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PRIMARY HEPATOCYTES AS A MODEL TO INVESTIGATE NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD): POTENTIAL THERAPEUTIC EFFECTS OF DIIODOTHYRONINES

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CONTENTS

INTRODUCTION

1. The Liver
   1.1 Rat Liver 4
   1.2 Human Liver 6
   1.3 Metabolic Functions 11

2. Thyroid Hormones and Diiodothyronines
   2.1 Synthesis and metabolism 17
   2.2 Hypothalamus-Pituitary-Thyroid (HPT) Axis 21
   2.3 Physiological Effects 21
   2.4 Way of Action 23
   2.5 Diiodothyronines 27

3. Non-Alcoholic Fatty Liver Disease (NAFLD)
   3.1 General features 28
   3.2 Pathogenesis 33
   3.3 Insights on Insulin Resistance (IR) 39
      3.3.1 Insulin action: normal and pathological 39
      3.3.2 Insulin resistance 42
   3.4 mTOR: Structure and Functions 46
      3.4.1 Structure 46
      3.4.2 mTORC1 & mTORC2: upstream 48
      3.4.3 mTORC1 & mTORC2: downstream 52
      3.4.4 mTORC1 & mTORC2: metabolic functions and tissue-specific roles 55
   3.5 Role of Fructose in NAFLD 61

AIM OF THE WORK 65

MATERIALS and METHODS 71

RESULTS 84

FIGURES 90

DISCUSSION 109

REFERENCES 114

INTRODUCTION

1. THE LIVER

1.1 Rat Liver

Rat liver is constituted of four separate lobes, each one having different size and weight and named according to their position. The left lateral lobe is situated in left lateral position dorso-caudal to the median lobe and cranio-ventral to the caudate lobe and the stomach. This lobe has an average weight of 33 g, representing about 30% of the total liver mass, is associated to the base of the left portion of the median lobe by means of the thin vessels-enclosing pedicle that is in connection to the intrahepatic vena cava, and moreover interlobular ligaments link it with the superior caudate lobe. The median lobe, which accounts for about 40% (38 g) of the total liver weight, is placed under the diaphragm, fixed by means of falciform ligament that is extended from the xyphoid and diaphragm to the interlobular fissure. This lobe is characterised by a wide base, which surrounds about one half of the circumference of the vena cava, and is subdivided in a left part separated to the right part by a deep fissure. The right lobe is positioned on the right side of the vena cava, and is possible to distinguish two parts, the right superior and the right inferior lobe, the first representing 13% of the total liver mass, the latter about 6%. The caudate lobe, or Spiegel lobe, arranged on the left part of the vena cava, has an average weight of 7 grams, and consists of two parts, superior or upper caudate lobe, and inferior or lower caudate lobe.

The so-called paracaval liver should not be considered as a definite anatomic part of the liver, but rather as a functional assembly of the dorsal bottom portions of the right superior, inferior, and caudate lobes. It is bound to the dorsal portion of the diaphragm by thin ligaments, and covers the space from the pedicles of the diaphragm to the base

of the right inferior lobe. The paracaval liver as a whole represents about 2.5% - 3% of the total liver mass (Madrahimov N et al., 2006).

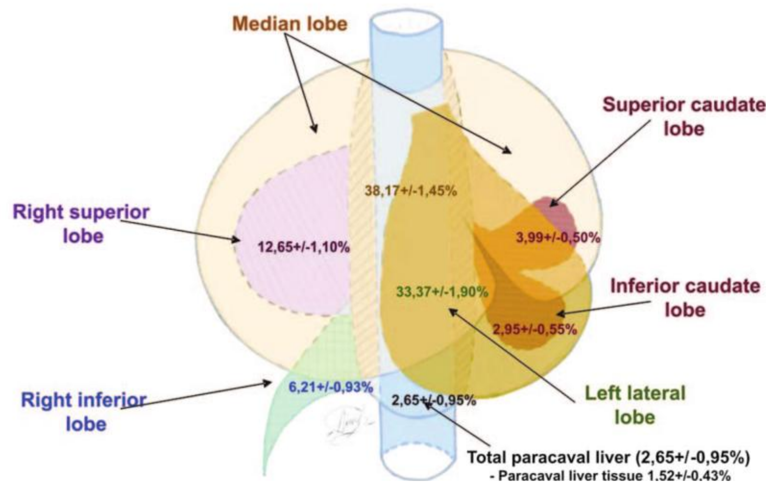

Figure 1 Rat liver lobes: weight distribution (from Madrahimov N et al., 2006).

About the circulatory system of the rat liver, on the functional point of view, two main venous systems are recognised, the portal system and the hepatic vein system. The first one has two main branches, the right portal vein that furnishes the right liver lobe, and the caudate portal vein, which is located on the left side and supplies the caudate lobe. A series of subsequent ramifications allow the portal vein to reach all lobes of the liver, as depicted below in Figure 2. The paracaval liver is furnished of portal blood from three distinctive sources, the left part from subdivisions of the caudate portal vein, the right one from the right portal vein, whereas the intermediate and cranial tissue from veins deriving from the dorsal wall of the main portal venous stem. The subdivisions of the hepatic artery parallel those of portal vein.

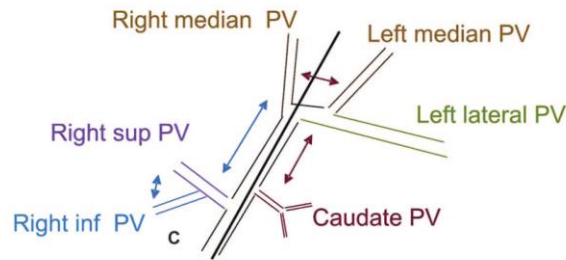

Figure 2 Rat liver: schematic representation of portal vein anatomy (from Madrahimov N et al., 2006).

The hepatic venous system collects blood from the left and the right part of the liver, by means of different veins as represented in Figure 3. The left hepatic vein, which is subdivided in a median and a lateral portion, drains the left lateral lobe and the left portion of the median lobe. The right median hepatic vein gathers the blood from right hemi liver, whereas the caudate vein does the same from the caudate lobe. Numerous small veins have the role to collect the blood from the paracaval liver, directing it into the intrahepatic part of the vena cava (Madrahimov N et al., 2006).

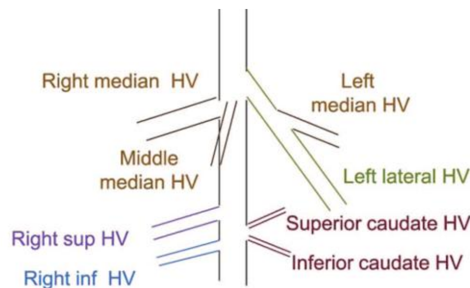

Figure 3 Rat liver: schematic representation of hepatic vein anatomy (from Madrahimov N et al., 2006).

1.2 Human Liver

In humans the liver is located, together with biliary tree and the gallbladder, in the right upper quadrant of the abdominal cavity, between the digestive tract and the remaining part of the internal organs. From the anatomical point of view, the human liver is positioned under the rib cage and covers the space between the right fifth

intercostal space and the midclavicular line, below the costal border. In normal adult people, the liver weight usually ranges between 1.4 to 1.6 kg, and traditionally was subdivided into four separate lobes, right, left, caudate and quadrate. About 25% of total blood pumped by the heart it is required to the liver to function properly, and it enters the liver for the most part (60%-70%) from the portal vein and for the remaining part (30%-40%) from the hepatic artery through the hilum. In the same district arrives the major bile duct, whereas the initial right and left branches of the portal vein, hepatic artery, and bile duct are positioned immediately external to the liver. The remaining part of the circulatory tree runs parallel wise inside the liver in the so-called portal tracts, giving rise to 17 to 20 orders of branches; it has been evaluated that almost all hepatic parenchyma is supplied thanks to about $4.5 \bullet 10^5$ terminal branches of the portal tract system. Septal venules transport portal vein blood inside parenchyma, whereas all the parenchyma, the principal bile ducts, the vasa vasorum of the major portal veins and hepatic veins, and the hepatic capsule are supplied by means of hepatic arteriolar twigs. Blood collected from all regions into ramification of the hepatic vein is brought outside the liver itself through the inferior vena cava. The circulatory system of the human liver is depicted in Figure 4.

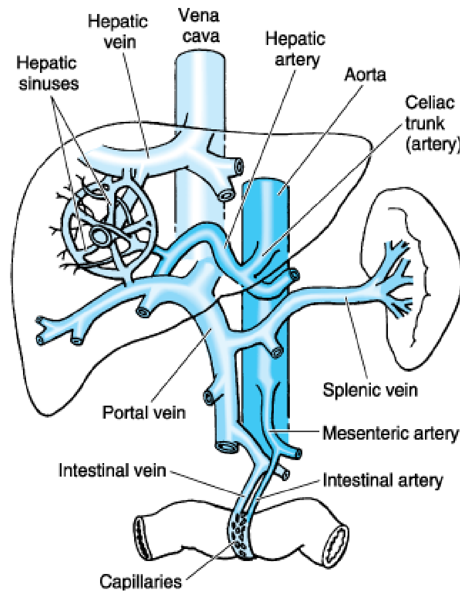

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Figure 4 Human liver: schematic representation of circulatory system.

About the microscopic organisation of the human liver, the first well recognised structures were the so-called lobules, 1-2 mm diameter roughly hexagonal assemblies, situated all-around the terminal hepatic veins, portal tracts being placed at the periphery of the lobule. In this way, it is possible to distinguish centrilobular hepatocytes, located near the terminal hepatic vein, and periportal hepatocytes, sited close to the portal tract. Anyway, considering that hepatocytes in close proximity to the terminal hepatic veins are very far from the blood supply, it has been suggested that they could be placed at the top of hypothetical triangular acini, the bases of which constituted by septal venules that enter from the portal vein spreading from the portal tracts. The parenchyma inside the acinus could be subdivided into three distinct zones: zone 1, nearest to the vascular supply, zone 3 being adjacent to the terminal hepatic venule, and zone 2 in the middle. This sort of “zonation” has been found to have great metabolic relevance, considering that several hepatic enzymes show a lobular gradient

of activity, and also a lot of types of hepatic injuries have a zonal pattern of manifestation (Jungermann K, Kietzmann T, 1996).

The typical structures of the hepatic parenchyma are the cribriform, anastomosing sheets or "plates" of hepatocytes, easily distinguishable as a sort of threads of cells in microscopic images. Hepatocytes placed in the near proximity of the portal tract have been named as the limiting plate, and they are characterised by the peculiarity to form a sort of irregular border around the mesenchyme of the portal tract. Moreover, the hepatocytes cords previously described are radially oriented around the terminal hepatic vein.

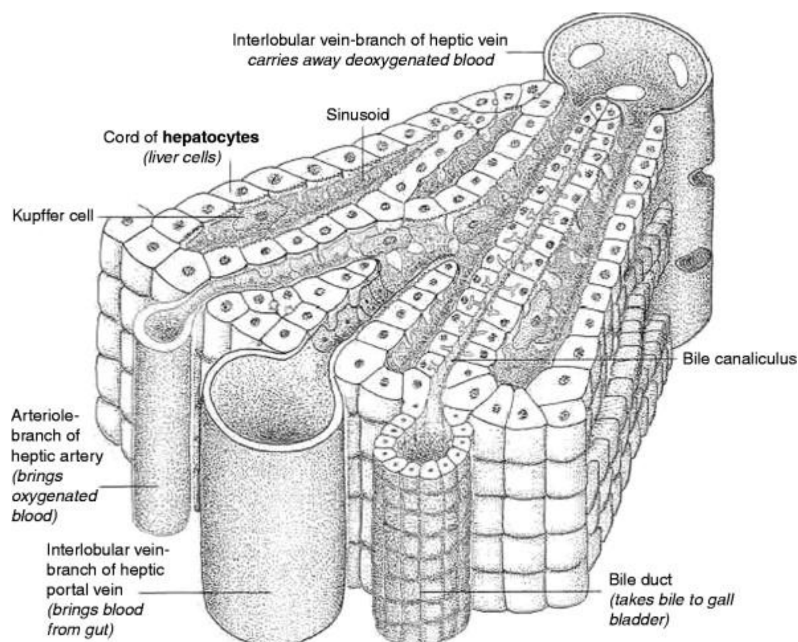

Figure 5 Human liver: schematic representation of microscopic organisation of hepatic parenchyma.

Vascular sinusoids are intercalated between the cords of hepatocytes, and are crossed by blood, which in turn finds the way out to the terminal hepatic vein passing through the orifices of the vein wall. Each sinusoid is delimitated by fenestrated endothelial cells that delineate the extra sinusoidal space of Disse, which is rich in hepatocytes microvilli. Kupffer cells, which belongs to the mononuclear phagocyte system, are

distributed in close proximity of the luminal face of endothelial cells, and the typical fat-containing perisinusoidal stellate cells are placed in a disperse manner inside the space of Disse. Stellate cells take part in the accumulation and metabolism of vitamin A, and when in presence of an inflammatory condition they acquire a myofibroblast phenotype, beginning to produce collagen.

Bile canaliculi are a sort of microscopic channels 1 to 2 µm in diameter connecting adjacent hepatocytes, shaped by grooves in the plasma membranes and demarcated from the vascular space by means of tight junctions. These intercellular spaces, which represent the final branches of the biliary tree, are filled with many microvilli; actin and myosin microfilaments that surround canaliculi provide the energy required to drive biliary fluid along the canaliculi, until the canals of Hering in the periportal region. Biliary fluid is then transferred to bile ductules, and finally to the terminal bile ducts within the portal tracts.

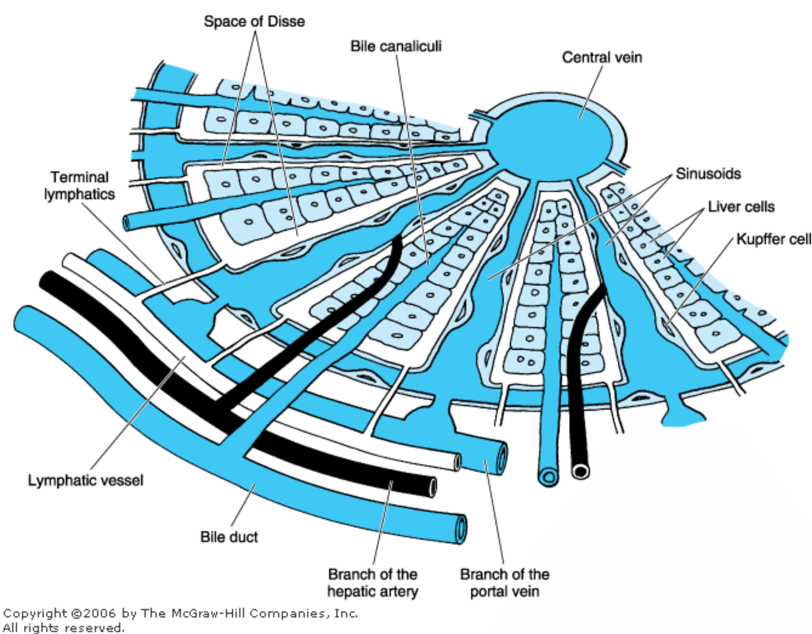

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Figure 6 Human liver: schematic representation of microscopic organisation of hepatic parenchyma, with particular reference to circulatory and biliary systems.

1.3 Metabolic functions

In Mammals the liver is probably the most important organ from a metabolic point of view, considering that it takes a fundamental part in the metabolism of all nutrient molecules and most hormones, in bile formation and excretion, in detoxifications reactions of many toxins and drugs (Stryer L, 1997).

Carbohydrates

All complex carbohydrates are catabolised to give glucose, fructose and galactose, and even if the liver has the ability to metabolise huge quantities of fructose, giving lactate as a final product, the most part of fructose and galactose are converted in glucose entering in its main metabolic pathway. In the liver (and adipose tissue as well) the phosphogluconate pathway is operative too, providing a cofactor essential for the fatty acids biosynthetic pathway (Stryer L, 1997).

The great majority of glucose introduced during a meal is converted and stored as glycogen, primarily inside the liver and to a lesser extent in skeletal muscle. Usually in Man, the maximum amount of glycogen that can be accumulated inside the liver is about 200 g, and when this threshold is surpassed, the liver gets start to the lipogenic pathway (Stryer L, 1997).

The liver and kidney play also an essential role considering their peculiar characteristic to possess the biochemical machinery needed to synthesise glucose starting from lactate, pyruvate, amino acids (principally alanine) and glycerol. From a physiological point of view this ability is of vital importance in maintenance of haematic glucose homeostasis, glucocorticoids, catecholamines, glucagon and thyroid hormone increasing gluconeogenesis, and insulin reducing it (Stryer L, 1997).

Lipids

The liver is able to stock excess of carbohydrates introduced with diet as glycogen, but when these stores become saturated, the lipogenic pathway start to work, converting carbohydrates and proteins as well into fatty acids. Hepatocytes can employ these fatty acids directly or store them, in synergy with white adipose tissue. Fatty acids can be utilised as fuel molecules from most cell types, with the exception of erythrocytes and renal medulla, which are strictly glucose-dependent. Brain has not a similar stringent dependence on glucose, considering that after several days without it, neurons show the ability to catabolise ketone bodies produced by the liver (Stryer L, 1997).

The final product of fatty acid oxidation is acetyl coenzyme A (acetyl-CoA), which in turn enters the citric acid cycle. Under conditions of excess of acetyl-CoA, the liver can produce the ketone body acetoacetic acid that can be used by other cell types as an energetic substrate, after reconversion into acetyl-CoA. Acetyl-CoA represents the principal building block necessary to synthesise cholesterol and phospholipids as well (Stryer L, 1997).

Proteins

The liver is certainly the key organ in protein metabolism, considering that if some block would occur in its functionality consequences at whole organism level could be fatal in few days. Four main stages can be summarised, i.e. deamination of amino acids, formation of urea, conversion between nonessential amino acids, production of plasma proteins (Stryer L, 1997).

Transamination reactions remove the amino group from amino acids giving the respective keto acids as a product. The great majority of amino acids introduced as protein from foods are deaminated at the liver level, only very little amounts being

processed in the kidneys, and the particular case of branched-chain amino acids (leucine, isoleucine, valine) that are catabolised almost exclusively in skeletal muscle.

For what concerns plasma proteins, excluding immunoglobulin, the liver synthesizes the remaining part. From a strictly quantitative point of view, albumin, α_1-antitrypsin and other proteases and elastases are the most abundant proteins produced by the liver. The function of liver in regulating plasma concentration of albumin is indeed very important, since this protein binds and transport fatty acids, several hormones and drugs, and keeps oncotic pressure in the plasma at normal values (Stryer L, 1997).

From a qualitative point of view, coagulation factors are without any doubt a class of essential proteins that, excluding factor VIII and von Willebrand factor, are all synthesized at the hepatic level. In particular it is meaningful to mention the vitamin K-dependent biosynthesis of prothrombin or factor II, and of factors VII, IX and X (Stryer L, 1997).

Other central proteins produced by the liver comprise complement, C-reactive protein, serum amyloid A, α_1-acid glycoprotein, but also transport proteins as transferrin, ceruloplasmin and haptoglobin, and protease inhibitors as α_1-antitrypsin, α_2-antiplasmin and antithrombin III. To be mentioned is plasma cholinesterase, the so-called pseudocholinesterase, which hydrolysing esters plays an essential role in the clearance of several local anaesthetics and succinylcholine (Stryer L, 1997).

Figure 7 summarises the principal metabolic pathways that takes place in the liver.

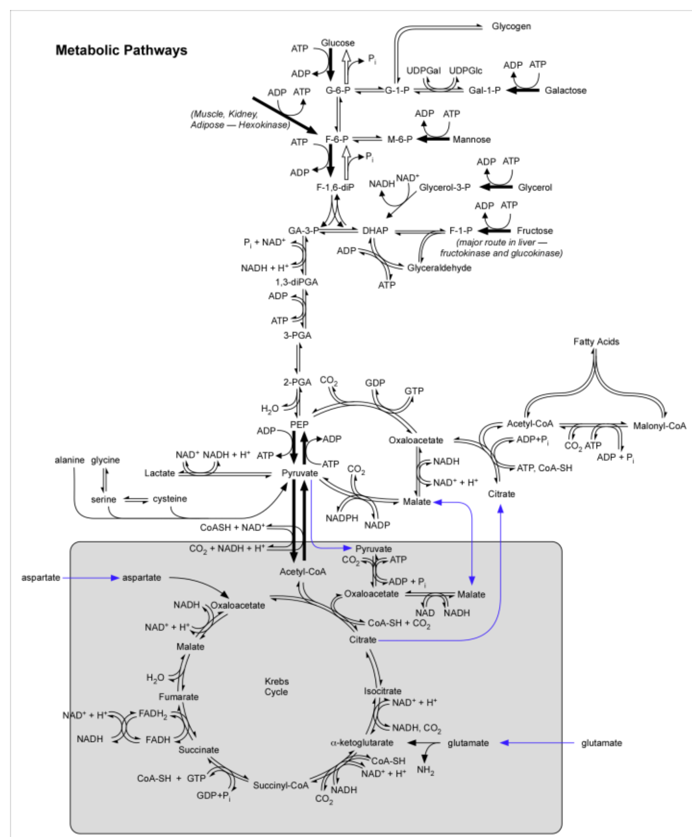


Figure 7 Human liver: schematic representation of metabolic pathways.

Drugs

The liver has certainly to be considered the principal site where exogenous molecules and most drugs are catabolised. At the hepatocyte level these substances are deactivated and transformed to obtain more water-soluble compounds, which are finally excreted through bile and/or urine.

Two main categories of reactions can be described in this regard, named phase I and phase II. Phase I reactions typically are characterised by the direct modification of reactive groups, thanks to mixed-function oxidases and/or cytochrome P-450 system, finally resulting in oxidations, methylations, deaminations, dealkylations and sulfoxidations. Striking examples of drugs cleared through this pathway are benzodiazepines, barbiturates, ketamine and ethanol, which are also able to induce the synthesis of the enzymes of the cytochrome P-450 system. Phase II reactions, on the

other hand, are characterised by conjugation reactions with glycine, taurine, and sulphate and glucuronide groups. After conjugation the product can be discarded through bile or urine (Stryer L, 1997).

Other metabolites

The liver performs several essential metabolic functions for what concerns vitamins, minerals and hormones. Vitamins A, B_{12}, E, D and K are stored mainly in the hepatocyte, whereas transferrin and haptoglobin, and ceruloplasmin are the key proteins that regulate iron and copper balance respectively. Regarding the role in hormones metabolism, well known is the function performed on thyroid hormone balance, considering that the great majority of triiodothyronine (T_3) is produced at the hepatic level, as well as all the steps required for its breakdown. Similarly, at the hepatic level takes place catabolism of glucagon, antidiuretic hormone, insulin and steroid hormones as oestrogens, cortisol and aldosterone (Stryer L, 1997).

Bile

Bile has a fundamental part in the processes of fat absorption and cholesterol, bilirubin and most drugs elimination. Phospholipids, conjugated bilirubin, cholesterol (0.3%), bile salts (10%) and other elements as mucus and pigments (3%), inorganic salts (0.7%), are secreted constantly into bile canaliculi. Bile is produced thanks to three principal mechanisms: a) Na^+/K^+-adenosine triphosphatase-mediated ion transport (bile salt-independent fraction), b) osmotic filtration induced by secretion of bile salts into canaliculi (bile salt-dependent fraction), c) secretin-mediated sodium and bicarbonate transport by ductules.

Bile produced in this way is directed into the duodenum through the common bile duct, formed by the joining of hepatic duct, which comes from connection of right and left hepatic ducts originating from hepatic lobules, with cystic duct from the

gallbladder. Sphincter of Oddi is deputed to control the biliary flow from the common bile duct into the duodenum. The gallbladder has the function to accumulate biliary fluid between meals, thanks to active sodium transport and passive water reabsorption. When cholecystokinin is liberated from the intestinal mucosa due to protein and fats, gallbladder is induced to shrink, sphincter of Oddi undergo to relaxation and bile is transferred into the small intestine.

From the functional point of view, the bile acids play an important role in facilitating the absorption of lipids operated by intestine as well as in helping emulsifying insoluble components of bile and elimination of cholesterol. The two most important bile acids, cholic acid and chenodeoxycholic acid, are conjugated with glycine and taurine and, as salts are secreted into bile. Inside the colon they are modified, by bacteria, to deoxycholic acid and lithocholic acid respectively, the so-called secondary bile acids. These ones play indeed an essential role in vitamin homeostasis, considering that any trouble affecting bile salts formation and/or secretion alters the normal process of absorption of fats and consequently of fat-soluble vitamins. Bilirubin is produced when the haeme ring is degraded inside Kupffer cells, due to the action of heme oxygenase that gives back biliverdin, carbon monoxide and iron; biliverdin in turn is converted to bilirubin by biliverdin reductase. Once produced bilirubin passes in the bloodstream, binds to albumin and thenceforth it enters inside hepatocytes, where the binding to intracellular proteins block it inside the cell. The hepatocyte possesses the enzymatic machinery needed to conjugate bilirubin with glucuronide, permitting the conjugated product to be excreted into bile canaliculi, only a minor fraction being reabsorbed into the blood. Once into the intestine, colonic bacteria transform about a half of bilirubin in urobilinogen, which in turn is partly

reabsorbed by the intestine and brought back to the liver (enterohepatic recirculation), and partially excreted through the kidney (Stryer L, 1997).

2. THYROID HORMONES and DIIODOTHYRONINES

2.1 Synthesis and metabolism

Thyroid gland is an endocrine gland, present in all Vertebrates, starting from adult Cyclostomata to Mammals and Man. Its microscopic anatomy is characterised by a vesicular structure, organised in oval or spherical cystic follicles, lined by a monostratified epithelium, composed by cells with different heights, often cubic in shape. The fundamental physiological characteristic of this gland is its peculiar ability to concentrate iodine and to use it to synthesize thyroid hormones (Padoa E, 1978).

Thyroid hormones are synthesized, with similar mechanisms in all Vertebrates, in the colloid, a sort of gel that fills the internal part of the thyroid follicles. Iodine, ingested with foods, is reduced to iodide ion in the intestine, enters the bloodstream and is absorbed into the epithelial thyroid cells. This process is mediated by the Na^+/I^+ transporter, which works using the high Na^+ extracellular concentration, which is produced by the action of Na^+/K^+ ATPase pump (Dai G et al., 1996).

Iodine is transported inside the thyroid follicle together with thyroglobulin, a 660-kDa glycoprotein, which contains 134-140 tyrosine residues. Iodine can be bound on about a quarter of these residues: this reaction that takes place at the interface between apical follicular cells and colloid, is catalysed by thyroid peroxidase (TPO), an haeme-based enzyme, localised in the apical plasma membrane of thyroidal epithelial cells. After that iodine has been covalently bound, thyroglobulin enters the colloid, where TPO also catalyse the coupling reaction between iodinated tyrosine residues: two diiodotyrosine lead to formation of thyroxine (3,5,3’,5’-tetraiodothyronine, T_4),

whereas one diiodotyrosine with one monoiodotyrosine give rise to 3,5,3'-triiodothyronine or T_3 (Yen PM, 2001).

After synthesis thyroid hormones are cut from thyroglobulin inside lysosomes of thyroidal epithelial cells, then diffuse outside lysosomes, and through epithelial cells basal membrane, enter the bloodstream (Taurog A, 1996).

In Man thyroid produces, every day, approximately 100 μg of thyroid hormones, the major part being represented by T_4 (≈ 90%) and its concentration in the serum is about 40 times higher with respect to T_3 (90 nM vs. 2 nM). Only the 0.03% of total T_4 found in serum is free, whilst the remaining part is bound to carrier proteins, as thyroxin-binding globulin (TBG), transthyretin (TTR), thyroxin-binding prealbumin (TBPA) and albumin; lesser amounts are bound to lipoproteins as well. For what concerns T_3, only about 0.3% is free in the serum, whereas the vast majority is transported by TBG, albumin, and lipoproteins. TBG is a 64-kDa tetrameric protein, and each monomer binds to one single thyronine (Robbins J, 2000).

In humans roughly 80% of plasmatic T_4 is coupled to TBG, 15% to TTR and 5% to albumin and lipoproteins, differently to T_3, 90% of which is coupled to TBG and the remaining 10% to albumin and lipoproteins (Choksi NY et al., 2003). In rodents TBG gene is expressed at very low levels and consequently almost all T_4 and T_3 are found bound to albumin and TTR (Kaneko JJ, 1989).

Once reached the target-organs thyroid hormones enter the cells through specific transporters (Hennemann G et al., 2001) or receptors for TBG and TTR (Robbins J, 2000).

The principal pathway leading to T_3 production is deiodination at position 5' of T_4 external ring, operated by deiodinases (Kohrle J, 2000). In man and rodents, about 10% of T_4 is deiodinated to T_3 before secretion by deiodinase type I (DI), whereas, for

the other 90%, deiodination takes place in the liver and in the kidney. In the brain, anterior pituitary gland, skeletal muscle, placenta, and in rodents also in brown adipose tissue, deiodinase type II (DII) is responsible for production of small amounts of T_3. Finally, deiodinase type III (DIII), localised in the brain, placenta, and foetal tissues, in man and rodents, is able to perform deiodination only on the inner ring of T_4 and T_3 molecules, giving rise to diiodothyronines and to 3,5', 3'-triiodothyronine or reverse T_3 (rT_3), which can be considered as the first step in the catabolic pathway of thyroid hormone (Wassen FW et al., 2004), even if some recent reports suggest that this molecule might have some biological effects (Cheng SY et al., 2010).

