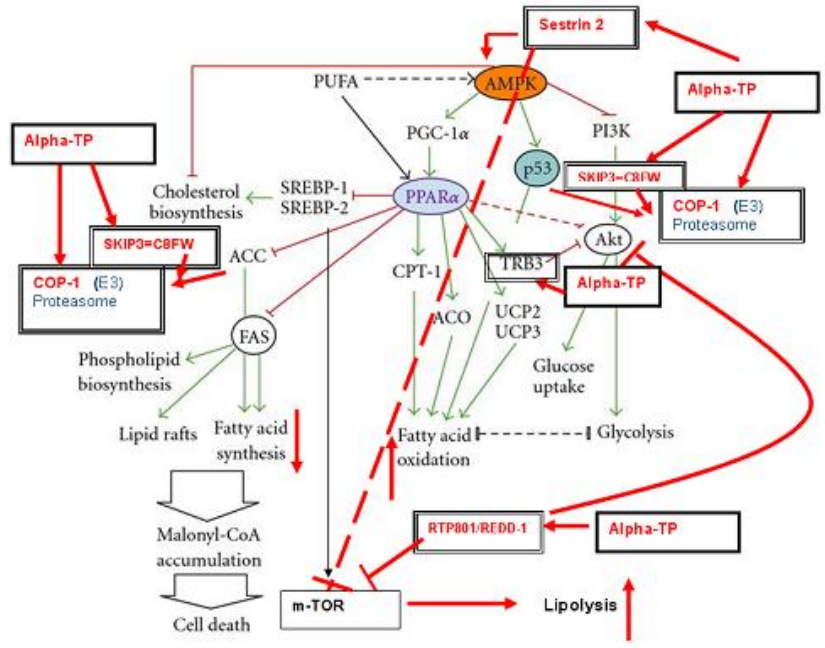
4. Inhibition of Cholesterol biosynthesis by increased expression of INSIG

5. TRB3 could suppression of adipocytes differentiation by negative regulating PPAR-alpha transcriptional activity (Takahashi, Ohoka et al. 2008).

All these pathways, activated by increased transcription of the mentioned genes, would result in an increase lipid degradation and inhibition of lipid synthesis. Due to its potent biological activities and the presence of amounts in the diet and our body, α-TP might strengthen our therapeutic arsenal .

More studies are, however, needed to substantiate this hypothesis.

Figure 6.1 Hypothetical scheme of *α*-TP on activity of Sestrin TRB3 and INSIG on lipid metabolism in NIH3T3-L1



In all cases, it is surprising that the effects of α-T are opposite to those of α-TP. In particular a clear dose-dependent up regulation of Sestrin by α-TP is observed in NIH3T3-L1 pre-adipocytes as well as a clear suppression by α-T in the same cells. Also TRB3 and INSIG transcription is up-regulated by α-TP and down regulated by α-T.

It appears therefore that in NIH3T3-L1 pre-adipocytes, the presence of α-T diminishes the transcription of fat catabolic enzymes while α-TP activates the transcription of the same genes therefore making this cell metabolically more active and less prone to accumulate lipid.
When the NIH3T3-L1 differentiated to adipocytes the opposite picture becomes visible. Sestrin transcription is clearly diminished by α-TP. The transcription of the other two genes appears also to be down regulated although to a lesser extent. On the other side α-T was capable of stimulating transcription of genes.
Consequently, also in adipocytes α-T appears to have an effect opposite to that of α-TP the former being efficient in up regulating the gene set intended to limit lipid accumulation.

The simplest conclusions that can be drown from the experiments presented here is that the presence of α-TP (but not α-T) in NIH3T3-L1 pre-adipocytes appears to activate a transcriptional gene set potentially preventing fat accumulation in these cells. In undifferentiated adipocytes, α-TP appears to be responsible for activation of those potentially protective genes. Using cell lines *in vitro* may not give reliable indications of the complex metabolic disorder resulting in obesity. However, *in vitro* results are important to understand, at least in part, the complexity of the disease. The use of high concentrations (micromolar) of α-TP may be criticized on the basis of the amounts physiologically found in plasma (nanomolar). However, the presence of large amounts of divalent cations needed in the incubation media for cell activities and survival makes the actual concentration of the free α-TP several orders of magnitude lower than the added amounts due to the sequestration properties of the divalent metal ions. Consequently, the used amounts of α-TP are comprised within a physiological range. Altogether, describing and understanding the effects of the two physiological compounds α-T and α-TP on single genes or a set of genes may be useful to focus upon some details of the complex picture of the control of pathological fat accumulation.
Clinical studies are, however, needed to substantiate this hypothesis.

CHAPTER 7
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I would like to thank my family.....

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