In adult men T_4 and T_3 serum half-lives are, respectively, 5-9 days and 1 day, whereas in rats, 12-24 hours and 6 hours (Choksi NY et al., 2003).

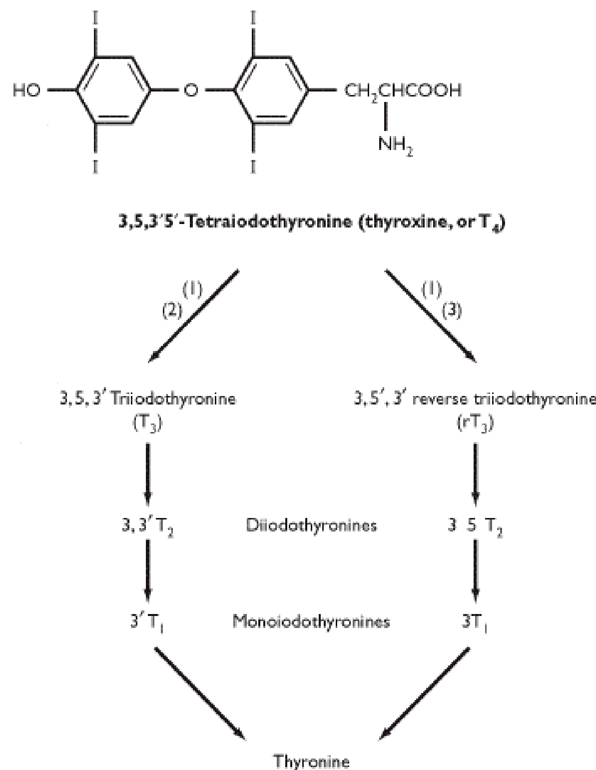


Figure 8 Thyroid hormone: schematic representation of catabolic pathways.

2.2 Hypothalamus-Pituitary-Thyroid (HPT) Axis

Thyroid hormones synthesis and secretion are tightly regulated by a negative feedback loop, involving the so-called Hypothalamus-Pituitary-Thyroid (HPT) Axis. This finely tuned control mechanism is achieved by the action of two hormones, Thyrotropin Releasing Hormone (TRH) produced by hypothalamus, and Thyroid Simulating Hormone (TSH), secreted by pituitary gland (Randall D et al., 1997).

Elevated concentrations of thyroid hormones in blood lead to inhibition of TRH production and consequently to a decrease of TSH secretion. It is well known that thyroid hormones are able to reduce the responsiveness of thyrotrophic cells to TRH, and that the ability of these cells to respond to TRH depends on their T_3 internal concentration. When T_3 levels are low, the number of TRH receptors increases inducing an increase in the synthesis of TSH, on the contrary, when in presence of higher T_3 concentrations, TRH receptors decrease as well as TSH production (Fliers E et al., 2006).

This regulation system is influenced by internal as well as external factors. Concerning the internal ones, somatostatin and dopamine negatively regulate TSH secretion in the hypothalamus (Samuels MH et al., 1992). One the other hand, among the external factors, one of the best known is the effect of changes in the atmospheric temperature in man as well as in rat: for example, in man, four days in a cold environment are able to induce an increase in the levels of TRH, TSH and as a consequence of circulating thyroid hormones, leading to a rise in basal metabolic rate and in heat generation (Eastman CJ et al., 1974). Similar results have been observed in rats, after 24h exposure to cold (Tuominen RH et al., 1985). Moreover, at the pituitary gland level, is well documented the inhibitory effect on TSH secretion induced by glucocorticoids

(Wilber JF, Utiger RB, 1969), and the stimulatory one provoked by oestrogens (Yarwood NJ et al., 1993).

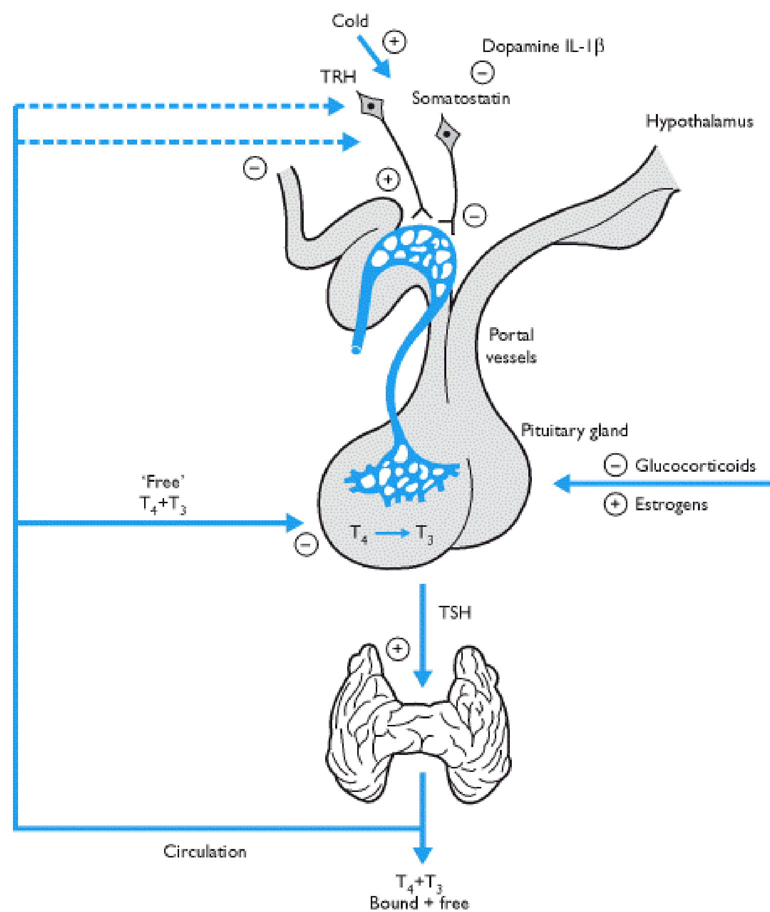


Figure 9 Thyroid hormone: schematic representation of Hypothalamus-Pituitary-Thyroid (HPT) axis.

2.3 Physiological Effects

In Mammals, thyroid hormones exert a wide variety of fundamental physiological effects including regulation of energetic metabolism, body temperature and growth, and bone metabolism. Essential are also actions exerted on cardiovascular and central nervous systems.

In almost all tissues, with the exception of brain, spleen and testicles, thyroid hormones are able to induce an acceleration of metabolic processes, due to an increase of mitochondria number and size and also to a stimulation of synthesis of respiratory chain enzymes (Wrutniak-Cabello C et al., 2001). Moreover an increase of Na^+/K^+

ATPase molecules has been observed, due to enhanced transcription coupled to augmented transport of primary transcripts from the nucleus to the cytoplasm (Harrison AP, Clausen T, 1998). It is useful to remember that 15% to 40% of the energy produced by cells is consumed to keep the electrochemical gradient across the cell membrane, so an intensification of activity of Na^{+}/K^{+} ATPase necessarily parallels with an increase of basal metabolic rate.

Important effects have been described on lipid and cholesterol metabolism at the hepatic level, where thyroid hormone is able to up regulate malic enzyme, glucose-6-P-dehydrogenase and fatty acid synthase (Oppenheimer JH et al., 1995), and also acid cholesterol ester hydrolase (Severson DL, Fletcher T, 1981) and LDL receptor (Scarabottolo L et al., 1986).

About the role of thyroid hormones in the regulation of carbohydrate metabolism, it has been shown that the great majority of the enzymes involved in the gluconeogenesis are stimulated (Feng X et al., 2000), together with GLUT4 glucose transporter gene expression (Chidakel A et al., 2005).

Thyroid hormone displays relevant physiological actions on bone metabolism as historically described in patients that develop hypothyroidism during childhood, which classically show delayed growth and short stature. Deleterious effects on bone mass have likewise been reported in hyperthyroidism, which induces lost of thickness and increase in porosity (Greenspan SL, Greenspan FS, 1999).

On the cardiovascular system thyroid hormones reduce systemic vascular resistance, and induce vasodilatation, leading to an increase of blood flow. Heart frequency, contractility and amount of blood pumped are as well stimulated, as shown examining hyper- and hypothyroid patients (Klein I, Ojamaa K, 1998).

Fundamental functions are played by thyroid hormones in correct development and functionality of brain, both in humans and rats. In Man, neonatal hypothyroidism results in mental retardation and neurological defects (Bernal J, 1999). In rats, neonatal thyroid hormones deprivation leads to a decrease in axonal growth and dendritic spread, in cerebral, watching and hearing cortex, in hippocampus and cerebellum (Rabie A et al., 1977). In adults the influence exerted by thyroid hormones is similarly very pronounced: hyperthyroidism is able to induce increase of CNS excitability, whereas hypothyroidism have the opposite effect, as shown in rats, where reduction in response to visual and acoustic stimuli, slowed reflexes, and smaller alpha waves in electroencephalogram have been well documented (Padoa E, 1978).

2.4 Way of Action

Genomic Effects

Once reached target cells thyroid hormones generate a lot of biological effects, some of which described above.

Historically it was shown that thyroid hormone actions are mediated by nuclear receptors (TRs), tightly associated with chromatin and with high affinity and specificity for T_3. Thyroid hormones enter into the target cell, where T_3 is produced by deiodination of T_4, and in the nucleus it binds to TRs, which are associated to Thyroid Response Elements (TREs), found in the promoter region of target genes. Formation of T_3-TR-TRE complexes is the first step in the process of regulation, positive as well as negative, of target genes under thyroid hormone control (Samuels HH et al., 1988).

TRs are members of the same nuclear receptors superfamily that includes steroid hormones, Vitamin D and retinoic acid. In humans, rat, mice, chicken and amphibian, two isoforms have been discovered, TRα and TRβ, codified by two separate genes,

located respectively on chromosomes 17 and 3 in man. Both isoforms bind T_3 (K_D = 10^{-9} and 10^{-10} respectively) and are involved in mediating gene expression induced by thyroid hormone. Moreover additional isoforms are produced by alternative splicing, giving rise to TRα1 and TRα2, the latter acting as transcriptional inhibitor, and to TRβ1 and TRβ2. TRα1 is expressed mainly in skeletal muscle and brown adipose tissue, whereas TRα2 is found more ubiquitously. TRβ1 is concentrated principally in brain, liver and kidney, whilst TRβ2 is located exclusively in the anterior pituitary gland and in specific hypothalamic areas (Lazar MA, Chin WW, 1990).

The mechanism that leads to transcription of the genes under thyroid hormone control is complex, and a comprehensive description is out of the aims of this thesis. Anyway it is important to underline some key aspects of this process. First of all, TRs bind to TREs as monomers, homodimers, but also as heterodimers with another important class of nuclear receptors, i.e. Retinoid X Receptors (RXRs). RXRs bind 9-cis-retinoic acid with high affinity, and form homodimers with Retinoid Acid Receptors (RARs), Vit. D Receptors (VDRs) and Peroxisome Proliferator Activated Proteins (PPARs) (Glass CK, 1994). This characteristic is important because in this way the amount of target genes that can be regulated by T_3 is largely increased, considering that heterodimers binds to TREs at different sites and with different orientation with respect to homodimers (Glass CK, 1994).

The best model known until now describing the transcriptional activation induced by thyroid hormones states that T_3 binding to its own nuclear receptor is able to remove a transcriptional block imposed by a co-repressor complex located on TRE. A lot of co-repressor and co-activator proteins have been identified to date, which probably widen the range of tissue specificity of the hormonal action (Yen PM et al., 2001).

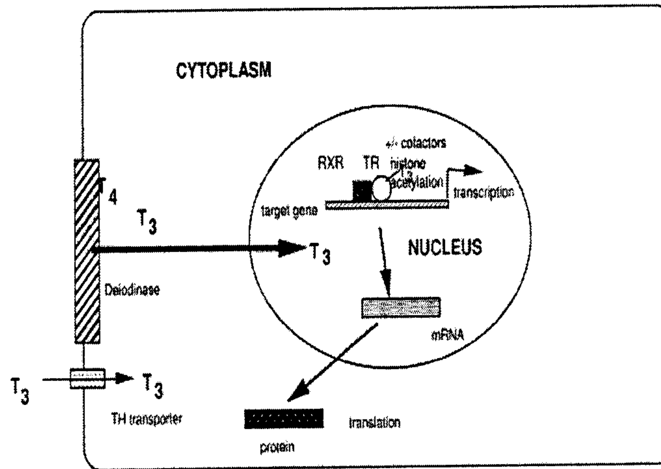


Figure 10 Thyroid hormone: schematic representation of genomic effects.

Nongenomic Effects

Until three decades ago the vast majority of scientific community was convinced that the only way by which thyroid hormones were able to trigger biological effects was through transcriptional activation of specific genes, i.e. through a genomic way.

Starting from the early '80s new ideas emerged from the work of several researchers, which introduce the concept that thyroid hormones could induce several important responses also without directly affecting gene expression, in the so-called nongenomic way. Typically these effects are characterised by rapid onset (seconds or minutes), and direct interaction of thyroid hormones, both T_3 and T_4, with plasma membrane proteins and/or specific receptors, resulting in allosteric activations and induction of cell signaling pathways. Some striking examples are represented by the direct stimulation by T_3 of human Ca^{2+}-ATPase activity in vitro (Davis FB et al., 1983) and 2-deoxyglucose uptake in rat thymocytes in vitro (Segal J, Ingbar SH, 1981), but several others have been described to date. More recently specific effects on ionic fluxes through the cell membrane have been reported, as for activation of inward rectifier potassium channels currents (Sakaguchi Y et al., 1996), Na^+/H^+ antiport (Incerpi S et

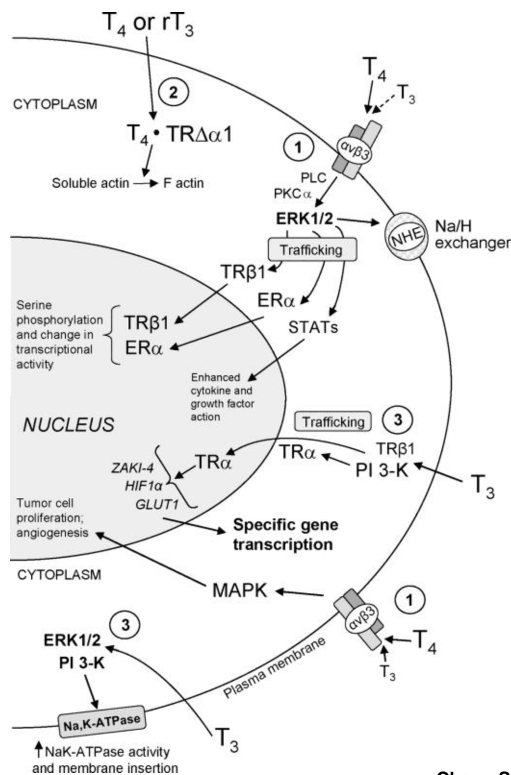
al., 1999) and Na^{+} and Ca^{2+} currents (Wang YG et al., 2003). Typically these actions show a time of onset that ranges between 30 sec to 20 min after hormonal stimulation and are observed using concentrations included in the interval 1 nM to 10 nM (Davis PJ et al., 2005).

Some specific biological actions have been discovered for T_4 as well, especially for what concerns several signaling pathways. For example induction of tyrosine phosphorylation and consequent translocation inside the nucleus of STAT3 transcription factor has been observed and shown to be dependent on PKC, MAPK and Raf-1 activation. Moreover a stimulation of interaction between MAPK and TRβ1 leading to phosphorylation of this receptor has been reported, which has been shown to be phosphorylated by p38 MAPK as well (Yen PM, 2001). Other reports have assessed the role of T_4 in the activation of PLC, leading to DAG production and PKC activation (Kavok N et al., 2001).

All these observations could suggest the existence of a membrane receptor for thyroid hormone mediating these effects, and, in fact, a high affinity binding-site has been identified on integrin αVβ3 in a fibroblast cell line (Bergh JJ et al., 2005). Since that discovery several other papers have reported and described biological effects due to the interaction with this integrin (Scarlett A et al., 2008; Yalcin M et al., 2010).

Interestingly, two recent papers suggested that thyroid hormones, and in particular T_3, may exert their effects through a novel nongenomic way, which is mediated by a cytoplasmic TRβ1 and finally leads to Akt activation (Verga Falzacappa C et al., 2007; Verga Falzacappa C et al., 2009).

In Figure 11 are represented several nongenomic effects triggered by T_4, T_3 and rT_3, which lead to the activation of specific signaling pathways.



Cheng S et al. Endocrine Reviews 2010;31:139-170

Figure 11 Thyroid hormone: schematic representation of nongenomic effects.

2.5 Diiodothyronines

Until two decades ago diiodothyronines, 3,5-diiodothyronine (3,5-T_2) and 3,3'-diiodothyronine (3,3'-T_2), were considered only a product of catabolism of thyroid hormone, without any significant biological effect. But from the mid '90s several reports began to describe some metabolic actions at the mitochondrial level, these molecules being able to rapidly stimulate oxidative rate in rat liver (Lanni A et al., 1992), by binding and stimulating activity of cytochrome c oxidase, as shown in rat hepatocytes *in vitro* (Lanni A et al., 1994), and confirmed on isolated enzyme as well (Goglia F et al., 1994). Moreover a calorigenic effect of both diiodothyronines was found to exist in rats *in vivo* (Lanni A et al., 1996). Indeed it was later demonstrated that 3,5-T_2 is able to bind to subunit Va of cytochrome c oxidase, completely abolishing allosteric inhibition, which usually takes places when [ATP]/[ADP] ratio is

high (Arnold S et al., 1998). Recently a direct role of 3,5-T_2 in increasing the activity of glucose-6-P-dehydrogenase has been assessed in rat (Lombardi A et al., 2000), supporting the idea of a biologically active molecule.

It is interesting to underline that no other physiological effects have been described to date for diiodothyronines, so they do not show most of the well-known effect of thyroid hormone described above. Only one report demonstrates that 3,5-T_2 displays some selective thyromimetic effects *in vivo* and *in vitro*, being able to suppress TSH secretion, even if 50% less with respect to thyroid hormone (Ball SG et al., 1997).

Moreover no receptors have been identified until now for diiodothyronines, nor nuclear, cytoplasmic, or plasma membrane ones. Three cytoplasmic proteins able to bind 3,5-T_2 have been identified in rat liver, but no insights about the role played by these proteins have been proposed (Moreno M et al., 2003).

3. NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

3.1 General features

Non-Alcoholic Fatty Liver Disease (NAFLD) is nowadays considered one of the most considerable pathologic conditions affecting the liver especially in western world, with the higher prevalence observed in United States of America, United Kingdom and north of Europe. Recent reports suggest that about 15% to 20% of population from different countries could be affected, with an increasing incidence of people at risk to develop this pathology (Anderson N, Borlak J, 2008). The frequency is much higher in obese population, for which epidemiological studies report percentages that ranges from 57.5% to 74% (Browning JD et al., 2004). NAFLD has been described in children as well, with 2.6% occurrence in non-obese subjects, which rises to 22.5% in obese ones (Angulo P, 2002).

From a clinical point of view, NAFLD can be diagnosed because usually patients show the same typical manifestations observed in Alcoholic Liver Disease (ALD), but without any excessive consumption of alcoholic beverages. According to some authors the amount of alcohol consumed should be lower than 20 g for women and 30 g for men to be in presence of NAFLD (Bellentani S et al., 1997).

For what concerns the diagnostic analysis that can be employed in NAFLD assessment, no one of the most common laboratory tests can provide a clear and definitive indication of this pathology, liver biopsy being the only instrument with which is possible to obtain a well-defined evidence of the presence of this condition (Angulo P, 2002). Several risk factors and clinical manifestations can anyway provide some useful indications about the possible presence of NAFLD. It has been reported that hyperlipidemia, obesity and type II diabetes are recurrently observed in patients with NAFLD. For example obese population (BMI≥30) has been found to have a higher incidence of a factor of about 5 with respect to non-obese people (Bellentani S et al., 2000). Moreover, a positive correlation between truncal obesity and NAFLD has been reported also in patients with regular BMI (Ruderman N et al., 1998). In about half of patients presenting hyperlipidemia analysed in one clinical study the presence of NAFLD was subsequently reported, and the same study established that hypertriglyceridemia is a more dangerous risk factor if compared with hypercholesterolemia (Assy N et al., 2000). About the clinical manifestations of NAFLD, it is important to underline that most people affected have no evident signs of the pathology, with the exception of a sense of fullness and/or mild ache on the right side of the upper part of abdomen, together with tiredness and hepatomegaly Some laboratory analysis can be useful to assess if a person is affected or not by NAFLD, such as serum levels of aminotransferase, alkaline phosphatase, γ-glutamyltransferase.

Aspartate aminotransferase and alanine aminotransferase levels are often higher than normal in NAFLD patients, as well as that of alkaline phosphatase and γ-glutamyltransferase, even if to a lesser extent with respect to ALD. Several other parameters are subjected to variations when in presence of NAFLD, such as elevated serum ferritin levels. Hypoalbuminemia, hyperbilirubinemia and a prolonged prothrombin time are often present when NAFLD is in the cirrhotic phase (Angulo P, 2002). Imaging techniques are employed as well in the diagnosis of NAFLD and may represent a valuable tool, with particular reference to ultrasonography, computed tomographic (CT) scanning, and magnetic resonance imaging and spectroscopy. Ultrasonography has been reported to have a very high sensitivity and specificity in identifying steatosis (above 90%), and fibrosis (above 85%) (Joseph AE et al., 1991). CT scanning and magnetic resonance imaging can be used in the assessment of fat infiltration inside the liver parenchyma, the first being eligible when steatosis is diffuse, which is the predominant condition in NAFLD, the second being more suitable when infrequently happens that steatosis has a focal pattern. Finally, magnetic resonance spectroscopy is commonly used to evaluate the amount of fat infiltrate inside the liver parenchyma (Angulo P, 2002).

From an histological point of view, the typical features observed after a liver biopsy, which characterise a liver as a NAFLD liver are steatosis, hepatocyte ballooning, glycogen nuclei, mixed inflammatory-cell infiltration, Mallory's hyaline, cell necrosis and fibrosis (Angulo P, 2002). The two following pictures below clearly illustrate some of the most typifying features of NAFLD. In Figure 12 [haematoxylin and eosin – 200X] is evident the presence of a macrovesicular steatosis, even if in some hepatocytes is possible to find both macro- and microvesicular one. The fat infiltrate may be found prevalently inside acinar zone 3 when the amount is not so abundant, but

increasing quantities of fat led to a more diffuse pattern of distribution. Hepatocyte ballooning is visible as well, consisting in a degeneration of the hepatocyte determined by an accumulation of intracellular fluid, which induces cell swelling that characteristically occurs in zone 3 close to steatotic hepatocytes. In this photo is also possible to see Mallory's hyaline bodies inside the hepatocytes undergoing ballooning degeneration in zone 3. These bodies are frequently (≈50%) observed in adult people affected by NAFLD but not specific, being present in ALD or other liver pathologic conditions as well. Clearly distinguishable is also the inflammatory infiltrate that usually consists of lymphocytes and neutrophils, and is localised in zone 3. It is interesting to underline that usually in adults the inflammatory infiltrate does not localise in portal tracts, whereas in children this is the typical pattern of infiltration. Furthermore in children Mallory's hyaline is usually very scarce or not present (Kleiner DE et al., 2005).

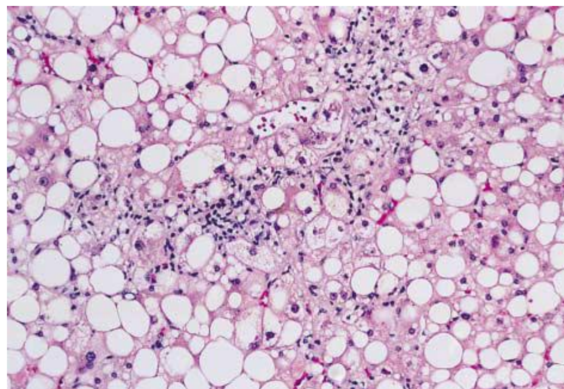


Figure 12 NAFLD: macrovesicular steatosis, inflammatory infiltrate, Mallory's hyaline and hepatocyte ballooning (from Angulo P, 2002).

Figure 13 [Masson's trichrome – 200X], illustrates a clear example of fibrotic liver, in which are evident the three typical patterns of fibrosis, i.e. perivenular, pericellular and perisinusoidal in zone 3. It is useful to highlight that in NAFLD as well as in ALD, collagen is put down initially in the pericellular space around the central vein and in

the perisinusoidal region inside the zone 3. Moreover in several regions it is possible to observe a typical pattern in collagen deposits, the so-called “chicken-wire” fibrosis. Together considered these features can be helpful to distinguish NAFLD- and ALD-induced fibrosis to a fibrosis provoked by other liver diseases, in which collagen deposition typically begins in the portal region (Angulo P et al., 2007).

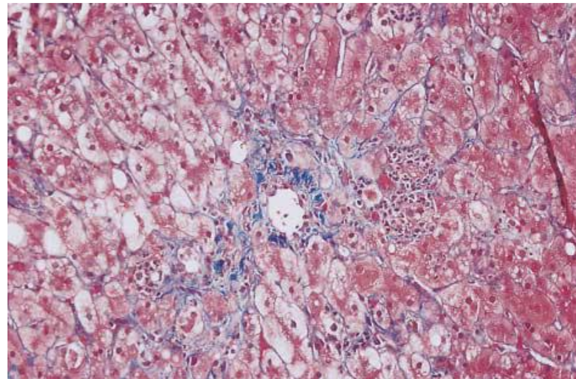


Figure 13 NAFLD: perivenular, pericellular and perisinusoidal fibrosis in zone 3 (from Angulo P, 2002).

In 1999 Brunt EM and co-workers (Brunt EM et al., 1999), proposed a valuable classification of NAFLD, according to degree of severity and localisation of steatosis, inflammation and fibrosis observed.

Grading for steatosis
Grade 1→<33% of hepatocytes affected
Grade 2→33% to 66% of hepatocytes affected
Grade 3→>66% of hepatocytes affected
Grading for steatohepatitis
Grade 1→mild
-Steatosis: predominantly macrovesicular, involves up to 66% of lobules
-Ballooning: occasionally observed; zone 3 hepatocytes
-Lobular inflammation: scattered and mild acute inflammation (polymorphonuclear cells) and occasional chronic inflammation (mononuclear cells)
-Portal inflammation: none or mild
Grade 2→moderate
-Steatosis: any degree; usually mixed macrovesicular and microvesicular
-Ballooning: obvious and present in zone 3
-Lobular inflammation: polymorphonuclear cells may be noted in association with ballooned hepatocytes; pericellular fibrosis; mild chronic inflammation may be seen
-Portal inflammation: mild to moderate
Grade 3→severe
-Steatosis: typically involves >66% of lobules (panacinar); commonly mixed steatosis
-Ballooning: predominantly zone 3; marked
-Lobular inflammation: scattered acute and chronic inflammation; polymorphonuclear cells may be concentrated in zone 3 areas of ballooning and perisinusoidal fibrosis

-Portal inflammation: mild to moderate
Staging for fibrosis
Stage 1→zone 3 perivenular, perisinusoidal, or pericellular fibrosis; focal or extensive
Stage 2→as above, with focal or extensive periportal fibrosis
Stage 3→bridging fibrosis, focal or extensive
Stage 4→cirrhosis

3.2 Pathogenesis

The pathological mechanisms that induce the development of NAFLD are so far not completely clarified and still matter of debate, even if some key factors seem to be elucidated. One fundamental condition that has to be satisfied is a significant accumulation of lipids inside the hepatocyte, principally as triglycerides. Several theories have been proposed to date, but now most researchers believe that the first step required for onset of NAFLD is certainly insulin resistance, according to what hypothesised by Day CP and James OFW in 1998. Insulin resistance, in turn, would lead to fat accumulation, and hence to steatosis, probably inducing modifications in the uptake, synthesis, oxidation or export of lipids, as shown in the following figure (Angulo P, 2002).

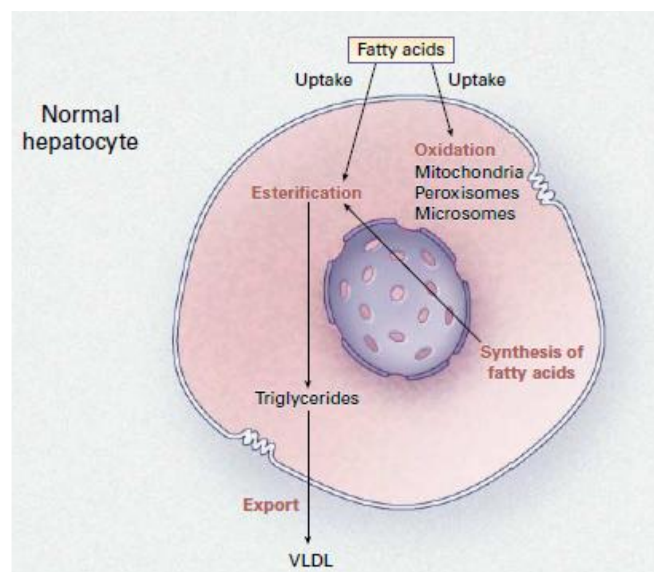


Figure 14 NAFLD: fatty acid metabolism in a normal hepatocyte (from Angulo P, 2002).

Onset of insulin resistance is indeed a complex topic to deal with, even if until now some points have been clarified. Several molecules, as reported in figure 15, can block insulin action. Once induced an insulin resistance condition, two main events are thought to be responsible for progression to a steatotic state: hyperinsulinemia and increase in lipolysis at the adipocyte level. At the hepatocyte level, hyperinsulinemia leads to an enhancement of glycolytic pathway, resulting in an increased synthesis of fatty acids. Moreover, the concomitant decrease in apolipoprotein B-100 (ApoB-100) synthesis has the final effect to raise the triglycerides content inside the cell. Increased lipolysis at the adipose tissue level determines the increase of the haematic concentration of fatty acids, which in turn are up taken by the liver. Such excess of fatty acids induces two parallel effects: an overwork of mitochondrial β-oxidation that leads to accumulation of fatty acids themselves, and a stimulation of synthesis of cytochrome P-450 2E1 and 4A that in turn are able to trigger the production of Reactive Oxygen Species (ROS), responsible for an extensive lipid peroxidation of cell membranes (Weltman MD et al., 1998; Leclercq IA et al., 2000).

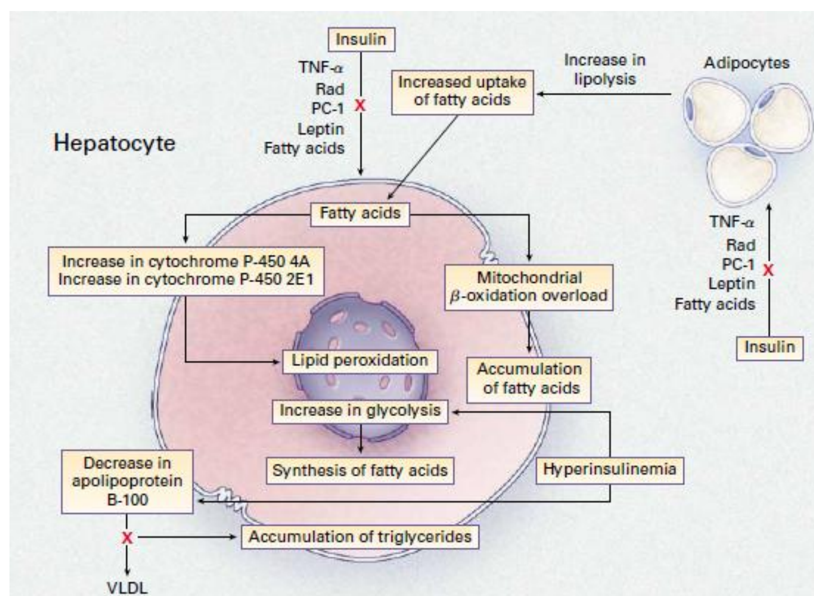


Figure 15 NAFLD: metabolic pathways in an insulin resistant hepatocyte (from Angulo P, 2002).

Another aetiology that has been proposed for onset of NAFLD involves deregulations of fatty acids oxidation pathways at the hepatocyte level. As illustrated in figure 16, the increased amount of fatty acids present in the insulin-resistant hepatocyte may result in three principal outcomes. First of all microsomal ω-oxidation is increased, leading to production of dicarboxylic acids, which are supposed to be cytotoxic and are targeted to peroxisomal β-oxidation. Peroxisomal β-oxidation is thought to have an extremely important role in the pathogenesis of NAFLD, if considered that the absence of peroxisomal fatty acyl-CoA oxidase has been shown to be responsible for the induction of extensive microvesicular steatosis and steatohepatitis as well. Furthermore if this enzyme does not work, much of the acyl-CoA produced by peroxisomes is no further processed and acts as a PPAR-α ligand, hyperactivating it, and inducing as a consequence the up-regulation of PPAR-α–controlled genes, as those involved in mitochondrial, peroxisomal and microsomal enzyme systems (Fan CY et al., 1998). An additional gene that has been proved to be under PPAR-α influence and that can be found in liver from NAFLD patients is uncoupling protein-2 (UCP-2). Its part in NAFLD progression it is not well defined, but some authors have proposed a role in raising the susceptibility of the fatty hepatocyte to secondary hits, such as TNF-α or endotoxins (Chavin KD et al., 1999).

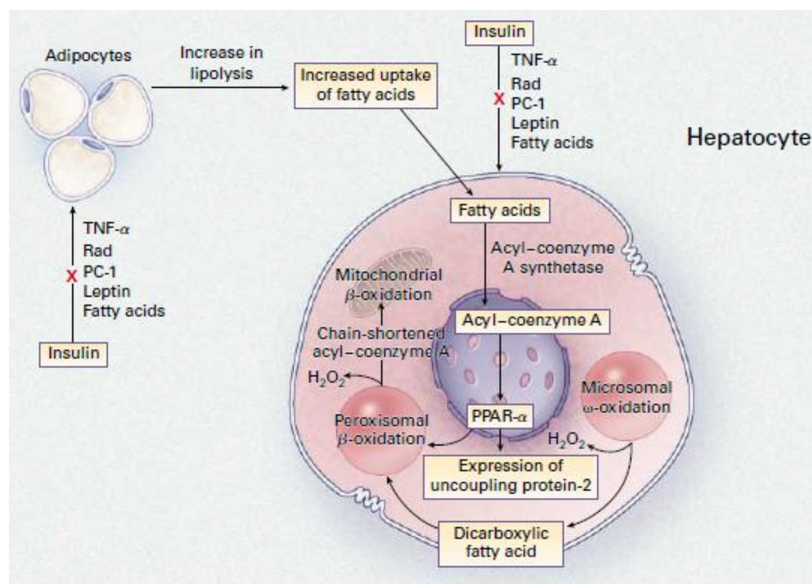


Figure 16 NAFLD: metabolic pathways in an insulin resistant hepatocyte, with particular reference to microsomal ω-oxidation and peroxisomal β-oxidation (from Angulo P, 2002).

According to what discovered until now, the progression from simple steatosis to steatohepatitis and eventually cirrhosis and fibrosis is largely due to raised intrahepatic level of reactive oxygen species (ROS), produced at the mitochondrial level as a consequence of the surplus of fatty acids. ROS in turn are able to trigger at least three different processes: lipid peroxidation, cytokine induction and Fas ligand (FasL) expression. The two main products derived from lipid peroxidation are malondialdehyde (MDA) and 4-hydroxynonenal (HNE) that cause several deleterious effects at the cellular level. Primarily they can induce cell death and are as well thought to be responsible for the formation of Mallory's hyaline, through protein cross linking (Zatloukal K et al., 1991). Secondarily, it has been shown that they are able to promote collagen deposition by activating hepatic stellate cells (HSC) (Leonarduzzi G et al., 1997). Thirdly, HNE behaves as a proinflammatory agent, showing a chemotactic action towards neutrophils (Curzio M, 1985).

For what concerns cytokine induction, there are several evidences reporting that ROS have a role in promoting the formation of TNF-α, TGF-β, and interleukin-8 (IL-8). TNF-α and TGF-β make caspase to be activated (Higuchi M et al., 1997; Inayat-Hussain SH et al., 1997), whereas TGF-β alone works as a cofactor, together with MDA and HNE, in the process of building up Mallory's hyaline, doing so by stimulating collagen production by HSC (Leonarduzzi G et al., 1997) and activating tissue transglutaminase, which cause cytoskeletal proteins to cross-link (Angulo P, 2002). Finally IL-8 is well known as a powerful chemoattractant molecule for neutrophils (Yoshimura T et al., 1987).

Several cooperating factors have been shown to trigger FasL expression at the hepatocyte level, which together led to an important increase in ROS concentration, by means of a sort of positive feedback. Two aspects need to be noticed: first, ROS-induced TNF-α inhibits action of respiratory chain at the mitochondrial level (Lancaster JR Jr et al., 1989), second, ROS reduce the antioxidant pool inside the hepatocyte, in this way causing more ROS to be produced (Sastre J et al., 1989). All these ROS has as a consequence the expression of FasL (Hug H et al., 1997), which can interact with Fas molecule present in the neighbouring hepatocytes, with the effect of a diffuse cell death.

Mitochondria seem to play a central role in the pathogenesis of NAFLD, if considered that in non-affected population and in the vast majority of patients with simple steatosis, this organelle show no significant abnormalities, but ultrastructural injuries, as linear crystalline inclusions inside megamitochondria, can be evidenced in patients with steatohepatitis (Sanyal AJ et al., 2001). Additionally, patients with steatohepatitis show alterations in liver ATP homeostasis, characterised by slowed rates of ATP synthesis, resulting in a severe ATP depletion (Cortez-Pinto H et al., 1999). As

explained in the previous paragraph this effect can be induced by an increased oxidative as well as inflammatory stress.

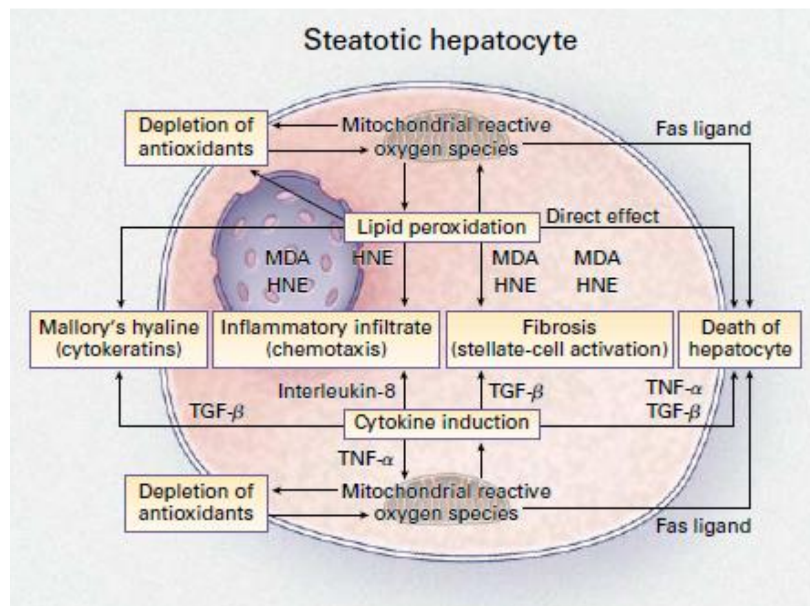


Figure 17 NAFLD: metabolic pathways in a steatotic hepatocyte (from Angulo P, 2002).

It is thus possible to state, according to Day CP and James OFW (Day CP, James OFW, 1998), that NAFLD pathogenesis can be described as a two-steps or "two-hits" process, the first being represented by onset of insulin resistance, which induces fat overload at the hepatic level. Secondarily, the oxidative stress generated by excess of lipids is able to trigger a series of linked events as lipid peroxidation, cytokine and FasL induction, which together can cooperate to induce steatohepatitis and finally fibrosis.

3.3 Insights on Insulin Resistance (IR)

Diabetes Mellitus (DM) can certainly be considered one of the principal metabolic diseases in the modern age. It has been estimated that actually more that 140 million people are affected worldwide and that this number is expected to double by 2025 (Zimmet P et al., 2001). As an outstanding example, it is useful to consider that in USA, some estimates report a probable number of 16 million affected subjects, with about 54000 deaths/year due to diabetes-related causes (Narayan KM et al., 2003).

Actually scientists and physicians agree on the assumption that DM can be better described classifying it in two major categories: Type I DM, in which pancreatic β-cells are destroyed due to an autoimmune reaction, and Type II DM, which is thought to be provoked by a peripheral resistance to insulin action, together with an insufficient secretion by pancreatic β-cells, the so-called "relative insulin deficiency". From an epidemiological point of view, Type I DM accounts for approximately 10% of all patients, whereas the remaining part should be included in Type II DM. A small number of cases, about 5%, is due to other causes, as for example, some monogenic forms such as Maturity-Onset Diabetes of the Young (MODY), Mitochondrial Diabetes, or several forms provoked by insulin gene or insulin receptor mutations. In addition other possible causes should be mentioned, such as exocrine pancreas defects, some endocrinopathies, such as Cushing syndrome, infections with cytomegalovirus and Coxsackie virus B, and several drugs (Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002).

3.3.1 Insulin action: normal and pathological

In normal, non-affected people, insulin plays an essential role in the process of maintenance and regulation of glucose homeostasis, together with its companion hormone, glucagon, through the well-known mechanism.

At the level of β cells of the pancreatic islets, insulin is synthesized as preproinsulin in the rough endoplasmic reticulum (RER), and directed to Golgi apparatus, where the mature insulin is produced and stored in secretory granules, together with C-peptide. When the haematic levels of glucose rise as a consequence of a meal, pancreatic β cells uptake glucose itself through GLUT-2 transporter. Glucose catabolism inside the cells leads to increased production of ATP that results in higher values of the cytoplasmic ATP/ADP ratio, which in turn are able to inhibit the ATP-sensitive K^{+}-channel present on plasma membrane, with depolarisation and Ca^{2+} influx through voltage-dependent channels. Higher $[Ca^{2+}]$ induces insulin release from β cells granules, the so-called immediate release of insulin. When the stimulus continues to be present, the β cell begins to actively synthesise insulin, and this kind of response is usually prolonged in time.

As mentioned before, insulin is fundamentally a hormone that exerts anabolic functions, being responsible for augmenting the uptake of glucose at the level of adipose tissue, striated and myocardial muscle, and liver.

Figure 18 summarises the physiological effects of insulin in the three aforementioned organs, in a normal condition, in fed and fasting states, and when Type II DM is present. When the system works properly, insulin secretion is stimulated after a carbohydrate (CHO in the figure) intake, as discussed before. At the level of skeletal muscle, glucose uptake is increased as well as glycogen synthesis, similarly to what happens in the liver, where glycogen synthesis and *de novo* lipogenesis are stimulated, whereas gluconeogenesis is inhibited. Lipogenesis is stimulated in white adipose tissue (WAT) as well, together with a down regulation of lipolysis. The primary event leading to insulin resistance and Type II DM development seems to be the ectopic intramyocellular lipid (IMCL) accumulation, which results in a blockage of glucose

uptake and glycogen synthesis. Secondarily, the excess of glucose present in the bloodstream after a meal is then directed to the liver, where contributes to increase intrahepatic triglycerides content, promoting a steatotic condition. Moreover, in the fasted state lipolysis is largely stimulated in WAT, and the surplus of circulating fatty acids is re-esterified in the liver, further contributing to augment steatosis (Samuel VT, Shulman GI, 2012).

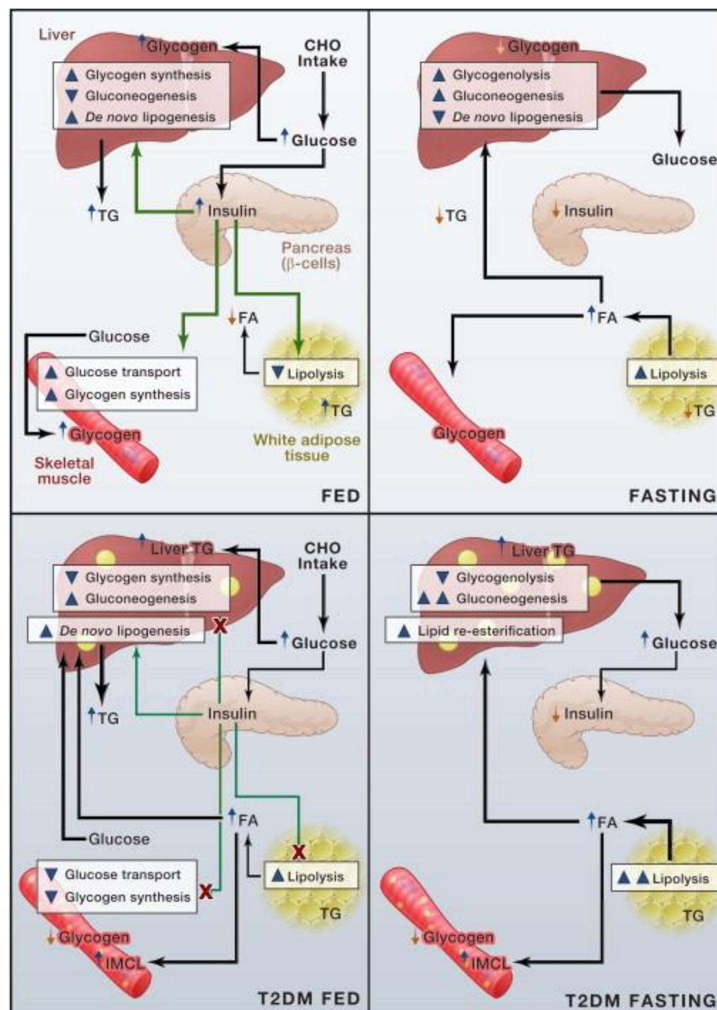


Figure 18 IR: schematic representation of insulin effect in normal and T2DM conditions (from Samuel VT, Shulman GI, 2012).

3.3.2 Insulin resistance

Onset of insulin resistance is certainly one of the most intriguing and challenging topics to deal with in cellular and molecular biology, considering that many cellular processes involved have been identified until now, but that many others remain obscure so far. At least two aspects seem to play a central role in contributing to insulin resistance development: ectopic lipid accumulation at skeletal muscle and liver level, as mentioned before, and the Unfolded Protein Response (UPR) or endoplasmic reticulum stress, whereas not completely clarified is which part inflammation has in the overall process.

For what concerns lipid accumulation, it is now completely defined that in both skeletal muscle and liver, this is the first step that is able to trigger insulin resistance. The mechanism proposed is quite similar in both tissues, as depicted in the next two pictures, representing skeletal muscle and liver respectively (Samuel VT et al., 2010).

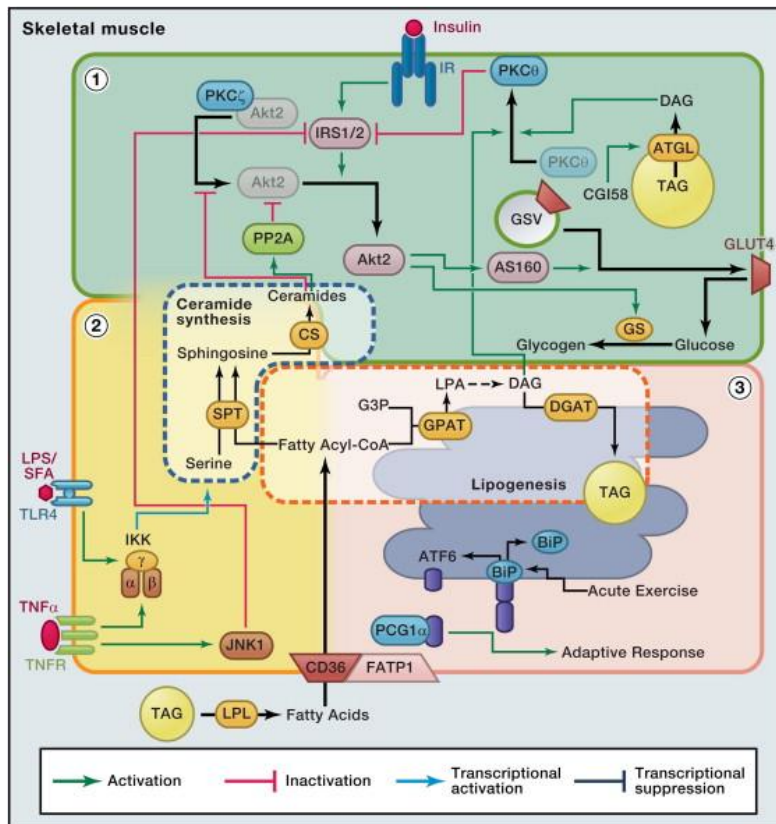


Figure 19 IR: schematic representation of signaling and metabolic pathways involved in onset of IR in skeletal muscle (from Samuel VT, Shulman GI, 2012).

In skeletal muscle, insulin binding to its own receptor (IR) present in the plasma membrane leads to activation of its tyrosine kinase activity, which result in phosphorylation of Insulin Receptor Substrate proteins 1 and 2 (IRS1/2). This event in turn is able to activate Akt2 by several steps that will be analysed deeper in the next paragraph. Activated Akt2 determines the translocation of GLUT4-containing storage vesicles (GSVs) to the plasma membrane and activation of glycogen synthase (GS). Several distinct but as well as overlapping biochemical deregulations can perturb this pathway. First of all, it has been shown that lipid accumulation is able to induce an increase in diacylglycerol (DAG) content, which is a well-known activator of protein kinase C (PKC) family that belongs to AGC super family of serine/threonine kinase. Indeed, now PKC family has been sub classified in three distinct groups: conventional

PKCs (cPKC: α, βI, βII, γ) that need both DAG and Ca^{2+} to be activated, novel PKCs (nPKC: δ, ε, η, θ) for which only DAG is required, and atypical PKCs (aPKC: ζ, λ) that are not dependent on DAG and/or Ca^{2+}. It has been demonstrated that in skeletal muscle raised levels of DAG lead to PKCθ activation, which in turn phosphorylates IRS-1 on Ser^{1101}, resulting in an inhibition of insulin signaling due to an impairment of the physiological Tyr phosphorylation (Li Y et al., 2004). Interestingly, a role in insulin resistance onset has been proposed for ceramides, which would be able to inhibit Akt2 activation by means of two different mechanisms. First, by activating protein phosphatase 2A (PPA2), which directly dephosphorylate Akt2, second by blocking the dissociation of the complex PKCζ•Akt2, in this way impairing Akt2 functionality (Teruel T et al., 2001; Powell DJ et al., 2003). A part in this movie has been suggested for inflammatory response as well, with particular attention to TNFα signaling. In fact, it has been shown that in obese subjects adipose tissue macrophages [ATMs] release TNFα, which in turn, through jun-N-terminal kinase 1 (JNK1) activation may block insulin signaling by phosphorylation of IRS1 at Ser^{307} residue (Hotamisligil GS et al., 1996; Aguirre V et al., 2002). For what concerns the function of UPR in the onset of insulin resistance, it has been assessed that an activation of this pathway may lead to an impairment of insulin signaling, even if a clear association between these two aspects has not been found yet. In mice it has been observed that a high-fat diet is able to induce the UPR, but the same has not been found in humans.

Interestingly, a possible involvement of UPR in the response of skeletal muscle to exercise has recently been reported, through activation of Peroxisome proliferator Gamma Coactivator 1α (PGC-1α) and ATF6α, but how this process could be linked to insulin sensitivity remains unclear (Samuel VT, Shulman GI, 2012).

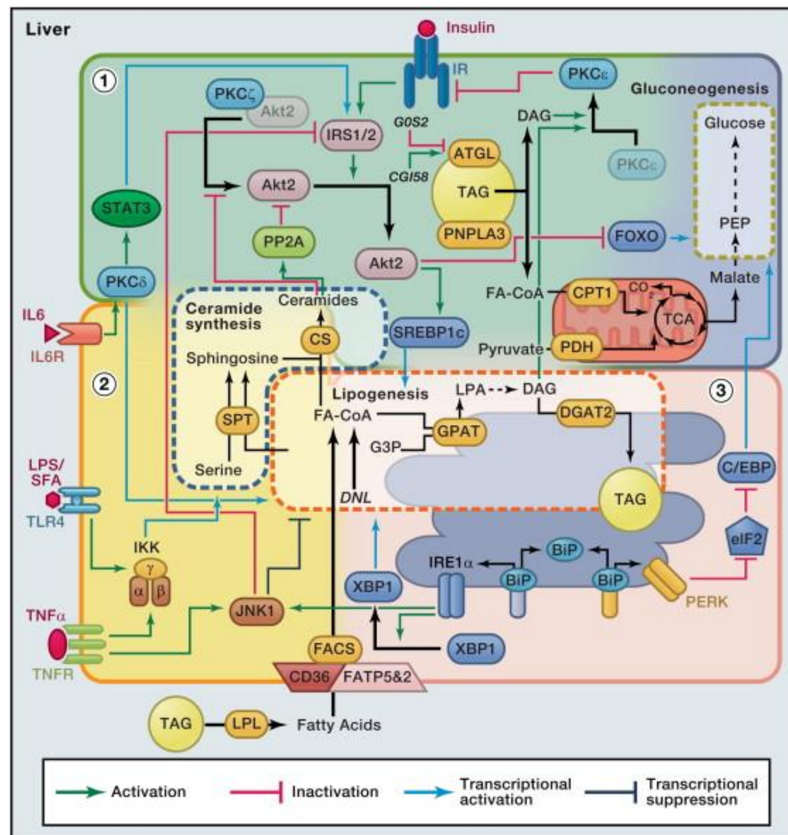


Figure 20 IR: schematic representation of signaling and metabolic pathways involved in onset of IR in the liver (from Samuel VT, Shulman GI, 2012).

Many aspects just described for skeletal muscle are quite similar in the liver as well, even if some differences exist. First of all, different PKCs, through slight dissimilar mechanisms, have been found to contribute to the development of insulin resistance, with respect to skeletal muscle. PKCε is usually found strictly associated with the IR in rats, and an activation of this kinase due to increased levels of DAG is supposed to lead to an impairment of IR activity. PKCδ has been reported as well to have a role in rat liver, and evidences collected until now permit to conclude that probably this kinase may be activated in two distinct ways: the first one classically by DAG, the second one being able to respond to the proinflammatory milieu that can arise as a consequence of a continuative hyperlipidemia, probably through IL6R or IKK-β. Similarly to what described in the skeletal muscle, ceramides induces the activation of

PP2A, which in turn dephosphorylates Akt2, impairing its functionality. Moreover ceramides are able to block the dissociation of the complex PKCζ•Akt2 that normally occurs after insulin stimulation, again impeding Akt2 effects (Teruel T et al., 2001; Powell DJ et al., 2003).

About the role played by UPR, most aspects resemble those observed in skeletal muscle, with only minor differences. On this regard, it is interesting to underline the role of transcription factor XBP1 (X-box Binding Protein 1), which is thought to exert a deleterious part in the onset of insulin resistance, increasing levels of intracellular DAG in this way stimulating PKCε activation (Jurczak MJ et al., 2012).

3.4 mTOR: Structure and Functions

The presence of efficient systems able to regulate and organise all the complex pathways involved in metabolic processes is indeed essential for every living organism. At the apex of the evolutionary tree, Mammals have developed several devices to cope with this matter, and one of most intensively studied in the last years as well as in the present days, is certainly the pathway activated by the protein kinase mammalian target of rapamycin or mTOR. So far several pathological conditions in humans have been shown to be induced by the non proper functioning of mTOR, including tumours, neurodegenerative processes, obesity and type II diabetes. For this reasons, many research groups worldwide are actually studying this topic, trying to find pharmacological approaches useful from a therapeutic point of view (Samuel VT, Shulman GI, 2012).

3.4.1 Structure

mTOR was originally discovered due to its inhibition operated by rapamycin, also named sirolimus, a macrolide synthesised by *Streptomyces Hygroscopicus* bacteria

that owns a typical antiproliferative action. Two studies published in 1993, for the first time identified two possible genes mediators of the cytotoxic effect of rapamycin in *S. cerevisiae*, named TOR1 and TOR2 (Cafferkey R et al., 1993; Kunz J et al., 1993). Biochemical approaches led rapidly to the characterisation of mTOR, of the 12-kDa FK506-binding protein (FKBP12) that is able to bind to rapamycin (Brown EJ et al., 1994; Sabatini DM et al., 1994; Sabers CJ et al., 1995) and also to the discover of the binding site for the complex rapamycin•FKBP12 (Chen J et al., 1995).

From a biochemical point of view, mTOR can be classified as an atypical Ser/Thr protein kinase, included in the broad family of phosphoinositide 3-kinase (PI3K)-related kinase. Usually it interacts with different proteins, which confer to mTOR peculiar characteristics that define its physiological functions. Depending on which proteins mTOR associates with, it forms two different complexes with different roles and sensitivity to rapamycin, termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). These two complexes are quite large, and so far six proteins have been discovered for mTORC1 and seven for mTORC2, in addition to the common catalytic subunit mTOR. Excluding mTOR, four components are shared by the two complexes, termed: mammalian lethal with sec-13 protein 8 (mLST8 or GβL), DEP domain containing mTOR-interacting protein (DEPTOR) and the Tti1/Tel2 complex. Specific proteins characterise the two assemblies, i.e. regulatory-associated protein of mTOR (raptor) and proline-rich Akt substrate 40 kDa (PRAS40) for mTORC1, and rapamycin-insensitive companion of mTOR (rictor), mammalian-stress activated map kinase-interacting protein (mSin1) and protein observed with rictor 1 and 2 (protor1/2) for mTORC2. The two aforementioned complexes greatly differ for the sensitivity to rapamycin, mTORC1 being strongly inhibited by acute treatment, differently to mTORC2, which seems to be targeted only by chronic exposures that

probably disassembly its structure. The biochemical and structural basis for this effect are until now not completely clear, even if it has been proposed a destabilising action on the complex as well as an allosteric inhibition of the kinase domain (Laplante M, Sabatini DM, 2012).

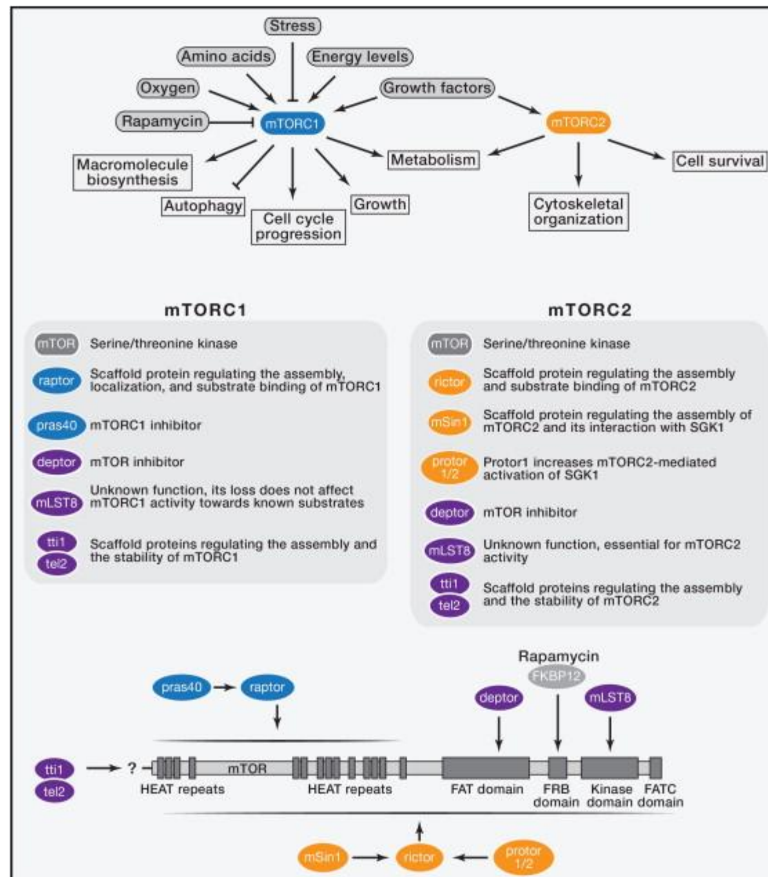


Figure 21 mTOR: schematic representation of mTORC1 and mTORC2 complexes (from Laplante M, Sabatini DM, 2012).

3.4.2 mTORC1 & mTORC2: upstream

From what depicted in Figure 21, it is clearly understandable that at the present time there is more information about mTORC1 with respect mTORC2, even though now many efforts are concentrated on mTORC2.

Several factors connected to energetic status of the cell have been shown to be responsible for the activation of mTORC1, i.e. oxygen, amino acids, energy levels as

well as growth factors, having as outcome the control of some of the most important cellular processes, as macromolecule biosynthesis, for instance lipid and proteins, cell cycle progression, autophagy, cell growth and metabolism.

For what concerns mTORC2, it is known that it is activated by growth factors, and that it regulates cell survival, metabolism and cytoskeletal organisation.

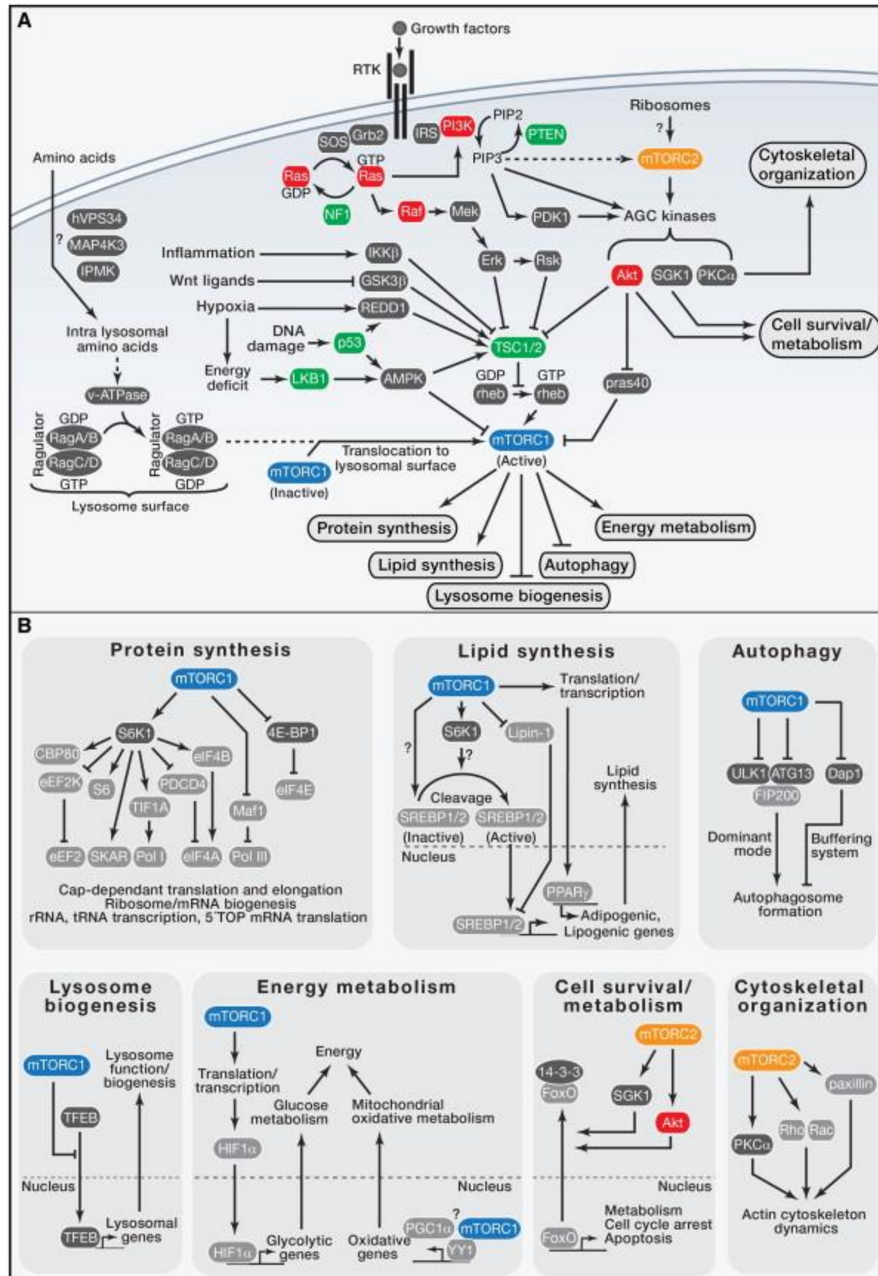


Figure 22 mTOR: schematic representation of signaling and metabolic pathways regulated by mTORC1 and mTORC2 complexes (from Laplante M, Sabatini DM, 2012).

Figure 22 summarises the state of art of what is known about mTORC1- and mTORC2-activated signaling pathways and the principal processes regulated by these two complexes.

A central role in the regulation of mTORC1 activity is undoubtedly played by TSC1/2 complex, an heterodimer of two distinct subunits, termed TSC1 (Tuberous SClerosis 1 or hamartin) and TSC2 (Tuberous SClerosis 2 or tuberin). TSC1/2 owns a GTPase-activating protein (GAP) activity, and it is able to inactivate Rheb (Ras homolog enriched in brain) GTPase favouring the formation of the GDP-bound form, in this way permitting a negative regulation of the mTORC1 activation. On TSC1/2 converge almost all the external stimuli that activate or inhibit activity of mTORC1, with the important exception of amino acids, which seem to act in a TSC1/2-independent manner. Among the best characterised pathways that can induce mTORC1 regulation through TSC1/2 at least five should be mentioned: growth factors, proinflammatory cytokines, Wnt pathway, together with hypoxia and DNA damage. Growth factors, insulin and insulin-like growth factor 1 (IGF1) being paradigmatic, classically activate Ras and PI3K signaling, resulting in the activation of extracellular-signal-regulated kinase 1/2 (ERK1/2), protein kinase B (Akt/PKB), and ribosomal S6 kinase (RSK1), which inactivate the TSC1/2 complex by phosphorylation, inducing as a consequence mTORC1 activation (Inoki K et al., 2002; Ma L et al., 2005). Interestingly Akt has been shown to activate mTORC1 directly, phosphorylating and inducing the dissociation of the PRAS40 inhibitory subunit from the complex (Vander Haar E et al., 2007). Proinflammatory cytokines, as for example TNFα, similarly stimulate mTORC1 by inactivation of TSC1/2, mediated by IKKβ phosphorylation of TSC1 (Lee DF et al., 2007). Wnt pathway, on the other hand, operates through inhibition of glycogen synthase kinase 3β (GSK3- β), which usually phosphorylates TSC2,

promoting the activity of the complex (Inoki K et al., 2006). As mentioned above, cellular stresses as hypoxia and DNA damage can affect mTORC1 functionality. Hypoxia and energy deficit inside the cell are sensed by adenosine monophosphate-activated protein kinase (AMPK), which in turn is capable of phosphorylating TSC2, inhibiting mTORC1, or similarly to Akt, can directly phosphorylate raptor subunit, inducing an allosteric inhibition of mTORC1. An emerging role in this picture is being assessed for transcriptional regulation of DNA damage response 1 (REDD1), which has been demonstrated to be able to stimulate TSC2 after activation by hypoxia, as well as by p53. p53 is also essential in the response to DNA damage, considering that a p53-dependent transcription mechanism induces the expression of Tsc2 and phosphatase and tensin homolog deleted on chromosome 10 (Pten) genes, with the resulting overall down regulation of the signaling pathway (Feng Z et al., 2005).

For what concerns the mechanism by which amino acids are able to activate mTORC1, several progresses have been made, even if the overall process is still very far to be completely clarified. At present it seems clear that all amino acids, but especially leucine and arginine, are necessary to activate mTORC1, even in presence of other stimuli, as growth factors, and that the mechanism is TSC1/2-independent. Recently a model has been proposed that involves Rag GTPases as the principal mediators of regulation of mTORC1 activity by amino acids. In Mammals exists four types of Rag proteins, RagA to RagD, which associate obligatory to form RagA/B and RagC/D heterodimers, with opposite nucleotide binding properties, that means that when one heterodimer is bound to GTP the other one is bound to GDP. In this context, amino acids stimulate RagA/B binding to GTP, permitting the interaction with raptor that determines the translocation of mTORC1 to the lysosomal surface, where Rag GTPase interacts with a multisubunit complex called Ragulator, necessary for mTORC1

activation (Sancak Y et al., 2008; Sancak Y et al., 2010). Once on the lysosomal surface, mTORC1 is probably activated thanks to the action of Rheb GTPase, even though more investigations need to verify this hypothesis (Laplante M, Sabatini DM, 2012). Interestingly, a recent paper suggests that amino acids, by accumulating inside the lysosomal lumen should be able to activate vacuolar H^+-adenosine triphosphate ATPase (v-ATPase), which in an unknown way would activate Ragulator and Rag GTPase (Zoncu R et al., 2011).

Less is known about mTORC2 activation if compared to mTORC1, even if some recent papers are shedding some light on this topic. First of all, about the effect of rapamycin, mTORC2 differently to mTORC1 is not affected by an acute treatment (Jacinto E et al., 2004), even if the situation is probably more complex, considering that it has been shown that in many cell types a prolonged treatment with rapamycin can interfere in an unknown way with mTORC2 assembly (Sarbassov DD et al., 2006). For what concerns mTORC2 activation, the mechanism remains at the present day quite obscure, being insensitive to stimulation by nutrients but responsive to growth factors such as insulin. A possible way of activation has been recently proposed, which recognises a direct role of ribosomes that seem to be able to directly activate mTORC2, after that insulin, through a PI3K signaling, promotes mTORC2 association with ribosomes themselves (Zinzalla V et al., 2011).

3.4.3 mTORC1 & mTORC2: downstream

Several essential cellular processes have been shown to be under the direct control of mTORC1, such as protein and lipid synthesis, energy metabolism, autophagy and lysosome biogenesis.

According to what has been discovered so far, protein synthesis is regulated by mTORC1 essentially by phosphorylation of two key proteins, i.e. the S6 kinase 1

(S6K1) and the translational regulators eukaryotic translational initiation factor 4E (elF4E)-binding protein 1 (4E-BP1). Phosphorylation of S6K1 leads to its activation, which finally results thanks to a series of effectors, in an increase of mRNA biogenesis, and translational initiation and elongation. Differently phosphorylation of 4E-BP1 has the result to block its binding to the cap-binding protein elF4E, favouring on the other that to elF4F, inducing in this way the formation of the complex necessary for the initiation of cap-dependent translation. Two additional factors play a role in this process: the regulatory element tripartite motif-containing protein-24 (TIF-1A), which upon activation by mTORC1 interacts with RNA Polymerase I (Pol I), stimulating ribosomal RNA (rRNA) transcription, and the Pol III repressor Maf1, which is inhibited by phosphorylation, allowing the transcription of 5S rRNA and transfer RNA (tRNA) (Ma XM, Blenis J, 2009; Kantidakis T et al., 2010).

The main way by which mTORC1 controls lipid biosynthesis is by inducing the expression and increasing the activity of PPARγ, the well-known central regulator of lipogenic genes. Importantly, several recent papers have pointed out that mTORC1 can regulate sterol regulatory element-binding protein 1/2 (SREBP1/2) activity, positively affecting its functions in an until now not well established way, even if a role for Lipin-1 has recently been proposed (Laplante M, Sabatini DM, 2009; Peterson TR et al., 2011).

For what concern energetic metabolism, is now well established that mTORC1 is able to positively affect transcription and translation of hypoxia inducible factor 1α (HIF1α), which positively regulates many genes involved in the glycolytic pathway. Several evidences demonstrate a positive role played by mTORC1 on oxidative metabolism as well, being able to augment mitochondrial DNA content and the expression of the genes of oxidative metabolism, presumably by favouring the

association inside the nucleus of PPARγ Coactivator 1α (PGC1α) and the transcription factor Ying-Yang 1 (YY1) (Düvel K et al., 2010).

Anabolic processes and cell growth are also stimulated by mTORC1 indirectly, through a negative regulation of autophagy and lysosome biogenesis. The first task is achieved in Mammals by means of the direct phosphorylation of the kinase complex ULK/Atg13/FIP200 (unc-51-like kinase 1/mammalian autophagy-related gene 13/focal adhesion kinase family-interacting protein of 200 kDa), whose activity is requested to start the autophagy process (Hosokawa N et al., 2009), and through the inhibition of the suppressor of autophagy DAP1(death-associated protein 1) (Koren I et al., 2010). Likewise mTORC1 inhibits lysosome biogenesis by phosphorylating the transcription factor EB (TFEB), in this way blocking its entry inside the nucleus and impeding the transcription of many genes that play essential roles for the proper functioning of these organelles (Settembre C et al., 2012).

Two main pathways downstream mTORC2 have been identified to date, both ones involving chains of phosphorylation events, but leading to different outcomes. The most characterised pathways are those involving Akt and serum- and glucocorticoid-induced protein kinase 1 (SGK1). mTORC2 directly phosphorylate Akt on the critical Ser473 residue, resulting in its full activation (Sarbassov DD et al., 2005), which affects several essential cellular processes, such as metabolism, growth and proliferation, survival and apoptosis, at level of many tissues, as will explained in greater detail in the next paragraph. Anyhow it is possible to say that when mTORC2 is defective, the consequent lack of Akt functionality results in impaired phosphorylation of several key Akt targets, such as forkhead box O1/3A (FoxO1/3A). Another important kinase under the direct influence of mTORC2 is SGK1, which modulates cell growth and ion transport, and that is able as well to phosphorylate

FoxO1/3A on the same residues phosphorylated by Akt, thus introducing an interesting double check on FoxO1/3A activity (Garcìa-Martinez JM, Alessi DR, 2008). Finally it is important to underline the role played by mTORC2 in the activation of PKC-α that together with paxillin and Rho and Rac GTPases plays a relevant role in the actin cytoskeleton organisation and dynamics (Jacinto E et al., 2004).

3.4.4 mTORC1 & mTORC2: metabolic functions and tissue-specific roles

Both mTORC1 and mTORC2 complexes have been shown to exert fundamental functions in the regulation of key metabolic processes, in at least four organs, i.e. white adipose tissue (WAT), muscle, pancreas (β-cells) and liver. Moreover, recent researches have assessed a new, important role in the regulation of energy balance at the hypothalamus level (Laplante M, Sabatini DM, 2012).

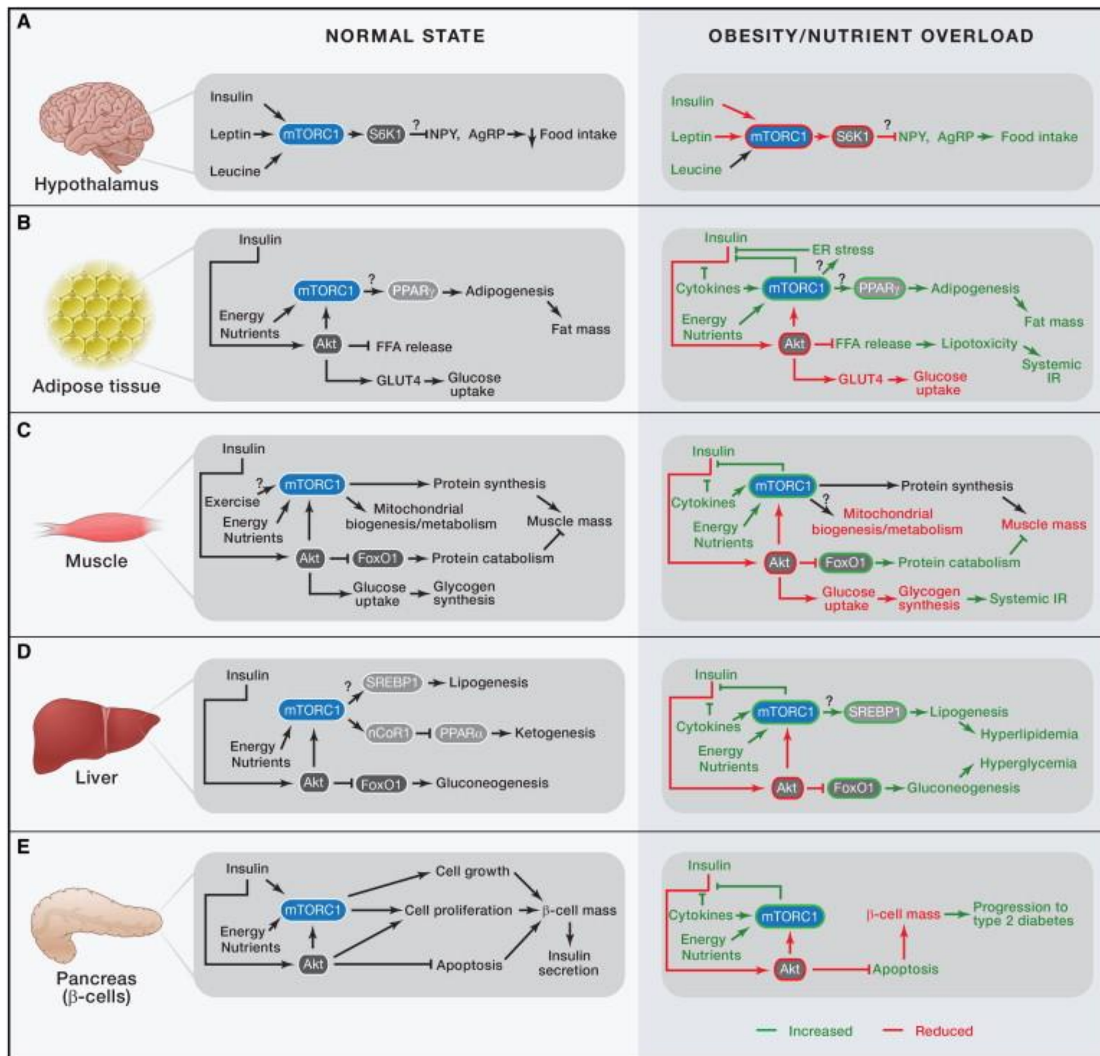


Figure 23 mTOR: schematic representation of signaling and metabolic pathways regulated by mTORC1 and mTORC2 complexes in hypothalamus, adipose tissue, muscle, liver and pancreas (from Laplante M, Sabatini DM, 2012).

The hypothalamus has been subjected to extensive research efforts, in the attempt to find potential pathways involved in the onset of obesity and metabolic unbalances, considering that this area of the brain is of pivotal importance in the processes of integration of signals brought by nutrient molecules present in the bloodstream, such as lipids, amino acids and proteins, and by hormones such as insulin and leptin (Schwartz MW, Porte Jr D, 2005). One specific area of hypothalamus, the arcuate nucleus (ARC), has been shown to be particularly important in this context, and to

express mTORC1, whose activation induced by leucine or leptin is able to reduce food intake, with a mechanism that probably through S6K1 activation, leads to a reduced expression of neuropeptide Y (NPY) and agouti-related peptide (AgRP). In pathogenic conditions, such as nutrients overload and obesity, mTORC1 is no more activated by leptin, inducing a condition of hyperphagia, opening the mind to speculations about the possible existence of genetic predispositions eventually responsible for an altered control of food intake (Cota D, 2009).

At WAT level, mTORC1 plays a key role in the regulation of adipogenesis, as demonstrated by the observation that when inactivated, this process is completely blocked, and when overactive adipogenesis is strongly activated (Laplante M, Sabatini DM, 2009). Two proteins under mTORC1 control are central in the development of adipose tissue, S6K1 that direct the step from embryonic stem cells to adipogenic progenitors through the regulation of early adipogenic transcription factors, and 4E-BPs under whose translational control is PPARγ, which determines the terminal differentiation process (Carnevalli LS et al., 2010). The obesity condition is usually associated with high circulating levels of nutrients, insulin and proinflammatory cytokines, which are able to stimulate mTORC1 activity, with the final result of further increasing adipose mass, and also contributing to insulin resistance onset, through an S6K1-mediated block of insulin signaling (Um SH et al., 2004). An increased activation of mTORC1 can also result in a high rate of protein synthesis that can lead to an augmented ER stress and UPR, processes known to be able to trigger or exacerbate an insulin resistance condition (Ozcan U et al., 2008; Hotamisligil GS, 2010). Interestingly, mTORC2 as well seems to give a positive contribution to the correct metabolic homeostasis of WAT and to maintain the proper lipid profile, as demonstrated by the fact that mTORC2 adipose-specific ablation results in a regular

adipose tissue mass, but the lack of Akt activity leads to an increased lipolysis and to a consequent augment of free fatty acids (FFA) in the bloodstream (Kumar A et al., 2010).

For what concerns muscle, an interesting recent topic is that concerning the relationship between exercise and thus mechanical contraction, and mTORC1. In fact, in an unknown way, these kind of mechanical stimuli result in mTORC1 activation, with a consequent increase of protein synthesis, which finally results in muscle hypertrophy (Philp A et al., 2011). It has been also shown that mTORC1 is involved in the increase of oxidative metabolism by inducing the expression of PGC1-α, and that the normal feedback loop that regulate IRS1 activity is strongly reduced in the absence of mTORC1, which results in an enhanced Akt activation and glycogen accumulation. Similarly, elevated levels of mTORC1 activation, induced by high fat feeding and obesity, are able to inhibit insulin signaling in a S6K1-dependent manner, with the final result of a reduced glucose uptake, contributing to systemic insulin resistance (Um SH et al., 2004). Paradoxically, when mTORC1 is activated as a consequence of obesity the effect on muscle mass is deleterious, and a reduction is observed, probably mediated by an increased expression of ubiquitin ligases by FoxO1 (Wang X et al., 2006). Moreover, high-fat diet, obesity and type 2 diabetes, conditions that usually results in high levels of mTORC1 activation, have as a typical outcome an impaired mitochondrial biogenesis and functionality (Sparks LM et al., 2005). A possible explanation for this apparent contradiction is that probably other signaling pathways, different from those regulated by mTORC1 control these processes (Laplante M, Sabatini DM, 2012). mTORC2 seems to have no effect on muscle mass, but an inhibition results in a diminution of glucose uptake and therefore in a mild systemic glucose intolerance (Kumar A et al., 2008).

Both mTORC1 and mTORC2 play critical roles in maintaining the proper functionality of pancreatic β-cells, in this way being essential to regulate insulin secretion and hence guaranteeing all metabolic profile. For what concerns mTORC1, it has been assessed that when constitutively active it is able to increase β-cells size and number and to decrease concentration of blood glucose and insulin, resulting in an improved glucose tolerance, probably through an S6K1-mediated mechanism. Interestingly mTORC1 is involved as well in the so called β-cells compensation process, usually observed in conditions of high-fat diet or obesity. In such pathological states, nutrient excess and peripheral insulin resistance represent stimuli to pancreas to produce more insulin, which results in a raised formation of new β-cells from progenitors, together with proliferation and hypertrophy of pre-existing ones, with the final consequence being represented by an elevation of insulin synthesis and release. This adaptation on long time spans has anyway deleterious consequences, from two different point of view: first, β-cells cannot sustain such a proliferative and productive stress consequently resulting in the development of type 2 diabetes, and second a prolonged activation of mTORC1/S6K1 signaling leads to a feedback inhibition of IRS1 and IRS2, and hence to a decline of insulin responsiveness in islets, which has also been observed to be associated to decrease in cell vitality and increased apoptosis (Shigeyama Y et al., 2008). Recently mTORC2 has been found to have a role in this story, as shown in rictor null mice that exhibit reduction in β-cell mass, β-cell proliferation, pancreatic insulin content, and glucose-stimulated insulin secretion, which result in mild hyperglycaemia and glucose intolerance. These changes have been associated to a decrease in Akt phosphorylation at Ser473 and increased abundance of FoxO1 (Gu Y et al., 2011).

In the liver several key metabolic pathways are controlled by mTORC1 ad mTORC2, being of essential importance in the normal homeostasis of this organ, but as well involved in the onset of several hepatic pathological condition, such as for example nonalcoholic fatty liver disease (NAFLD). As described above for muscle and adipose tissue, in a normal physiological state mTORC1 activation stimulates lipogenesis through a SREBP1c-mediated mechanism in a still unknown way, and inhibits ketogenesis by inducing nuclear accumulation of nuclear receptor corepressor 1 (NCoR1), which in turn blocks the activity of PPAR-α (Yecies JL et al., 2011; Sengupta S et al., 2010). On the other hand, in a condition of nutrient overload and obesity, mTORC1/S6K1 activity is increased, which results in an inhibition of IRS1 activity with the consequent development of insulin resistance (Tremblay F et al., 2007). Interestingly, the high rates of activation of mTORC1 due the elevated levels of proinflammatory cytokines and nutrients typically found in insulin resistant conditions could explain why lipogenesis remains very active aggravating further the pathological profile (Peterson TR et al., 2011). For what concerns the role played by mTORC2, several evidences suggest that a blockage of PI3K/Akt signaling results in an increased gluconeogenesis, contributing to hyperglycaemia and to onset of hyperinsulinemia. In addition, a recent paper shed some light on these topics recognising the essential role of mTORC2 in driving lipogenesis independently to Akt2 activation, whereas gluconeogenesis seems to be mTORC2–independent (Yuan M et al., 2012).

3.5.1 Role of Fructose in NAFLD

In the last years many researchers and clinicians focused their attention on the role played by fructose in metabolic diseases, such as obesity, type 2 diabetes, metabolic syndrome, as well as in liver specific pathologies, one of the most extensively studied being certainly Non Alcoholic Fatty Liver Disease (NAFLD). This huge debate about the role of fructose probably raised as a consequence of the recent increased diffusion of sweetened foods and beverages, such as cereal and chocolate bars or drinks such as regular sodas, energy and sport drinks. These aliments are typically produced using High Fructose Corn Syrup (HFCS), which is a sweetener obtained by corn starch to yield glucose, and then by processing glucose to have a syrup containing variable percentage of fructose, the most common used being the so called HFCS55, that means 55% fructose-45% glucose, about the ratio found in sucrose (White JS, 2008). From a strictly biochemical point of view, fructose is theoretically capable of having some adverse effect, especially for what concerns triglyceride levels, considering its peculiar hepatic metabolism, which is prone to induce the formation of VLDL-triglyceride (Henry RR, Crapo PA, 1991). However, no evidences have been collected so far showing that fructose could have harmful effects in healthy subjects consuming an isocaloric, balanced diet, which also include fructose. Data obtained from epidemiological studies seem to assess a role of fructose-containing beverages in increasing body weight and obesity, but considering that these beverages are often associated with other sort of junk, high-fat foods, such as processed meats and fried potatoes, in a context of an elevated total energy intake, scarce consumption of fresh vegetables and fruits and physical activity, it is difficult to determine the real contribution fructose in these observations (Mozaffarian D et al., 2011). Indeed, another recent study demonstrated that fructose was able to induce an increase in body

weight only when associated with a hypercaloric diet, not when in the context of an isocaloric, balanced diet (Sievenpiper JL et al., 2012 [b]). This perspective is further confirmed by a paper that collects many evidences showing no effects of fructose on body weight and plasma triglycerides levels when consumed in amounts typically associated with a normal isocaloric Western diet, in both healthy, normal weight individuals as well as in overweight, obese people (Dolan LC et al., 2010 [a]; Dolan LC et al., 2010 [b]). Several clinical studies seem to confirm these epidemiological observations, revealing that fructose can be considered seriously harmful only when consumed at very high doses, and particularly in overweight or obese subjects. In particular, it has been reported that fructose overconsumption was able to alter lipid metabolism, inducing dyslipidemia and increase in blood glucose, without affecting insulin sensitivity, in the case of a moderate overfeeding (Lê KA et al., 2006). Employing higher fructose concentrations for a longer period coupled to a hypercaloric diet, slightly different but still coherent results were obtained, with a moderate augment of circulating triglycerides and ectopic fat deposition, together with a decrease in hepatic insulin sensitivity. These values were significantly higher in healthy offspring of patients with type 2 diabetes, a subgroup of individuals prone to develop metabolic disorders (Lê KA et al., 2009). Moreover, when compared to glucose overfeeding in a context of a hypercaloric diet in healthy males, fructose overfeeding resulted in comparable or lighter effects in all the metabolic parameters taken in account (Ngo Sock ET et al., 2010). Shifting attention to the liver, it seems likely that the augmented hepatic fat deposition observed during fructose overload trials might be explainable admitting a stimulation of de-novo lipogenesis, together with an inhibition of lipid oxidation (Lê KA et al., 2009). Interestingly, in rodent studies it has been shown that fructose was responsible for an increased hepatic

inflammation, in this way favouring the transition from a steatotic condition to steatohepatitis. In fact, in mice it has been demonstrated that a high-fructose diet was able to induce a TNFα-mediated steatosis and insulin resistance, together with an increased neutrophil infiltration, suggesting that fructose may be involved in the onset of steatohepatitis as a proinflammatory agent (Kanuri G et al., 2011). Similarly it has been shown that a high fat coupled to a high fructose diet determined an increased hepatic ROS production and a NASH-like phenotype with significant fibrosis (Abdelmalek MF et al., 2010). The mechanism of fibrosis may involve an increased hepatic oxidative stress associated with hepatic macrophage aggregation, which in turn induces a TGFβ1-signaled collagen deposition and histologically evident hepatic fibrosis (Kohli R et al., 2010). Hence it seems probable that fructose, when consumed in excess, might have a proinflammatory effect at the hepatocyte level that, if associated with the other typical risk factors such as calories surplus coupled with high prevalence of fats in the diet and scarce physical activity, may constitute an additional stimulus to progression to NASH. Interestingly, a recent study carried out in mice suggested that a chronic intake of high doses of fructose was able to induce a huge bacterial overgrowth at the intestine level, that together with an increased permeability, finally resulted in an endotoxin-dependent activation of hepatic Kupffer cells, which can contribute to the onset of hepatic insulin resistance through a TNFα-mediated mechanism (Spruss A et al., 2009).

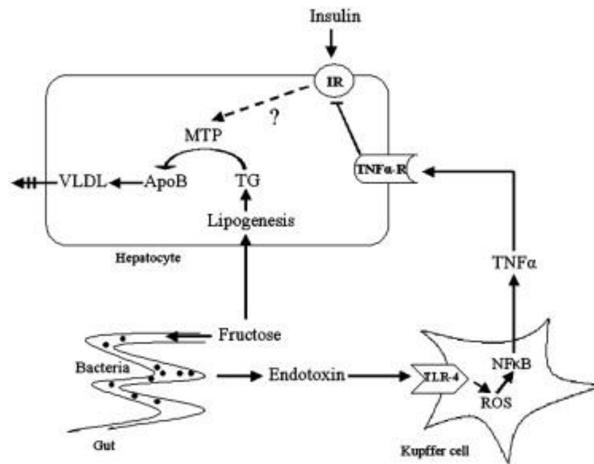


Figure 24 Fructose & NAFLD: schematic representation of the possible role played by fructose in the induction of NAFLD (from Spruss A, Bergheim I, 2009).

Finally it is useful to underline that until now there are no convincing evidences supporting the idea that fructose could in some way weaken the control of food intake, inducing a hyperphagic feeding behaviour (Soenen S et al., 2007; Moran TH, 2009). Anyhow, a recent research pointed out that carbohydrates in liquid form, such as those found in sugar- or HFCS-sweetened beverages, produced less satiety if compared with solid carbohydrates, leading to an increase in total long-term energy intake (Pan A, Hu FB, 2011).

AIM OF THE WORK

Non-Alcoholic Fatty Liver Disease (NAFLD) is nowadays considered one of the most diffused pathologic conditions affecting the liver especially in western world, where recent reports suggest that about 15% to 20% of the population could be affected, with an increasing incidence of people at risk to develop this pathology (Anderson N, Borlak J, 2008). The frequency is much higher in obese population, for which epidemiological studies report percentages that ranges from 57.5% to 74%. NAFLD has been described in children as well, with 2.6% occurrence in non-obese, which rises to 22.5% in obese ones (Angulo P, 2002).

The pathological mechanisms that induce the development of NAFLD are so far not completely clarified and still matter of debate, even if some key factors have been identified. Several theories have been proposed to date, but now most researchers believe that the first step required for NAFLD to develop is onset of insulin resistance, according to what hypothesised by Day CP and James OFW in 1998. Insulin resistance, in turn, would lead to an increase in fat accumulation, and hence to a pathological steatosis, probably inducing modifications in the uptake, synthesis, oxidation or export of lipids (Angulo P, 2002).

Onset of insulin resistance is certainly one of the most intriguing and challenging topics to deal with in cellular and molecular biology, considering that many cellular processes involved have been identified until now, but that many others remain obscure so far. For what concerns the liver, the first step that is able to trigger insulin resistance is lipid accumulation due to a dietary fat overload.

In the liver, insulin binding to its own receptor (IR) present in the plasma membrane leads to activation of its tyrosine kinase activity that result in phosphorylation of

Insulin Receptor Substrate proteins 1 and 2 (IRS1/2), event which in turn is able to activate Akt2 by means of several steps. Activated Akt2 determines the activation of glycogen synthase (GS), suppression of gluconeogenesis, and promotion of de novo lipogenesis through SREBP1c activation. Several distinct as well as overlapping biochemical deregulations can perturb this pathway. First of all, it has been shown that lipid accumulation is able to induce an increase in diacylglycerol (DAG) content, which is a well-known activator of protein kinase C (PKC) family. In the liver raised levels of DAG lead to activation of PKCε, which is supposed to lead to impairment of IR activity, and of PKCδ that has been reported as well to play a role, probably by influencing lipid homeostasis. Interestingly PKCδ seems to be able to respond to the proinflammatory milieu that can arise as a consequence of a continuative hyperlipidemia, probably through IL6R or IKK-β. Ceramides as well are probably involved through the activation of PP2A, which in turn dephosphorylate Akt2, impairing its functionality. Moreover ceramides are able to block the dissociation of the complex PKCζ•Akt2 that normally occurs after insulin stimulation, again impeding Akt2 effects.

So far several pathological conditions have been shown to be induced by the non proper functioning of the mTOR-activated pathways, including tumours, neurodegenerative processes, obesity and type 2 diabetes. For this reasons, many research groups worldwide are actually studying this topic, trying to find pharmacological approaches useful from a therapeutic point of view.

From a biochemical point of view, mTOR can be classified as an atypical Ser/Thr protein kinase, included in the broad family of phosphoinositide 3-kinase (PI3K)-related kinase. Usually it interacts with different proteins, which confer to mTOR peculiar characteristics that define its physiological functions. Depending on which

proteins mTOR associates with, it forms two different complexes with different roles and sensitivity to rapamycin, termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Laplante M, Sabatini DM, 2012).

At the present time more data have been collected about mTORC1 with respect mTORC2, even though now many efforts are addressed on mTORC2.

Several factors connected to the energetic status of the cell have been shown to be responsible for the activation of mTORC1, i.e. oxygen, amino acids, energy levels as well as growth factors, having as an outcome the control of some of the most important cellular processes, such as macromolecule biosynthesis, for instance lipid and proteins, cell cycle progression, autophagy, lysosome biogenesis, cell growth and metabolism.

For what concerns mTORC2, it is known that it is activated by growth factors such as insulin, and that it regulates cell survival, metabolism and cytoskeletal organisation.

Two main pathways downstream mTORC2 have been identified to date, both ones involving chains of phosphorylation events, but leading to different outcomes. The most characterised pathways are those involving Akt and serum- and glucocorticoid-induced protein kinase 1 (SGK1). mTORC2 directly phosphorylates Akt on the critical Ser473 residue, resulting in its full activation (Sarbassov DD et al., 2005), which in turn affects several essential cellular processes, such as metabolism, growth and proliferation, survival and apoptosis, at the level of many tissues. It is possible to say that when mTORC2 is defective, the consequent lack of Akt functionality results in impaired phosphorylation of several key Akt targets, such as forkhead box O1/3A (FoxO1/3A).

In the liver several key metabolic pathways are controlled by mTORC1 ad mTORC2, being of essential importance in the normal homeostasis of this organ, but as well

involved in the onset of several hepatic pathological conditions, such as for example nonalcoholic fatty liver disease (NAFLD). In a normal physiological state mTORC1 activation stimulates lipogenesis through a SREBP1c-mediated mechanism in a still unknown way, and inhibits ketogenesis by inducing nuclear accumulation of nuclear receptor corepressor 1 (NCoR1), which in turn blocks the activity of PPAR-α (Yecies JL et al., 2011; Sengupta S et al., 2010). On the other hand, in a condition of nutrient overload and obesity, mTORC1/S6K1 activity is increased, which results in an inhibition of IRS1 activity with the consequent development of insulin resistance (Tremblay F et al., 2007). Interestingly, the high rates of activation of mTORC1 due to the elevated levels of proinflammatory cytokines and nutrients typically found in insulin resistant conditions could explain why lipogenesis remains very active aggravating further the pathological profile (Peterson TR et al., 2011). For what concerns the role played by mTORC2, several evidences suggest that a blockage of PI3K/Akt signaling results in an increased gluconeogenesis, contributing to hyperglycaemia and to onset of hyperinsulinemia. In addition, a very recent paper shed some light on these topics recognising the essential role of mTORC2 in driving lipogenesis independently of Akt2 activation, whereas gluconeogenesis seems to be mTORC2–independent (Yuan M et al., 2012).

In the last years many researchers and clinicians focused their attention on the role played by fructose in metabolic diseases, such as obesity, type 2 diabetes, metabolic syndrome, as well as in liver specific pathologies, the most extensively studied being certainly Non Alcoholic Fatty Liver Disease (NAFLD). This huge debate about the role of fructose probably rose as a consequence of the recent increased diffusion of sweetened foods and beverages, which are produced typically using High Fructose Corn Syrup (HFCS) (White JS, 2008).

Several clinical studies seem to agree with the observation that fructose can be considered seriously harmful when consumed at very high doses, and particularly in overweight or obese subjects. In particular, it has been reported that fructose overconsumption was able to alter lipid metabolism, inducing dyslipidemia and increase in blood glucose, without affecting insulin sensitivity, in the case of a moderate overfeeding (Lê KA et al., 2006). Employing higher fructose concentrations for a longer period coupled to an hypercaloric diet, slightly different but still coherent results were obtained, with a moderate augment of circulating triglycerides and ectopic fat deposition, together with a decrease in hepatic insulin sensitivity. These values were significantly higher in healthy offspring of patients with type 2 diabetes, a subgroup of individuals prone to develop metabolic disorders (Lê KA et al., 2009). Moreover, when compared to glucose overfeeding in the context of a hypercaloric diet in healthy males, fructose overfeeding results in comparable or lighter effects in all the metabolic parameters taken in account (Ngo Sock ET et al., 2010). Shifting attention to the liver, it seems likely that the augmented hepatic fat deposition observed during fructose overload trials might be explainable admitting a stimulation of de-novo lipogenesis, together with an inhibition of lipid oxidation (Lê KA et al., 2009). Nonetheless until now no evidences have been collected about the role played by fructose per se in an *in vitro* system, without any systemic or physiological influence, and no information is present on the possible pathways it might activate.

Considering this background, aim of this work was to develop an *in vitro* model for NAFLD, with particular reference to the first “hit”, that is onset of steatosis and insulin resistance. Moreover, we tested the effects on steatosis and insulin resistance of 3,5-diiodothyronine and 3,3’-diiodothyronine, trying to assess potential mechanisms of action, considering that, as described in the introduction, these molecules were shown

to influence lipid metabolism in other experimental systems and might, due to their similarity with T_4 and T_3, activate cellular signaling pathways. The experiments described in this work were performed in two different experimental systems, rat primary hepatocytes cultured on collagen-coated dishes, and human primary hepatocytes cultured on Matrigel-coated dishes. In the first, the effect of oleic acid and fructose on triglycerides accumulation and insulin resistance induction was taken in account, together with the effect of 3,5-diiodothyronine on the same parameters. To evaluate the induction of insulin resistance, three markers were used, i.e. the levels of expression of p85α subunit of PI3K, PTEN and Akt. Moreover we looked at Akt phosphorylation as well, to evaluate both insulin resistance and the effect of 3,5-diiodothyronine. In the second experimental model used, attention was concentrated on the analysis of the effects of 3,5-diiodothyronine and 3,3'-diiodothyronine as lipid lowering agents, and on their ability to activate insulin signaling, trying to discover the molecular signaling pathways involved. In particular, the effect on Akt and FoxO1 phosphorylation was analysed as markers of activation of the insulin pathway, and the possible involvement of mTORC2 as a mediator of the action of these two molecules was studied as well.

MATERIALS and METHODS

1. MATERIALS

1.1 Chemicals

All chemicals were purchased from SIGMA, ALDRICH, SIGMA-ALDRICH and MERCK.

1.2 Antibodies

Rat primary hepatocytes

Primary antibodies were purchased from Cell Signaling TECHNOLOGY®: PI3 Kinase p85 (19H8) [cat. #4257], Akt (pan) (11E7) Rabbit mAb [cat. #4685], PTEN (D5G7) Rabbit mAb [cat. #5384] and from SIGMA-ALDRICH®: Rabbit Anti-Actin [cat. #A2066].

Human primary hepatocytes

Primary and secondary antibodies were purchased from Cell Signalling TECHNOLOGY®: Akt2 (D6G4) Rabbit mAb [cat. #3063]; Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb [cat. #4060]; FoxO1 (C29H4) Rabbit mAb [cat. #2880]; Phospho-FoxO1 (Ser256) Antibody [cat. #9461]; Rictor (53A2) Rabbit mAb [cat. #2114]; mTOR (7C10) Rabbit mAb [cat. #2983], Anti-rabbit IgG, HRP-linked Antibody [cat. #7074].

1.3 Solutions

Rat primary hepatocytes

Hank I and Hank II

A stock solution was prepared in the following way: 140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4 \bullet 7H_2O$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 6 mM $NaHCO_3$, 3 mM Hepes. Using this stock solution, Hank I and Hank II were then made in this manner:

Hank I adding 2% Bovine Serum Albumin (BSA), 0.06 mM EGTA and Hank II with 0.5 mM $CaCl_2 \cdot 2H_2O$ and collagenase; pH of both solutions should be set to 7.4.

Krebs-Henseleit

A stock solution was prepared in the following way: 240 mM NaCl, 9.6 mM KCl, 2.4 mM $MgSO_4 \cdot 7H_2O$, 0.34 mM Na_2HPO_4, 2.4 mM KH_2PO_4, 3.9 mM $CaCl_2 \cdot 2H_2O$, 50 mM $NaHCO_3$, 3 mM Hepes. This stock solution has to be completed with 2% Bovine Serum Albumin (BSA) and 12.5 mM Hepes to obtain the working solution.

Phosphate Buffered Saline (PBS)

PBS was prepared with 170 mM NaCl, 3.35 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.68 mM $CaCl_2 \cdot 2H_2O$, 0.49 mM $MgCl_2 \cdot 6H_2O$.

Tris Buffered Saline

50 mM Tris, 150 mM NaCl, pH 7.4 with HCl

Human primary hepatocytes

Perfusion buffer 1 (P1 medium)

P1 medium was prepared adding 0.5 mM EGTA (234.2 mg/L), 0.5% w/v BSA, and 50 µg/mL ascorbic acid in Ca^{2+}- and Mg^{2+}- free Hank's Balanced Salt Solution (HBSS) without phenol red.

Perfusion buffer 2 (P2 digestion medium)

P2 digestion medium was prepared dissolving 0.025% w/v collagenase XI (250 mg/L and 0.5% w/v BSA in Eagle's Minimum Essential Medium with Earle's salts (EMEM). This buffer is typically used 100 mL/10 g liver.

2. METHODS

2.1 Adult Rat Primary Hepatocytes

Hepatocytes were obtained with a perfusive enzymatic method, according to the procedure originally described by Moldeus and colleagues (Moldeus P et al., 1978), and successively revised by De Colli and colleagues (De Colli M et al., 1981).

Briefly, rats were anesthetized by intraperitoneal administration of sodium pentobarbital (5 mg/100 g body weight). The liver was perfused firstly with a calcium-free Hank's balanced salt solution containing 2% BSA and 0.6 mM EGTA acid, and secondly with Hank's solution containing 4 mM calcium chloride and 0.04% collagenase. Hepatocytes were released into a Krebs-Henseleit buffer with 2% BSA and subsequently seeded in 35 or 60 mm collagen-coated dishes.

2.2 Foetal Rat Primary Hepatocytes

Hepatocytes were obtained using a non-perfusive enzymatic method, according to the procedure described by Conti DeVirgiliis and colleagues (Conti DeVirgiliis L et al., 1981).

Briefly, livers extracted from rats at 21th day of intrauterine life, were cut in very small pieces until achieving a homogeneous appearance. This homogenate was then washed, once at room temperature under hood and trice for 10' at 37°C in a tempering bath under 95% O_2 and 5% CO_2 with Hank I solution, to remove calcium and blood. Washed homogenate was subsequently subjected to four enzymatic digestions, 10' each, with Hank II solution containing collagenase and $CaCl_2 \bullet 2H_2O$, in the same tempering bath at 37°C under 95% O_2 and 5% CO_2. At the end of every digestion, the supernatant was collected, diluted with Krebs-Henseleit solution and then centrifuged two times for 5' at 4°C. Cells collected after centrifugations were diluted in 5 mL

Krebs-Henseleit, counted in a Thoma-Burke chamber after Trypan Blue staining and finally plated in 35 mm collagen-coated dishes.

2.3 Human Primary Hepatocytes

Human Primary Hepatocytes were isolated by means of a perfusive enzymatic method, as originally described by Ballet F and colleagues (Ballet F et al., 1984), and subsequently revised by LeCluyse EL and Alexandre E (LeCluyse EL, Alexandre E 2010).

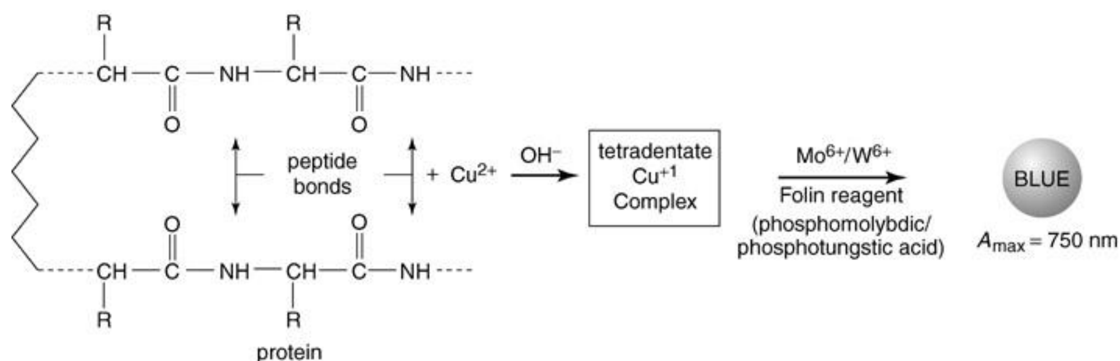
Adult human livers are usually obtained from patients undergoing surgical liver resection due to metastatic tumours, from brain-dead-but-beating-heart donors, or from dead people.

All the perfusion apparatus, including tempering water baths, heating devices and peristaltic pumps, and hence the entire procedure should be performed inside a biosafety cabinet. One to three vessels were cannulated and connected to a peristaltic pump, and the liver was placed into a tank containing warm HBSS until it was completely submerged. The tissue was perfused with P1 medium, then with P1 medium without EGTA and finally with P2 digestion medium for 20-25 minutes. After complete digestion was achieved, as revealed by softening and structural breakdown of the parenchyma, and enlargement of the tissue, the liver was disaggregated by chopping with scissors, releasing the hepatocytes which were in turn filtered, using two layers of gauze, centrifuged at 50 g for 5 minutes and washed twice with EMEM. The cells were counted with a Thoma-Burke chamber after Trypan Blue staining and then plated in 6-well plate culture dishes precoated with Matrigel [BD Matrigel™ Basement Membrane Matrix, 10 mL vial, Cat. 354234], $1.5 \cdot 10^6$ per well.

Hepatocytes were cultured in Swedish Media from William's E medium (WE-SWE), obtained supplementing William's E medium with 2 mM Glutamine, 20 mM Hepes, 10 nM Insulin, 0.01 M Gentamicin, 0.055 μM (0.05 μg/mL) and 100 nM Dexamethasone. All concentrations reported should be read as final concentrations in the culture medium.

2.4 Protein determination

Protein concentration was assessed according to the method originally described by Lowry OH et al. (1951) and later reviewed by Peterson GL (1979). This assay is based on a two step reaction that finally leads to development of a blue colour, first step being represented by the interaction between protein and copper in an alkaline medium, followed by reduction of Folin phenol reagent, which was described for the first time by Folin O, Ciocalteu V in 1927. The reactive component of this mixture is the phosphomolybdic-tungstic mixed acid that is the final chromogenic in the assay and includes the following chemical species: 3 H_2O, P_2O_5, 13/14 WO_3, 5/4 MoO_3, 10 H_2O. Colour production is essentially provoked by amino acids tyrosine and tryptophan, even if also cystine, cysteine and histidine give some contributes. These amino acids react with the Folin reagent, inducing the loss of 1,2, or 3 oxygen atoms, generating one or more possible reduced species, which give raise to the typical blue colour with maximum absorbance at λ=750 nm and minimum absorbance at λ=405 nm. Copper by chelating the peptide structure seems to facilitate the electron transfer to the chromogenic species, especially near to amino acids functional groups, in this way improving the sensitivity of the test.



Adult and Foetal Rat Primary Hepatocytes

A standard curve was constructed using Bovine Serum Albumin (BSA) 0.1 mg/mL. Briefly, an aliquot of each sample, variable from 5 to 20 µL depending on the experiment, was diluted to 200 µL DH_2O. Subsequently 1 mL solution "A" + "B" (100/1 v/v) was added, and after 10 minutes incubation, 100 µL solution "C" was inserted. After 30 minutes of incubation, absorbance was measured at λ=660 nm employing a Thermo Spectronic "GENESYS 10-S UV scanning" [Cat. 335907-02] spectrophotometer.

Solution "A": NaK tartrate 200 mg/L, Na_2CO_3 20 g/L, NaOH 4 g/L dissolved in DH_2O; Solution "B": $CuSO_4$ 0.1 g/L dissolved in DH_2O; Solution "C": Folin-Ciocalteu, diluted in a 1:1 ratio with DH_2O.

Human Primary Hepatocytes

Protein determination was performed employing the Bio-Rad DC Protein Assay Reagents Package [Cat. 500-0116], which includes: REAGENT A, an alkaline copper tartrate solution and REAGENT B, a dilute Folin Reagent. Protein concentration measurement was realised on 96 well microplates, using a Bio-Rad Protein Standard I (Lyophilized Bovine Plasma Gamma Globulin) [Cat. 500-0005] to build a standard curve, and absorbance was evaluated using a "Tecan Infinite 500" plate reader and "Magellan7" software.

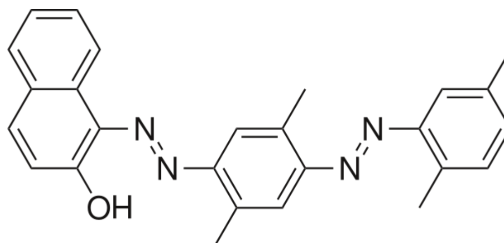
2.5 Oil Red O staining (ORO)

Oil Red O (ORO) (1-(2,5-dimethyl-4-(2-5-dimethylphenyl) phenyldiazenyl) azonapthalen-2-ol, or Solvent Red 27, Sudan Red 5B, C.I. 26125, $C_{26}H_{24}N_4O$) is a lysochrome (fat-soluble dye) diazo dye used for staining of neutral triglycerides and lipids. The basis for these dyes colouring fats resides in their capacity to dissolve into them, in other words the fat acting as a solvent for the dye. In fact lysochromes are mostly insoluble in strongly polar solvents, such as water, somewhat more in less polar solvents, such as ethanol, but are quite strongly soluble in non-polar solvents, such as xylene. Triglycerides, being non-polar compounds, dissolve them quite well, as do other lipids that show fatty components.

ORO has the appearance of a red powder with maximum absorption at 518 (359) nm. It belongs to the same family of dyes that includes Sudan II, Sudan III, Sudan IV, and Sudan Black B, which are characterized to the high affinity to fats, and then widely used to show neutral lipids, triglycerides and lipoproteins. Anyway, due to its deeper red colour resulting in a better quality of the staining, ORO has generally replaced Sudan dyes.

Typically, after the cell culture medium was removed, dishes were washed with PBS and incubated for 10 minutes with 10% formalin in 10X PBS. Formalin was then discarded and after another 1 h incubation with fresh 10% formalin, cells were washed twice with DH_2O and one time with 60% 2-propanol for 5 minutes. Cells were let to dry under hood, subsequently incubated 10 minutes with ORO, and then washed four times with DH_2O. Photos were acquired, ORO was eluted with 100% 2-propanol and absorbance was measured at λ=500 nm, using 100% 2-propanol as a blank.

Oil Red O [SIGMA Cat. O0625] was preliminary dissolved as follows: 0.35% w/v in 100% 2-propanol. Then this stock solution was diluted 60% v/v in DH_2O to obtain the working solution used during experiments.



2.6 Neutral Red staining Cytotoxicity Assay

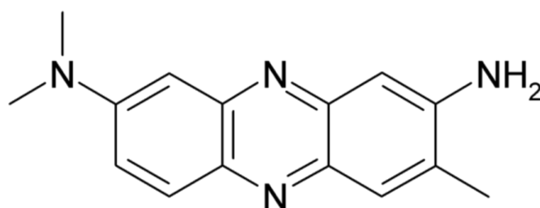
Neutral Red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) or toluylene red, Basic Red 5, C.I. 50040, $C_{15}H_{17}ClN_4$) is a slightly cationic dye that easily passes through cell membranes and localises especially inside lysosomes. The acidic milieu typical of these organelles favours the development of a red staining (pH<6.8 give rise to red, pH>6,8 produces orange-yellow).

Toxic treatments are able to induce alterations and damages to cell membranes, including lysosomal ones, resulting in the lost of any acidic compartment inside the cells: in these condition neutral red develops an orange-yellow staining.

This background draws the rationale for the use of this molecule as a cell vitality test, also considering that it is no harmful for health, quite cheap, and easy to perform.

Usually 1 mg/mL solution in DH_2O of Neutral Red [SIGMA Cat. N7005] is prepared and added to culture medium to achieve a final concentration of 10 μg/mL in the dish. After 1h incubation at 37°C, culture medium was discarded, and cells were washed twice with PBS. Neutral Red was eluted using acetil-ethanol, prepared by mixing glacial acetic acid with 50% v/v ethanol/DH_2O in 1:99 ratio.

Absorbance was measured at λ=540 nm, using acetil-ethanol as a blank, and values were normalised for protein content.



2.7 Lipid extraction and triglycerides measurement

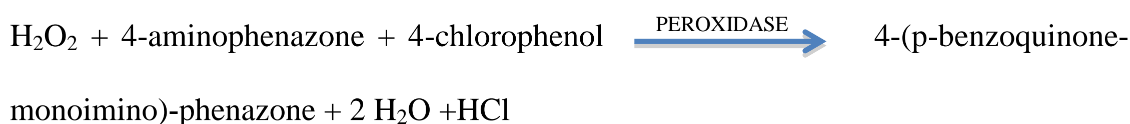
After the cell culture medium was discarded, dishes were washed twice with PBS and let dry at room temperature under hood. Lipids were extracted employing a mixture Hexan:Isopropanol (3:2), in two consecutive times 1 h and 30 minutes each respectively, and plates were let dry under hood until residual solvent was completely evaporated. Protein concentration was evaluated with the Lowry procedure, after dissolving cells in 1 M NaOH overnight.

The lipid extract was dried under N_2 flow at 40°C using a needle analytical evaporator [The MEYER N-EVAP. ANALYTICAL EVAPORATOR Organomation ASSOCIATE INC. P.O., Box 159 South Berlin MA 01549], then dissolved in $CHCl_3$ and dried again under N_2 flow at 40°C. The extract was suspended in $CHCl_3$+1% Triton X-100 and dried again. DH_2O was finally added until the solution is completely clear. Triglycerides content was evaluated with an enzymatic colorimetric test [TG Triglycerides GPO-PAP – Roche/Hitachi Cat. 12016648] based on the following reactions:

Triglycerides + $3H_2O \xrightarrow{LPL}$ glycerol + 3RCOOH

Glycerol + ATP $\xrightarrow{GK}$ glycerol-3-phosphate + ADP

Glycerol-3-phosphate + $O_2 \xrightarrow{GPO}$ dihydroxyacetone phosphate + H_2O_2



H_2O_2 + 4-aminophenazone + 4-chlorophenol $\xrightarrow{\text{PEROXIDASE}}$ 4-(p-benzoquinone-monoimino)-phenazone + 2 H_2O +HCl

This method is derived from that described by Wahlefeld AW et al., 1974, and utilises a microorganisms-derived lipoprotein lipase to completely hydrolyse triglycerides to glycerol, which in turn is oxidised to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide is subsequently involved in a peroxidase-catalysed reaction with 4-aminophenazone and 4-chlorophenol, the product of which is a red/orange dye (Trinder endpoint reaction). The colour intensity is directly proportional to the triglycerides concentration and can be evaluated reading absorbance at λ=492 nm using a "Tecan Infinite 500" plate reader and "Magellan7" software, utilising a standard curve constructed using the "Precinorm 120214" standard.

2.8 Transfection with siRNAs

Human primary hepatocytes cultured on Matrigel were transfected with two siRNAs specific for subunit Rictor of mTORC2 [Cell Signaling TECHNOLOGY®: SignalSilence® Rictor siRNA I, cat. #8649 and SignalSilence® Rictor siRNA II, cat. #8622], and with a scrambled siRNA to verify the efficiency of transfection [Cell Signaling TECHNOLOGY®: SignalSilence® Control siRNA, cat. #6568]. Transfection was performed using Lipofectamine® RNAiMAX Reagent [Invitrogen™ cat. 13778-030], diluted with Opti-Mem I [Invitrogen™ cat. 11058-021]. To achieve a good efficiency of transfection cell density at plating was set to $0.75 \cdot 10^6$/mL, 8 μL Lipofectamine®/well and 10 μL siRNAs/well were used.

2.9 Immunoelectrophoretic analysis

Rat primary hepatocytes

After the cell culture medium was removed, dishes were washed twice with ice-cold PBS, and cells were scraped, collected in PBS and centrifuged at 2200 rpm for 8 minutes. Pellet was then solubilised in a lysis buffer containing 10% SDS, 0.125 M Tris/HCl pH 6.8, 1% PMSF, 1% Leupeptin, 1% Sodium Orthovanadate ($NaVO_4$).

Samples were prepared for western blotting using a loading buffer containing 40% SDS (8%) [Sodium dodecyl sulphate SIGMA Cat. L4509], glycerol (40%) [Glycerol SIGMA Cat. G6279], β-mercaptoethanol (20%) [2-Mercaptoethanol SIGMA Cat. M3148], bromophenol blue (0.04%) [Electrophoresis Purity Reagent Bromophenol Blue, Bio-Rad Cat. N. 161-0404], 0.0315 M Tris/HCl pH 6.8.

Polyacrylamide gels were prepared using the following solutions:

Resolving gel: 3 M Tris/HCl pH 8.8; Stacking gel: 0.5 M Tris/HCl pH 6.8; Acrylamide: 30% Acrylamide/Bis Solution, 29:1 (3,3% C) Acrylamide:N,N'-Methylenbisacrylamid Electrophoresis Purity Reagent [Bio-Rad Cat. 161-0156]; TEMED: N,N,N',N'-Tetramethylethylene-diamine, for electrophoresis, approx. 99% [SIGMA Cat. T9281]; APS: Ammonium Persulfate [Bio-Rad Cat. 161-0700]; protein denaturing agent: 10% SDS.

Proteins were separated in a Bio-Rad Mini-PROTEAN® 3 Cell using the following running buffer: 10X Tris/Glycine/SDS Buffer for SDS-PAGE applications [Bio-Rad Cat. 161-0772]. Gels were blotted onto nitrocellulose membranes using the iBlot™ Dry Blotting System [Invitrogen™ Cat. IB1001], and subsequently stained with a 1% Ponceau Red solution in acetic acid [Ponceau S, practical grade – SIGMA Cat. P3504] to verify the blotting efficiency. After 1 h incubation at room temperature in blocking solution (5% defatted dry milk in TBS), membranes were incubated overnight at 4°C

with the primary antibody of interest. Secondary antibody incubation was performed for 1h at room temperature and bands revelation achieved using an ECL system [Thermo SCIENTIFIC SuperSignal® West Dura Extended Duration Substrate, Cat. 34075]. Densitometries were realised utilising ImageJ software, free downloadable on http://rsb.info.nih.gov/ij/.

Human primary hepatocytes

After the cell culture medium was removed, 6-well plates were washed twice with ice-cold PBS, and cells were scraped, collected in PBS and centrifuged at 2200 rpm for 8 minutes. Pellet was then solubilised in a lysis buffer containing 10% SDS, 0.125M Tris/HCl pH 6.8, 1 tablet of Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets [Roche Cat. 11 836 170 001], 1 tablet of PhosSTOP – Phosphatase Inhibitor Cocktail Tablets [Roche Cat. 04 906 845 001].

Samples were prepared for western blotting using Laemmli Sample Buffer [Bio-Rad Cat. 161-0737] following producer's directions for use.

Proteins were separated with Novex® 10% Tris-Glycine Gel 1.0 mm x 10 wells [Invitrogen™ Cat. EC6075BOX], in an Invitrogen™ XCell *SureLock*™ Mini-Cell [Cat. EI0001], using the following running buffer: 10X Novex® Tris-Glycine SDS Running Buffer [Invitrogen™ Cat. LC2675]. Gels were blotted onto Nitrocellulose Membrane [Invitrogen™ Cat. LC2001], using an Invitrogen™ XCell II™ Blot Module [Cat. EI9051], filled with a transfer buffer prepared as follows: 4% 25X Novex® Tris-Glycine Transfer Buffer [Invitrogen™ Cat. LC3675], 20% Methanol, 76% DH_2O. After Ponceau Red staining, performed using SIGMA-ALDRICH Ponceau S solution (0.1 % w/v in 5% acetic acid) [Cat. P7170], membranes were destained with 0.5 M NaOH and subsequently incubated for 30 minutes at room temperature with a blocking solution [Thermo SCIENTIFIC Starting Block™ T20 (PBS) Blocking Buffer, Cat. 37539].

Primary antibody incubation was carried out overnight at 4°C, whilst secondary antibody incubation was performed for 1h at room temperature; bands were revealed using an ECL system [Thermo SCIENTIFIC SuperSignal® West Dura Extended Duration Substrate, Cat. 34075], and chemiluminescence was measured with the Bio-Rad CHEMIDOC™ XRS System Universal Hood II and analysed employing the Bio-Rad Quantity One® Software.

2.10 Statistical analysis

Statistical calculations and graphing were performed using Graph Pad 4.01 software. The data were analysed for normality with the Kolmogorov-Smirnov test, and significance was assessed with a one-way ANOVA followed by Tukey's HSD test for multiple comparisons. The data were considered significant when p value <0.05.

RESULTS

The experiments described in this thesis have been performed employing two different experimental systems, rat primary hepatocytes (RPH) at adult and foetal stage cultured on collagen-coated dishes, and human primary hepatocytes (HPH) cultured on Matrigel-coated dishes. Although presenting several points of contact, such results will be hence described separately through this section.

RPH

As previously described, the aim of the study in RPH was to establish an *in vitro* model for the first "hit" of NAFLD and to evaluate in this system the role of fructose and the potential therapeutic role of 3,5-T_2.

As a steatogenic stimulus we used oleic acid, considering that in several other systems it was employed for such purpose (Vidyashankar S et al., 2013). Figure 1 shows the dose-response effect of oleic acid at concentrations ranging from 100 μM to 500 μM on triglycerides accumulation and cell viability, after 24 h of treatment. Histograms clearly demonstrate the effect of oleic acid in inducing triglycerides accumulation, with the maximum result obtained at 500 μM, with nearly double triglycerides content with respect to control. On the other hand, no significant detrimental effects were found on cell viability, regardless of the concentration used, permitting us to choose the concentration of 500 μM as an excellent compromise between cell viability and induction of triglycerides accumulation.

For what concerns the effect of fructose, Figure 2 reports results of a dose-response analysis, obtained treating hepatocytes with concentrations ranging from 5 mM to 200 mM. The range 5 mM to 20 mM is supposed to be physiological, whereas concentrations above these values should be considered strongly supraphysiological

(Segal MS et al., 2007). Our results clearly show that fructose exhibits a huge effect on triglyceride accumulation, comparable to that of oleic acid at the concentration of 50 mM, but no significant effects were observed in the range 5 mM to 20 mM. Interestingly, at higher concentrations, i.e. 100 nM and 200 nM, the amount of stored triglycerides was lower with respect to what observed at 50 mM, and this probably makes sense, if these data are compared with the effect of fructose on cell viability, as shown in the panel B of the figure. In fact is clearly evident as fructose was able to induce a reduction in cell viability, starting with a modest effect at 20 mM, until a minus 70% at 200 mM. For all of these reasons we decided to exclude the two highest concentrations from the following experiments.

In order to assess the effect of oleic acid and fructose when administered together, trying to simulate the typical condition of a western cafeteria diet, we treated hepatocytes for 24 h with 500 µM oleic acid and 5 mM to 50 mM fructose. Figure 3 clarifies that, also when administered in conjunction with oleic acid, fructose was not able to induce a large increase in triglycerides accumulation if compared with oleic acid alone. Noteworthy, fructose kept its ability to induce cell death, even if the presence of oleic acid worsen such effect, as evidenced in the panel B of the figure.

Considering that until now our model was able to reproduce steatosis without affecting cell viability, we turned our attention on insulin resistance, trying to evaluate if a 24 h treatment with oleic acid and fructose could be able to induce such a condition. For this purpose we considered two markers, i.e. p85α subunit of PI3K and PTEN, which in two different systems, one *in vivo* and one *in vitro*, have been shown to increase when in presence of insulin resistance conditions (Mauvais-Jarvis F et al., 2002; Shen YH et al., 2006; Lee SK et al., 2011).

For what concerns p85α, Figure 4A shows how 24 h treatment with 500 μM oleic was able to induce an increased expression of this subunit, whereas panel B suggests that fructose did not change p85α levels. In this perspective, Figure 5 demonstrates the positive effect of 24 h treatment with 500 μM oleic acid on PTEN expression, and these results together with those described previously strongly made us confident that the system we built could be considered with good approximation a steatotic and insulin resistant system.

On this system we analysed the effect of 3,5-T_2 on oleic acid-induced triglycerides accumulation, as reported in Figure 6. To investigate this aspect we utilised two different approaches, the first one consisting in treating hepatocytes with oleic acid and 3,5-T_2 contextually for 24 h, the second one in testing the effect of 24 h treatment with 3,5-T_2 after 24 h incubation with oleic acid. The results obtained using these two different approaches are reported in panel A and panel B respectively, and clearly demonstrate that 3,5-T_2 in the range of physiological concentrations, i.e. 1 nM and 0.1 nM, strongly reduced the triglycerides content, both if administered contextually with oleic acid or in a condition when triglycerides accumulation is well-established. These observations permit us to say that 3,5-T_2 is able to prevent triglycerides storage inside the hepatocyte, and that it can revert the accumulation induced by oleic acid as well.

Once assessed the role of 3,5-T_2 as a lipid-lowering agent, the next step was to analyse the possible role played in the insulin signaling pathway, and for this reason the effect on phosphorylation of Akt at Ser473 residue was examined, considering that this phosphorylation is one of the key steps downstream the insulin receptor. The results obtained, which are reported in Figure 7, are indeed very interesting for at least two reasons. First of all, as reported in panel B, 3,5-T_2 induced Akt phosphorylation comparably to insulin, and when this insulin-induced phosphorylation was inhibited

after 24 h incubation with oleic acid, 3,5-T_2 was still capable to drive this phosphorylation, suggesting that most probably 3,5-T_2 follows a different pathway with respect to insulin. Secondly, as reported in panel A, we found that after 24 h oleic acid load, the level of total Akt was increased of about 50% if compared to control, similarly to what shown for p85α and PTEN. Actually we are not able to explain this result, or to say if and in which way it could be interpreted as a sign of insulin resistance, but we can consider it as an interesting future topic of investigation.

To conclude the description of results obtained in RPH, now several data obtained using foetal RPH will be reported, which are not conclusive and still opened to further investigations, but anyway interesting, considering that they show several differences with respect to what found in adult cells. For example, as reported in Figure 8 A and B, oleic acid seemed to be less steatogenic in foetal cells, but also more toxic, and 3,5-T_2 appeared completely ineffective in preventing triglycerides accumulation, even if not toxic. On the other hand, similarly to what already demonstrated in adult hepatocytes, 24 h incubation with 500 µM oleic acid was able to stimulate synthesis of p85α subunit of PI3K, as represented in Figure 9 A, whereas 24 h incubation with 10 mM fructose did not affect such synthesis, as reported in Figure 9 B.

HPH

The experiments that will be described now with HPH were performed employing HPH cultured on Matrigel-coated dishes. Matrigel is a complex mixture of extracellular matrix proteins, originally isolated from the Engelbreth-Holm-Swarm mouse (EHS) sarcoma (Kleinman HK et al., 1982). This substrate allows to keep better in culture all the liver-specific characteristics of hepatocytes, such as morphology and several metabolic pathways (Ben-Ze'ev A et al., 1988), as described elsewhere in this thesis, giving the opportunity to work with a system resembling in most aspects a real

liver. Such morphological differences with respect to the usual collagen-grown hepatocytes is clearly noticeable in Figure 10, which reports two photos that were taken at two different magnification, 20X (A) and 40X (B).

Utilising this system we tried to analyse the effect of both diiodothyronines, 3,3'-T_2 and 3,5'-T_2 as lipid lowering agents, evaluating the total triglyceride content after 24 h, 48 h and 72 h of treatment, using decreasing concentrations of the two diiodothyronines, starting with 100 nM until 0.1 nM. These experiments were carried out using hepatocytes obtained from two different patients, one female and one male. Figure 11 reports the results acquired with the female patient, which show a reduction in the triglycerides content after the treatment with both diiodothyronines. An analogous result was found in the male patient, after 24 h and 72 h, as evidenced in Figure 12.

Once clarified that both diiodothyronines were able to reduce triglyceride content, attention was focused on their potential role in the stimulation of insulin signaling pathway, similarly to what already found in RPH. For such purpose we considered two well-known markers of this pathway, which are Akt and FoxO1. Again we utilised cells obtained from one male and one female patient, and for brevity one single experiment for each patient will be reported, considering that we obtained comparable results in both patients. Figure 13 reports the effect of 10 min. stimulation with 3,5'-T_2 and 3,3'-T_2 on phosphorylation of Akt at Ser473 residue in a female patient (VF8, from the place of isolation), and the graphs clearly demonstrated the huge increase in phosphorylation obtained, in the order of 200% with respect to control. Figure 14 reports the results obtained with the male patient (HF162, from the place of isolation), obtained blotting samples for phosphorylation of FoxO1 at Ser256, which is known to drive the proteasomal degradation of FoxO1, permitting the transcription of insulin-

responsive genes (Zhang X et al., 2002; Matsuzaki H et al., 2003). Similarly to what reported for Akt, an increase in the phosphorylation in the order of 150-200% with respect to control was found, strongly suggesting a direct positive role of diiodothyronines in the activation of insulin signaling.

Considering the data reported for RPH, which demonstrated that 3,5-T_2 was able to induce the phosphorylation of Akt in an insulin-resistant system, the question on which pathway diiodothyronines might use to stimulate such activation was taken in account. Among all the possible pathways that could be used, my idea was that a probable mediator might be mTORC2, for all the reasons already specified in the introduction and in the aim of the work. To test this hypothesis, Rictor subunit of mTORC2 complex was silenced using a siRNA approach, then hepatocytes were stimulated with 3,3'-T_2 for 10 min. and samples were blotted for Akt phosphorylated at Ser473 residue. As reported in Figures 15 and 16, the phosphorylation of Akt at Ser473 residue was completely blocked after treatment with siRNA(I) and siRNA(I+II), whereas only slightly reduced after silencing with siRNA(II) alone. Finally, to verify the efficiency of transfection, three different approaches were used, as shown in the last three figures. First of all cells were silenced with a scramble siRNA (control siRNA), to confirm that after such a silencing phosphorylation of Akt were still present, and indeed it was the case, as clearly demonstrated in Figure 17. Hence, to substantiate further this observation, the levels of Rictor and mTOR before and after silencing were evaluated, and results obtained confirmed that silencing was successfully achieved, and also that siRNA(I) resulted much more efficient if compared with siRNA(II).

Figure 1

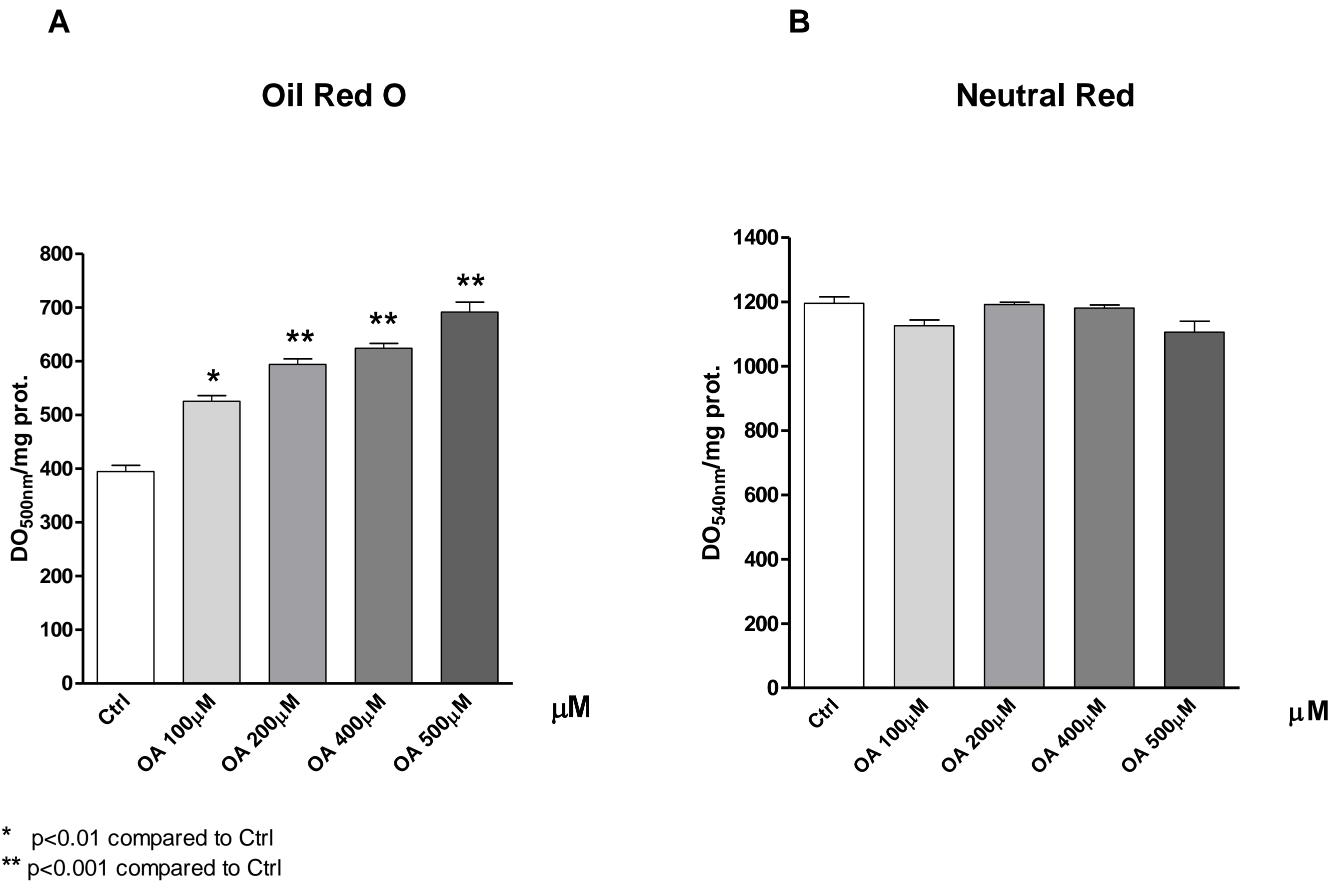


Fig.1 Effect of Oleic Acid (OA) on triglycerides accumulation and cell viability in rat primary hepatocytes, assessed using Oil Red O staining (A) and Neutral Red staining (B). Cells were treated for 24 h with OA and then analysed according to the respective protocols, as described in Materials and Methods section. Data represent at least four different experiments performed in duplicate.

Figure 2

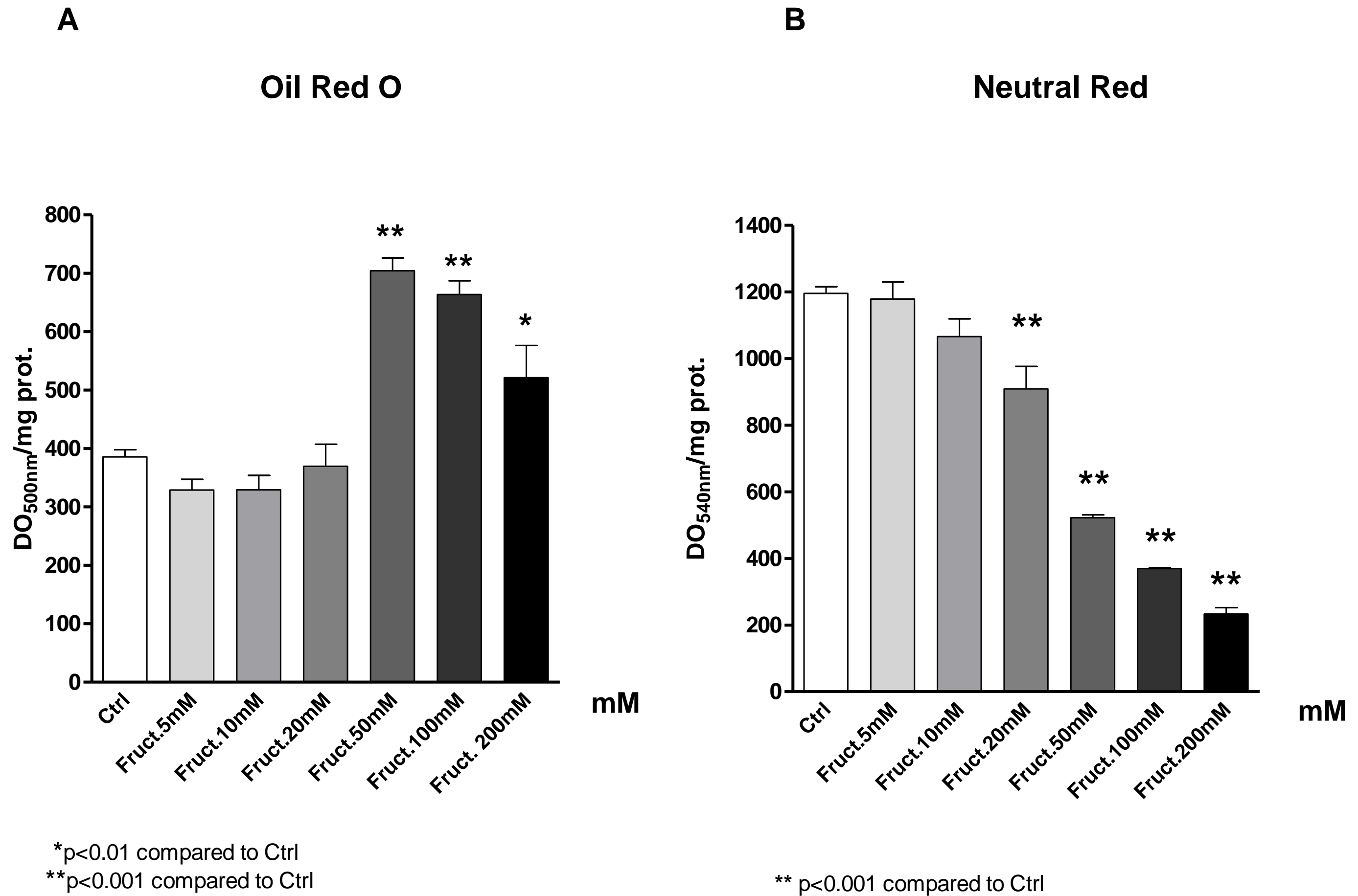


Fig.2 Effect of Fructose (Fruct.) on triglycerides accumulation and cell viability in rat primary hepatocytes, assessed using Oil Red O staining (A) and Neutral Red staining (B). Cells were treated for 24 h with Fruct. and then analysed according to the respective protocols, as described in Materials and Methods section. Data represent at least four different experiments performed in duplicate.

Figure 3

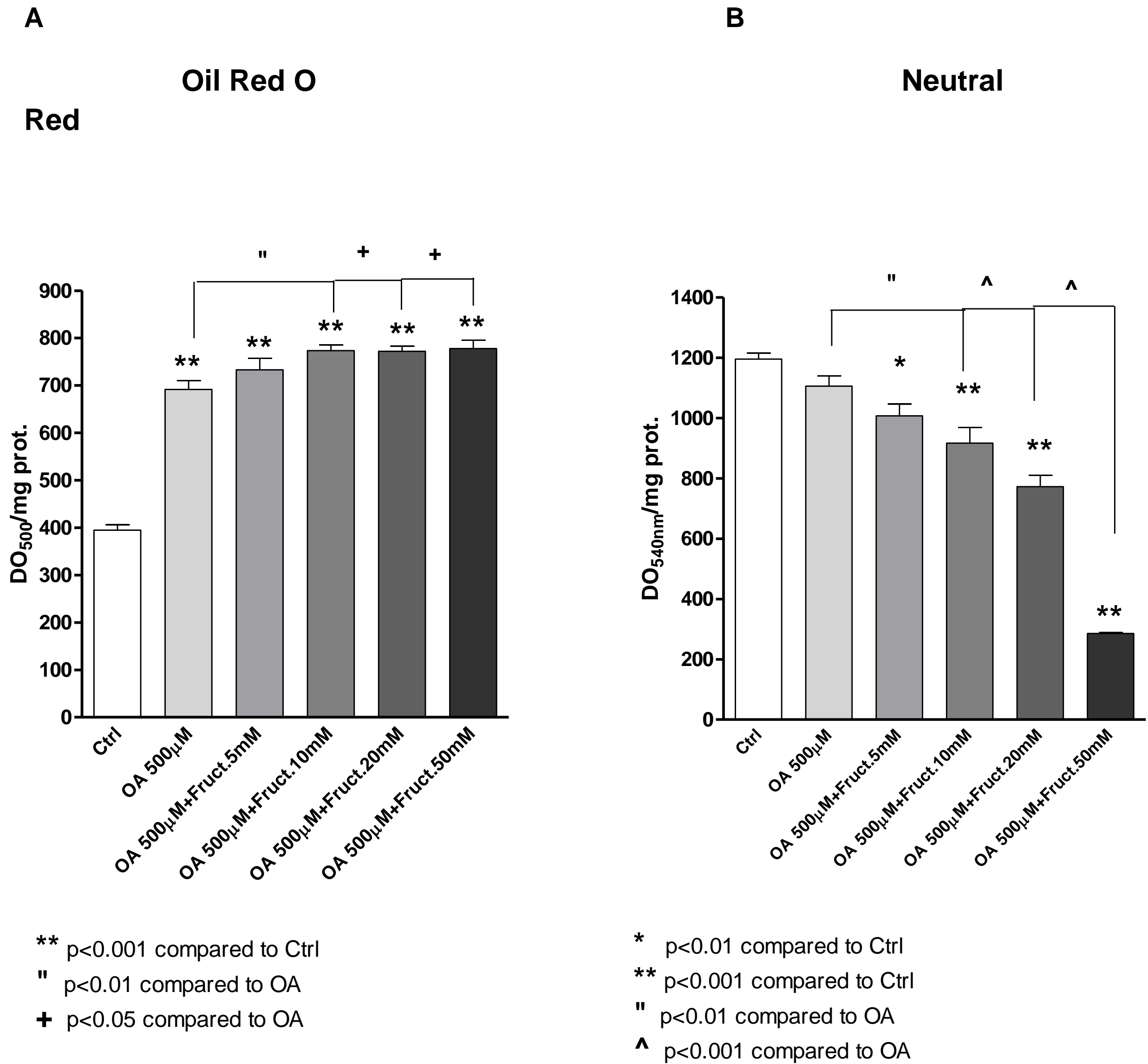


Fig.3 Effect of Oleic Acid (OA) plus Fructose (Fruct.) on triglycerides accumulation and cell viability in rat primary hepatocytes, assessed using Oil Red O staining (A) and Neutral Red staining (B). Cells were treated for 24 h with OA plus Fruct. and then analysed according to the respective protocols, as described in Materials and Methods section. Data represent at least four different experiments performed in duplicate.

Figure 4

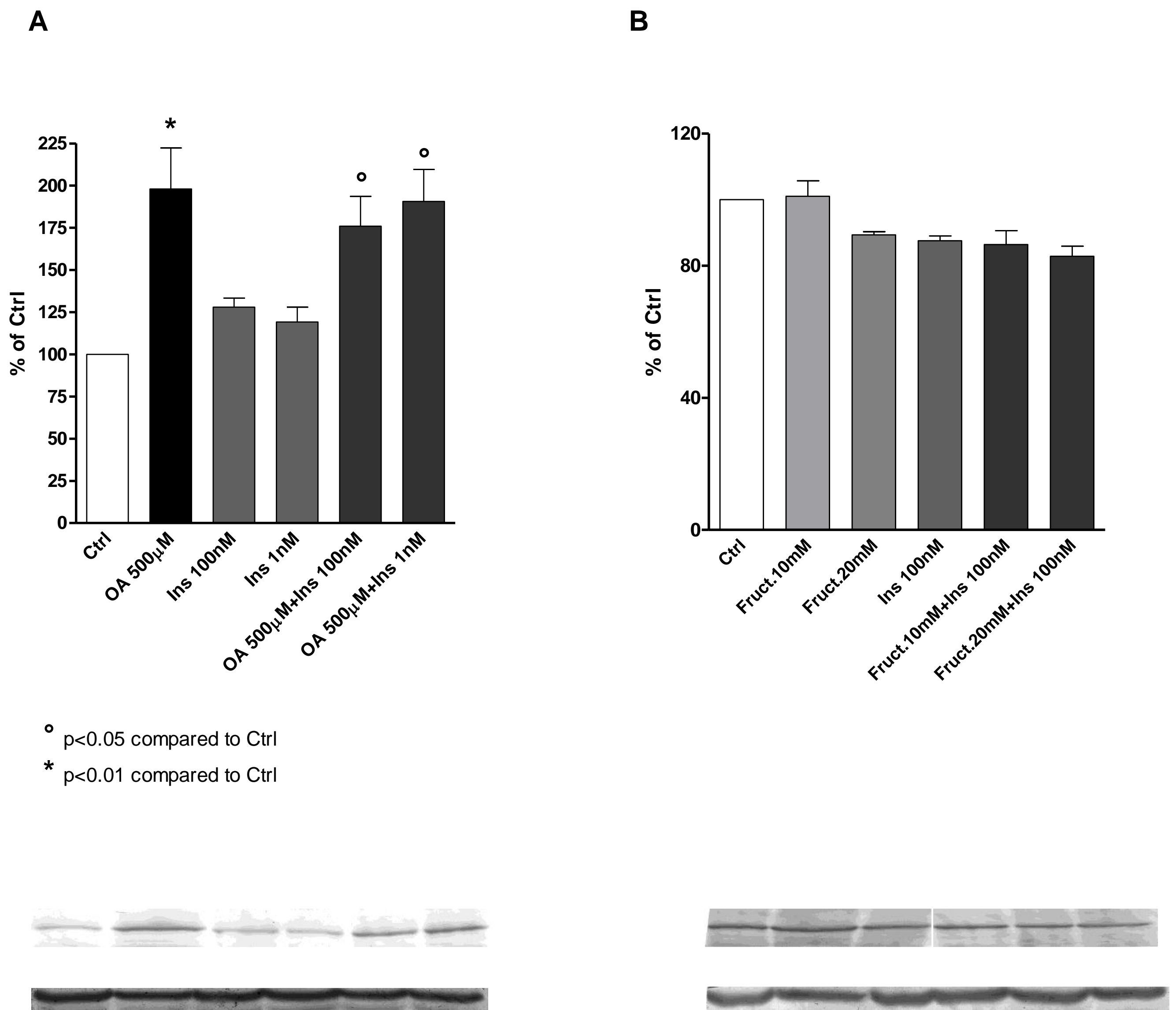
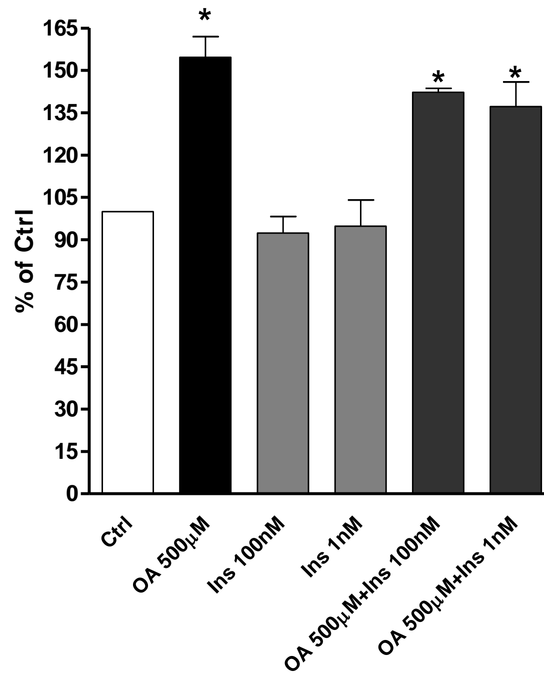


Fig.4 Effect of Oleic Acid (OA) (A) and Fructose (Fruct.) (B) on p85 levels in rat primary hepatocytes, assessed by Western Blot. Cells were treated for 24 h with OA or Fruct. Western Blot was performed according to the procedure described in Materials and Methods section. Data represent at least three different experiments performed in duplicate.

Figure 5



* p<0.01 compared to Ctrl

Fig.5 Effect of Oleic Acid (OA) on PTEN levels in rat primary hepatocytes, assessed by Western Blot. Cells were treated for 24 h with OA. Western Blot was performed according to the procedure described in Materials and Methods section. Data represent at least three different experiments performed in duplicate.

Figure 6

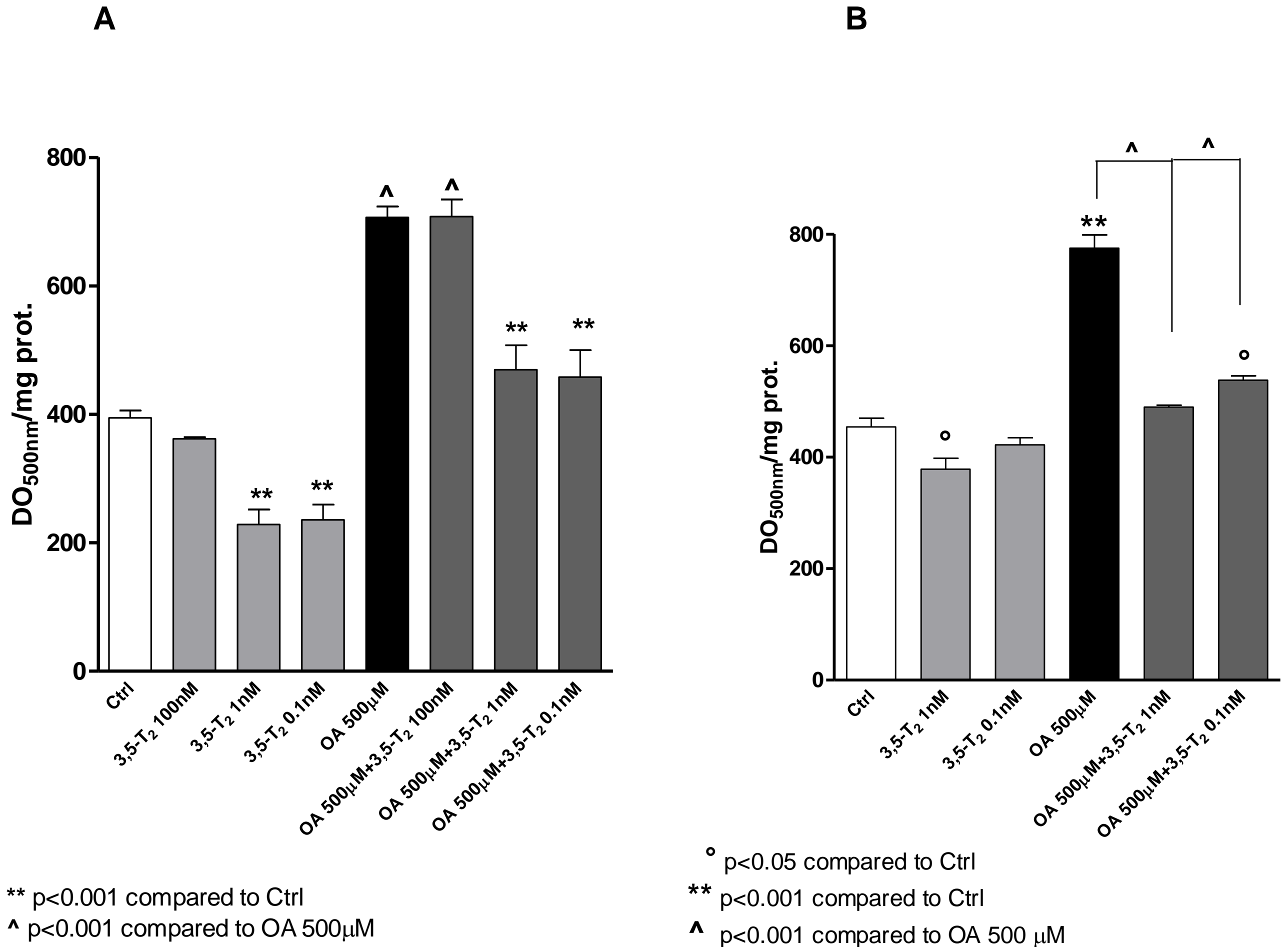


Fig.6 Effect of 3,5-Diiodothyronine (3,5-T_2) on Oleic Acid (OA)-induced triglycerides accumulation in rat primary hepatocytes, assessed using Oil Red O staining. Cells were treated for 24 h with OA plus 3,5-T_2 (A), or treated for 24 h with OA and subsequently with 3,5-T_2 for additional 24 h (B) and then analysed according to the respective protocol, as described in Materials and Methods section. Data represent at least four different experiments performed in duplicate.

Figure 7

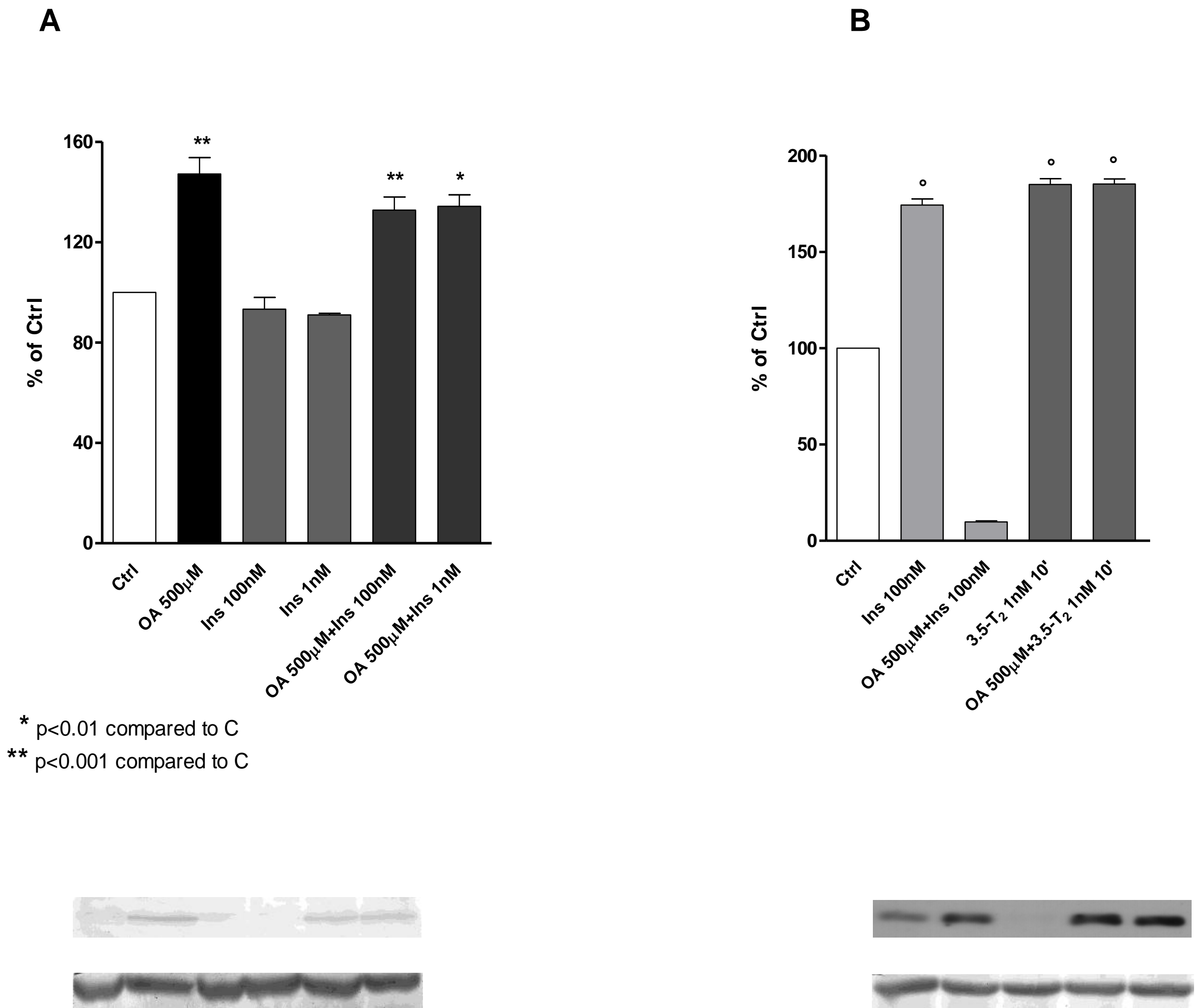


Fig.7 Effect of Oleic Acid (OA) and 3,5-T_2 on Akt phosphorylation at Ser473 residue in rat primary hepatocytes, assessed by Western Blot. Cells were treated for 24 h with OA and then stimulated for 15’ with Insulin (Ins) 100 nM and for 10’ with 3,5-T_2 1 nM. Western Blot was performed according to the procedure described in Materials and Methods section. (A) Akt total; (B) Akt-P(Ser473). Data represent at least three different experiments performed in duplicate.

Figure 8

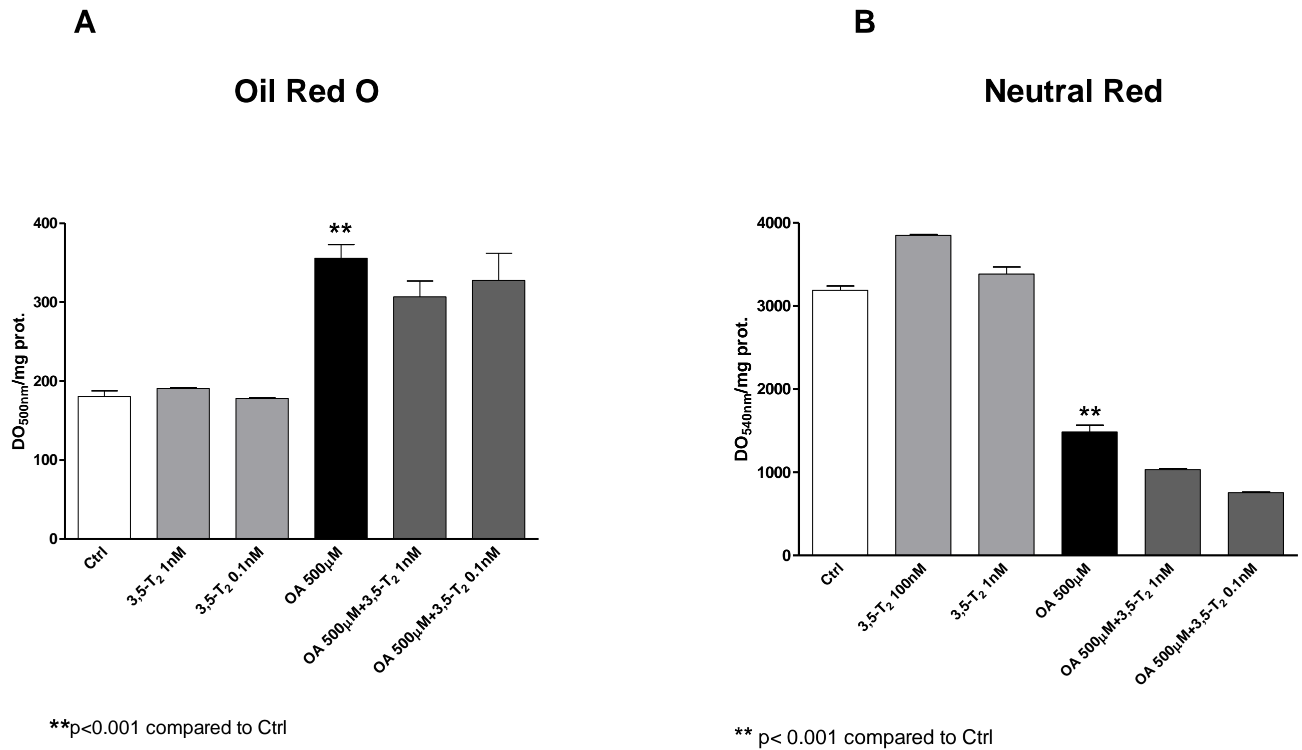


Fig.8 Effect of 3,5-Diiodothyronine (3,5-T_2) on Oleic Acid (OA)-induced triglycerides accumulation and cell viability in foetal rat primary hepatocytes, assessed using Oil Red O staining (A), and Neutral Red Staining (B). Cells were treated for 24 h with OA plus 3,5-T_2 and then analysed according to the respective protocols, as described in Materials and Methods section. Data represent at least four different experiments performed in duplicate.

Figure 9

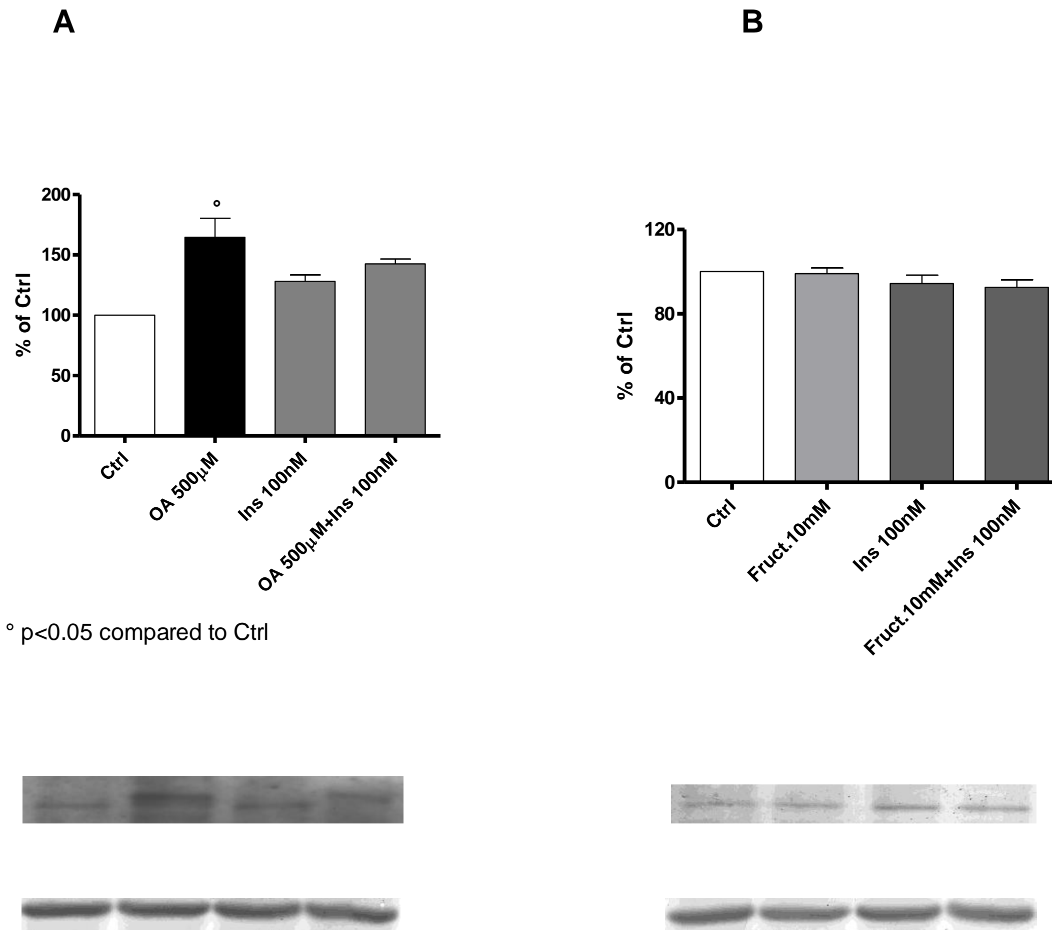
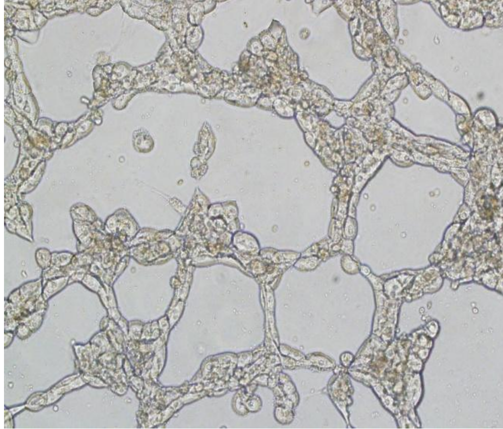


Fig.9 Effect of Oleic Acid (OA) (A) and Fructose (Fruct.) (B) on p85 levels in foetal rat primary hepatocytes, assessed by Western Blot. Cells were treated for 24 h with OA or Fruct. Western Blot was performed according to the procedure described in Materials and Methods section. Data represent at least three different experiments performed in duplicate.

Figure 10

A



B

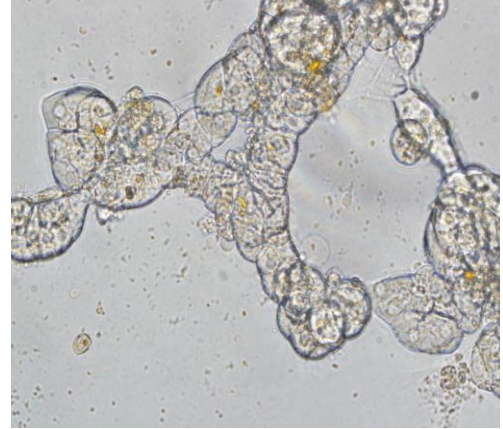


Fig.10 Examples of Human Primary Hepatocytes (HPH) cultured on Matrigel-coated dishes. Photos were taken at two different magnification, 20X (A) and 40X (B).

Figure 11

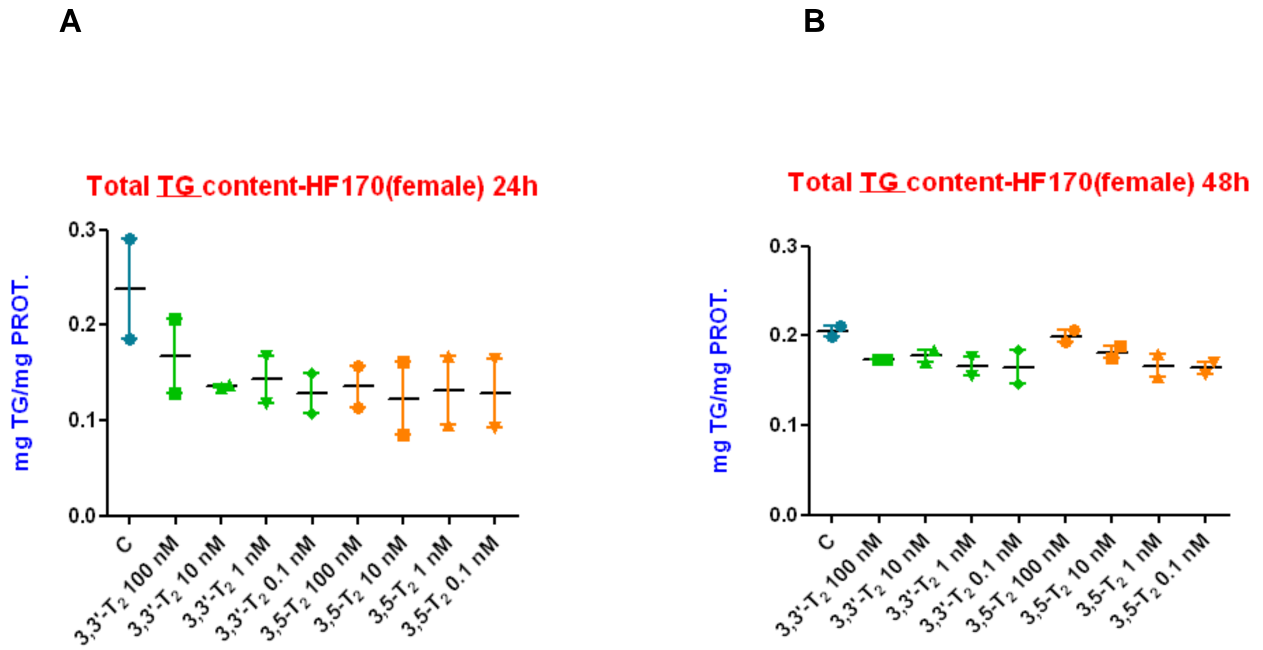


Fig.11 Effect of 3,3'-Diiodothyronine (3,3'-T_2) and 3,5-Diiodothyronine (3,5-T_2) on total triglycerides level in human primary hepatocytes, assessed using a triglycerides determination kit as described in Materials and Methods section. Cells were treated for 24 h (A) and 48 h (B) with 3,3'-T_2 or 3,5-T_2 before extracting lipids, as explained in Materials and Methods section.

Figure 12

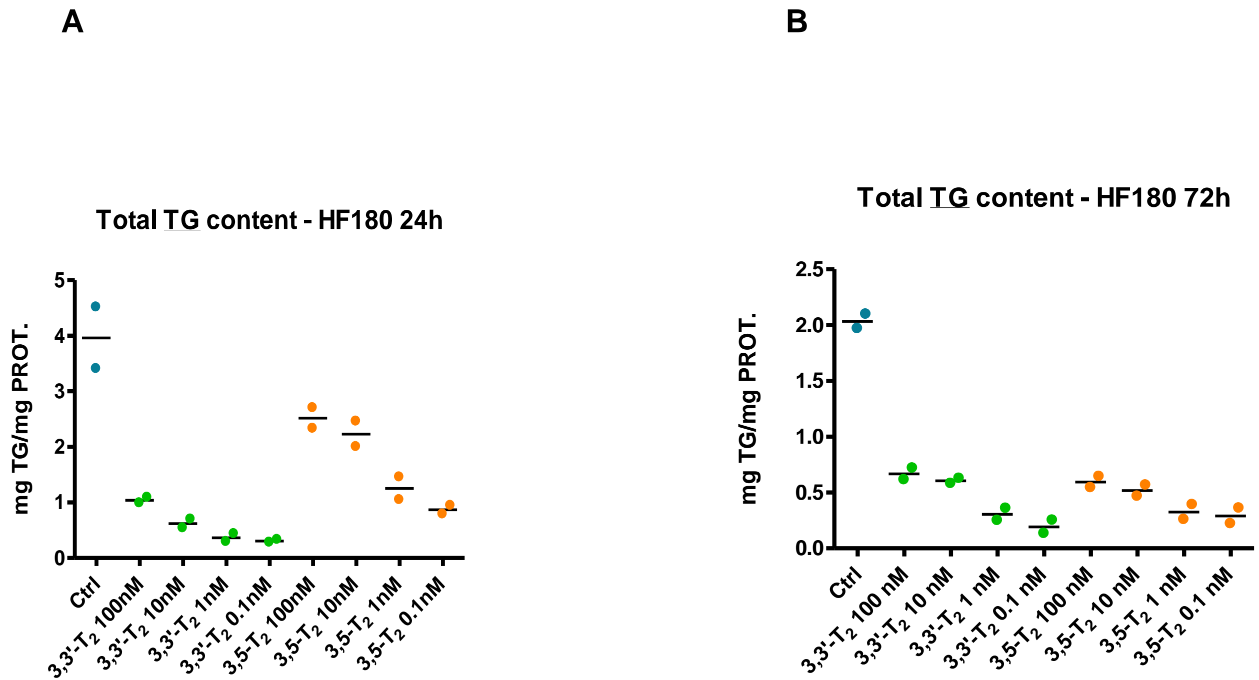


Fig.12 Effect of 3,3'-Diiodothyronine (3,3'-T_2) and 3,5-Diiodothyronine (3,5-T_2) on total triglycerides level in human primary hepatocytes, assessed using a triglycerides determination kit as described in Materials and Methods section. Cells were treated for 24 h (A) and 72 h (B) with 3,3'-T_2 or 3,5-T_2 before extracting lipids, as explained in Materials and Methods section.

Figure 13

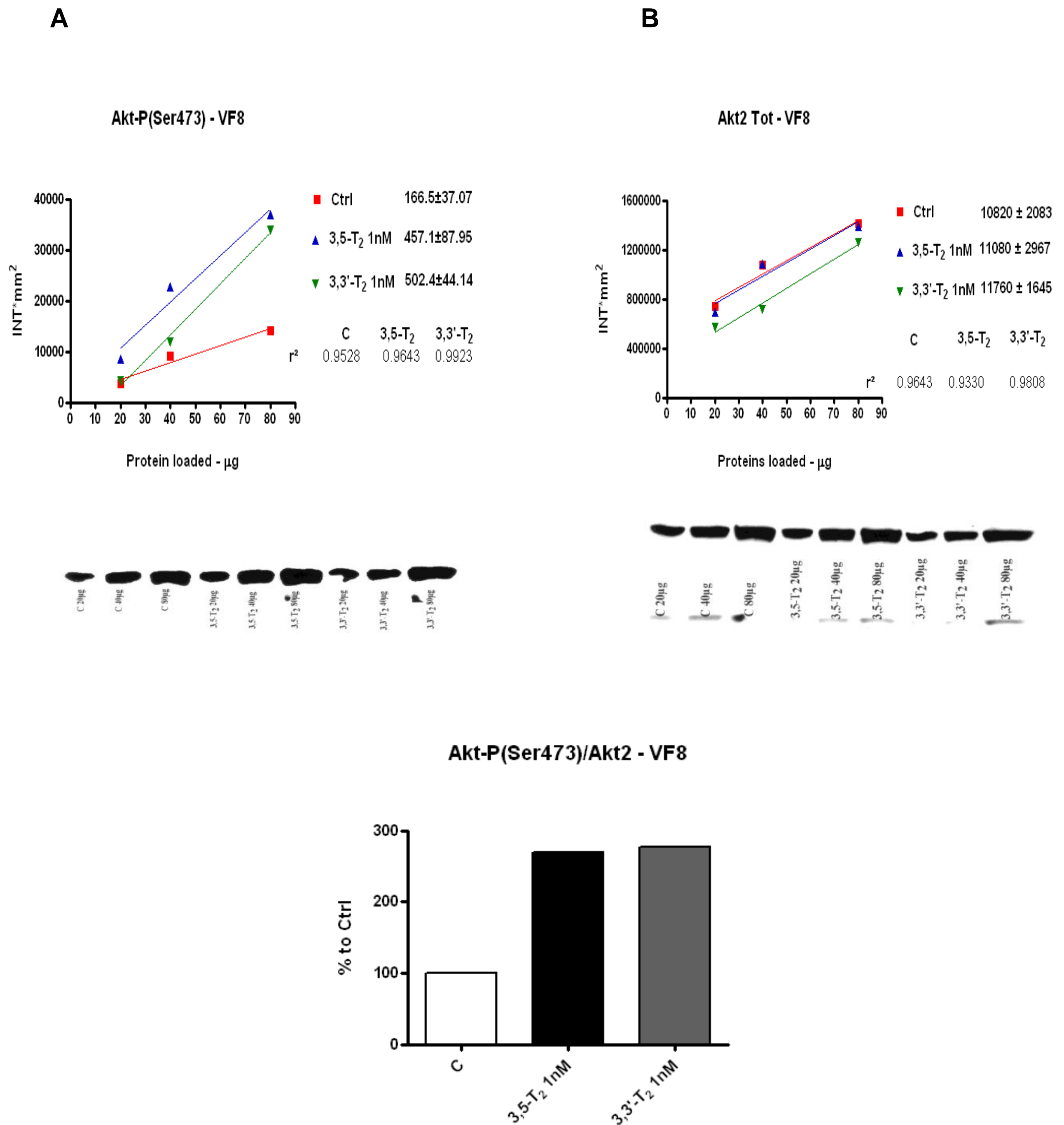


Fig.13 Effect of 3,5-Diiodothyronine (3,5-T_2) and 3,3'-Diiodothyronine (3,3'-T_2) on Akt phosphorylation at Ser473 residue in human primary hepatocytes, assessed by Western Blot. Cells were treated for 10' with 1 nM 3,5-T_2 and 3,3'-T_2. Western Blot was performed according to the procedure described in Materials and Methods section.

Figure 14

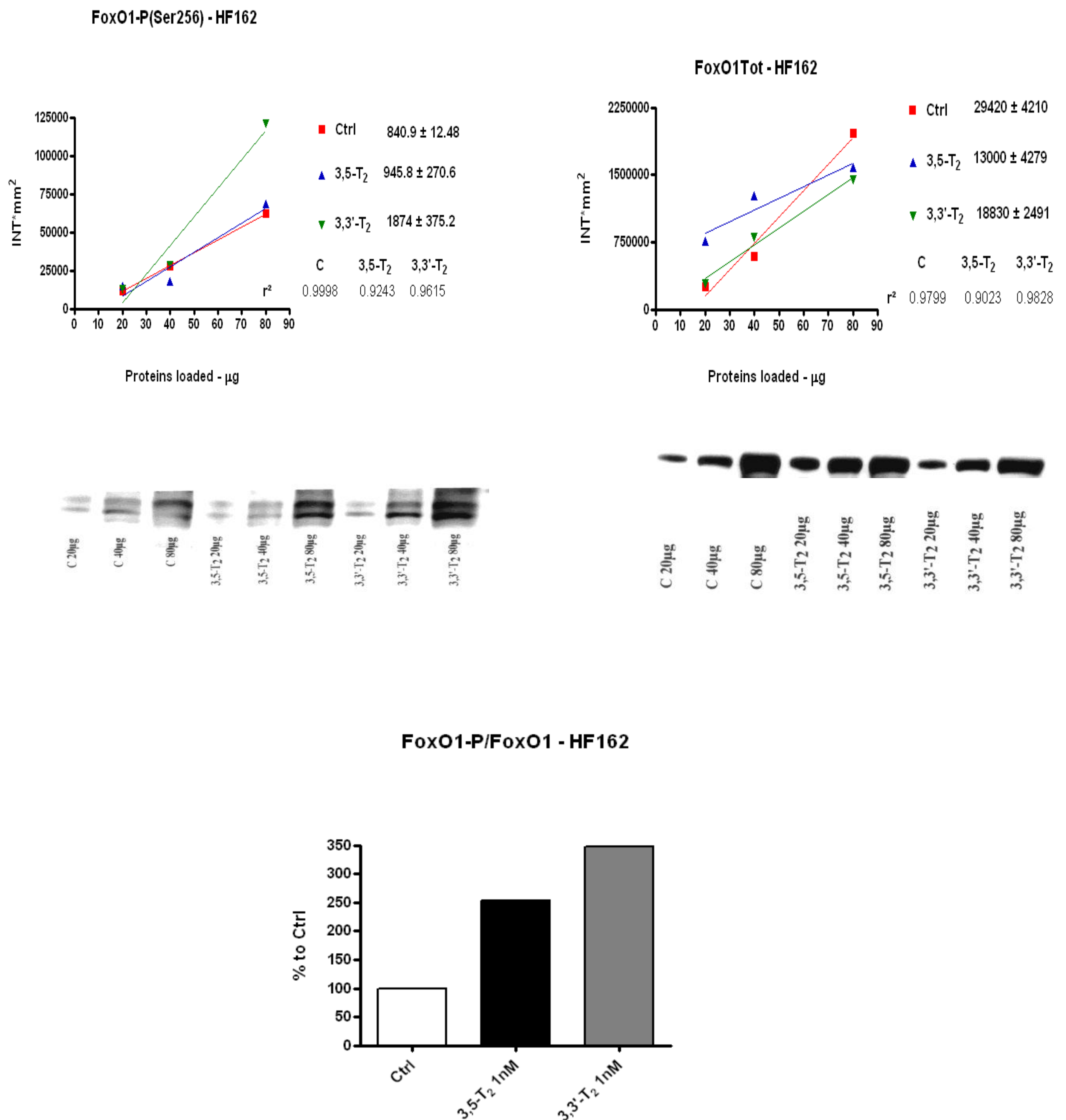


Fig.14 Effect of 3,5-Diiodothyronine (3,5-T_2) and 3,3'-Diiodothyronine (3,3'-T_2) on FoxO1 phosphorylation at Ser256 residue in human primary hepatocytes, assessed by Western Blot. Cells were treated for 10' with 1 nM 3,5-T_2 and 3,3'-T_2. Western Blot was performed according to the procedure described in Materials and Methods section.

Figure 15

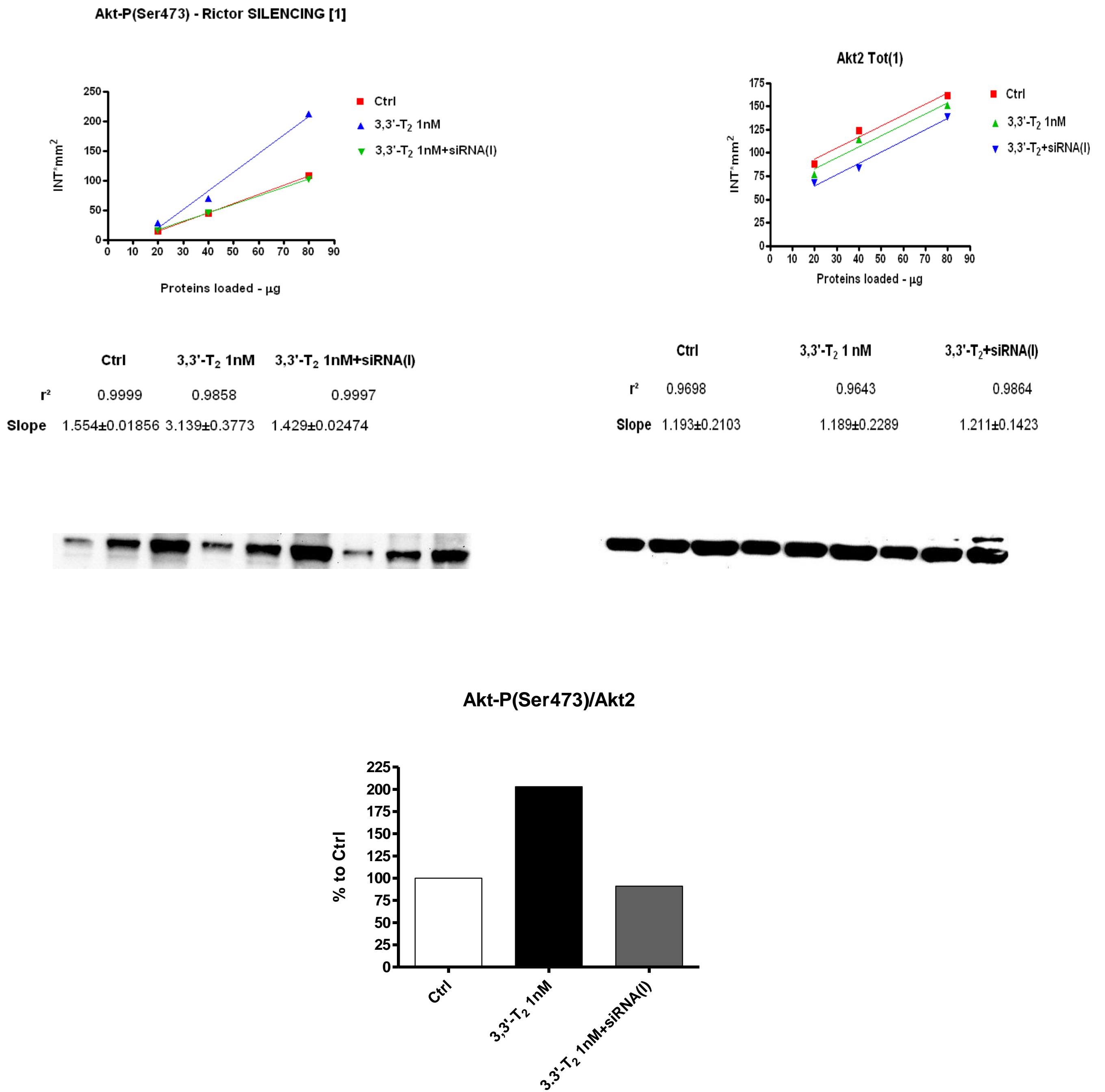


Fig.15 Effect of 3,3'-Diiodothyronine (3,3'-T_2) on Akt phosphorylation at Ser473 residue in human primary hepatocytes after siRNA silencing of Rictor subunit of mTORC2 complex, assessed by Western Blot. Cells were silenced with siRNA(I) and then treated for 10' with 3,3'-T_2 1 nM. Gene silencing and Western Blot were performed according to the procedure described in Materials and Methods section.

Figure 16

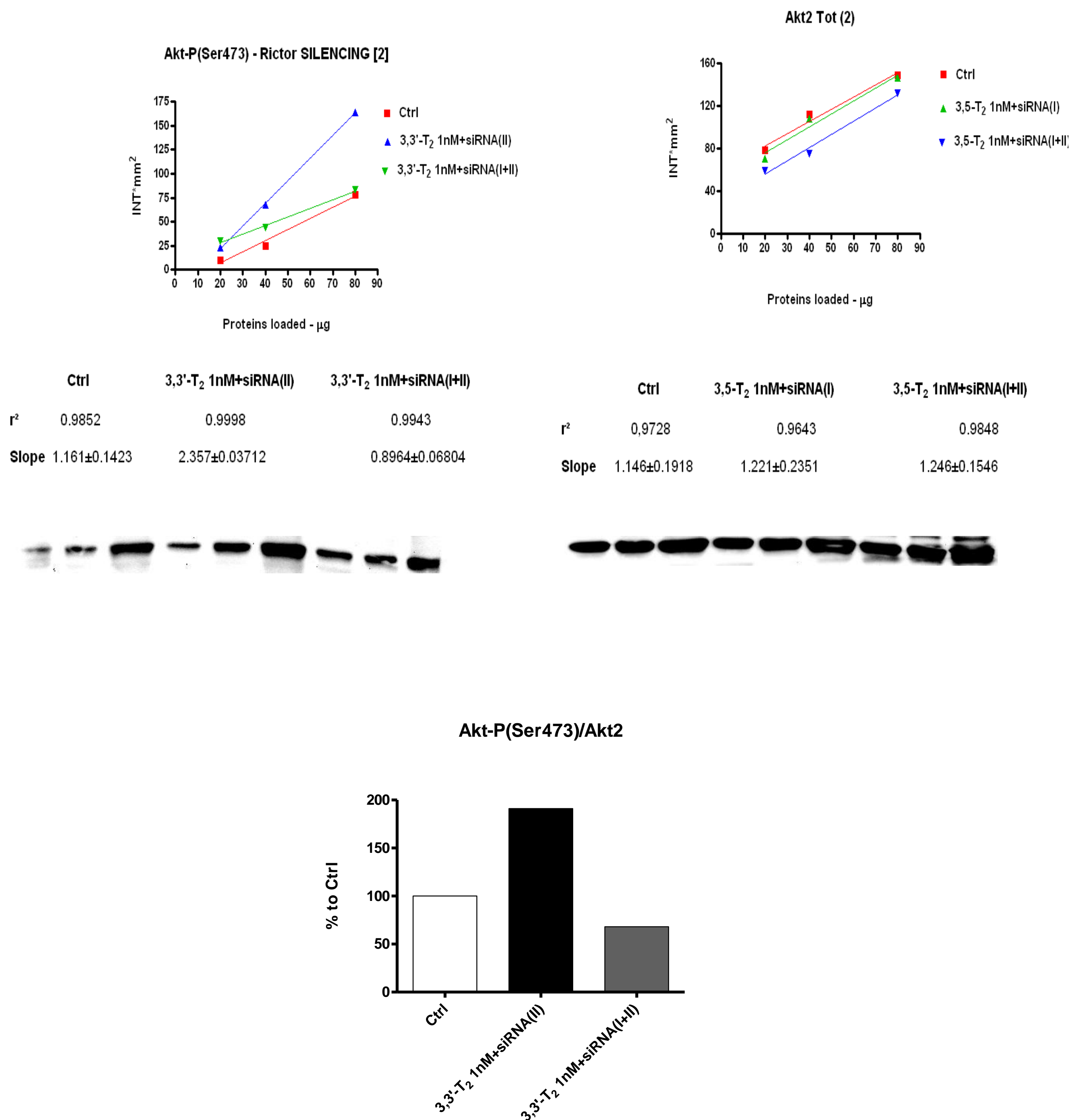


Fig.16 Effect of 3,3'-Diiodothyronine (3,3'-T_2) on Akt phosphorylation at Ser473 residue in human primary hepatocytes after siRNA silencing of Rictor subunit of mTORC2 complex, assessed by Western Blot. Cells were silenced with siRNA(II) and siRNA(I+II), and then treated for 10' with 3,3'-T_2 1 nM. Gene silencing and Western Blot were performed according to the procedures described in Materials and Methods section.

Figure 17

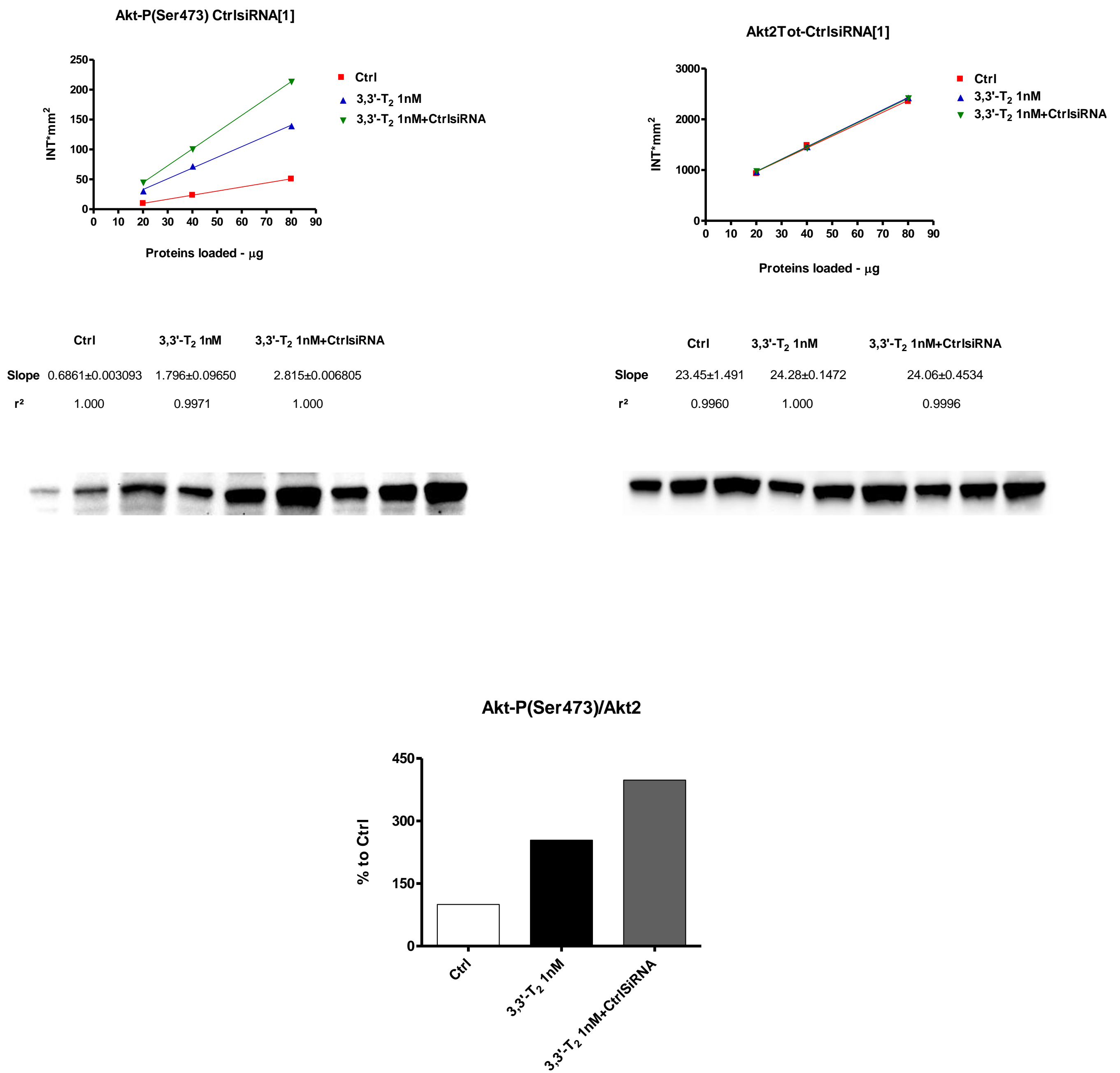
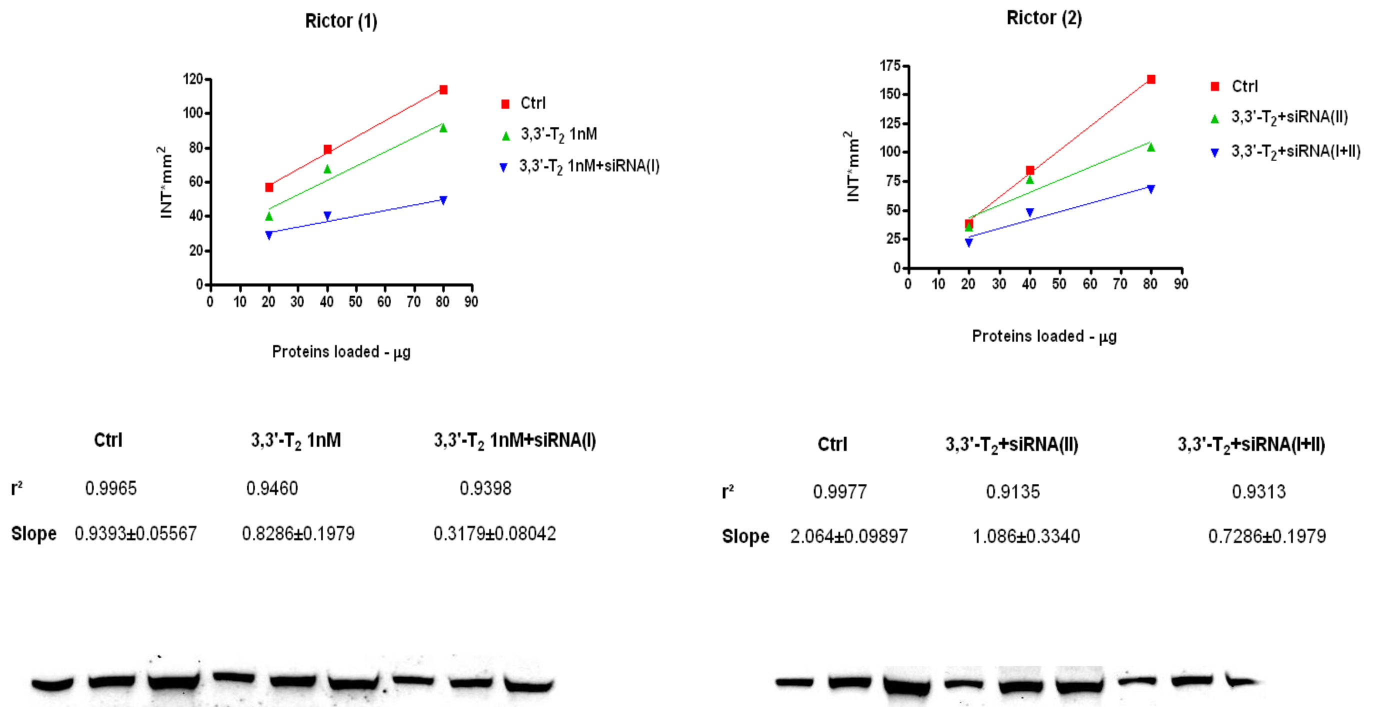


Fig.17 Effect of 3,3'-Diiodothyronine (3,3'-T_2) on Akt phosphorylation at Ser473 residue in human primary hepatocytes after treatment with a CtrlsiRNA (scramble), assessed by Western Blot. Cells were silenced with the CtrlsiRNA and then treated for 10' with 3,3'-T_2 1 nM. Gene silencing and Western Blot were performed according to the procedures described in Materials and Methods section.

Figure 18

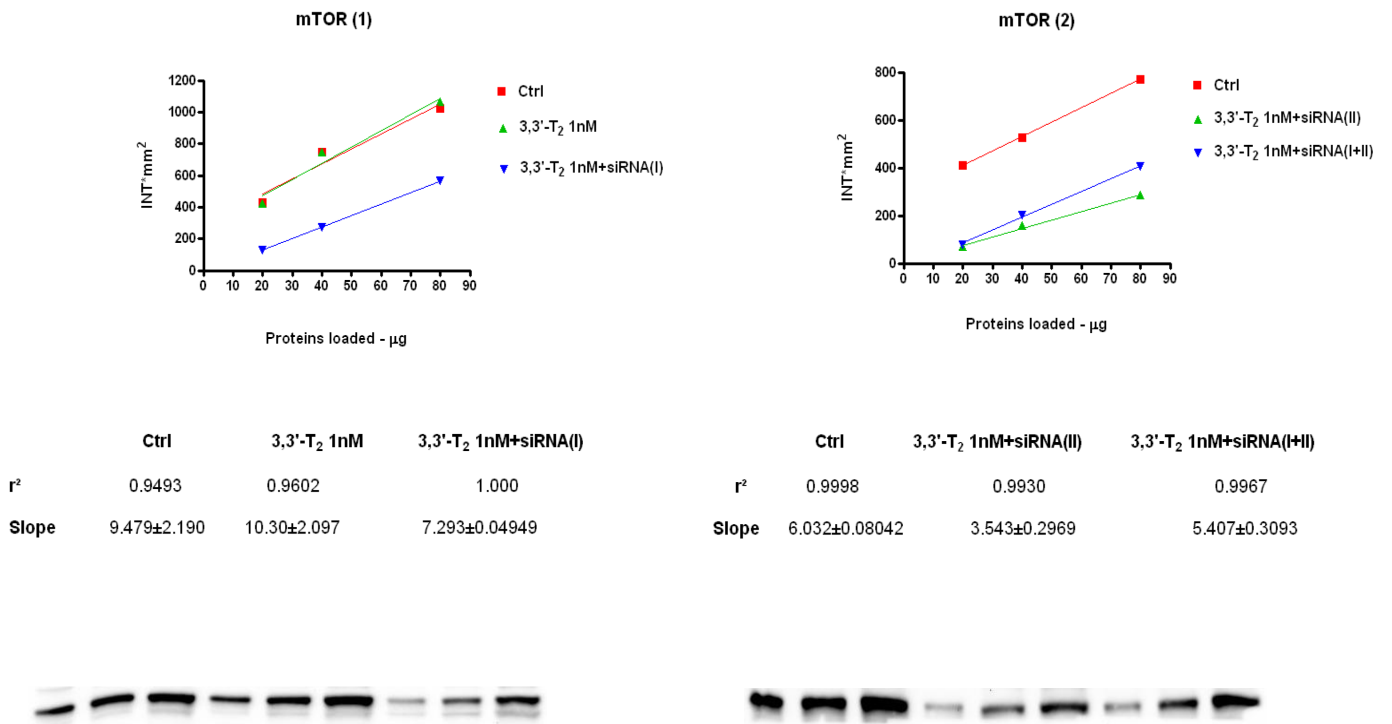


	Ctrl	3,3'-T_2 1nM	3,3'-T_2 1nM+siRNA(I)
r^2	0.9965	0.9460	0.9398
Slope	0.9393±0.05567	0.8286±0.1979	0.3179±0.08042

	Ctrl	3,3'-T_2+siRNA(II)	3,3'-T_2+siRNA(I+II)
r^2	0.9977	0.9135	0.9313
Slope	2.064±0.09897	1.086±0.3340	0.7286±0.1979

Fig.18 Effect of 3,3'-Diiodothyronine (3,3'-T_2) and on the levels of Rictor subunit of mTORC2 in human primary hepatocytes after siRNA silencing of Rictor subunit of mTORC2 complex, assessed by Western Blot. Cells were silenced with siRNA(I), siRNA(II) and siRNA(I+II), and then treated for 10' with 3,3'-T_2 1 nM. Gene silencing and Western Blot were performed according to the procedures described in Materials and Methods section.

Figure 19



	Ctrl	$3,3'$-T_2 1nM	$3,3'$-T_2 1nM+siRNA(I)
r^2	0.9493	0.9602	1.000
Slope	9.479±2.190	10.30±2.097	7.293±0.04949

	Ctrl	$3,3'$-T_2 1nM+siRNA(II)	$3,3'$-T_2 1nM+siRNA(I+II)
r^2	0.9998	0.9930	0.9967
Slope	6.032±0.08042	3.543±0.2969	5.407±0.3093

Fig.19 Effect of 3,3'-Diiodothyronine (3,3'-T_2) on the levels of mTOR in human primary hepatocytes after siRNA silencing of Rictor subunit of mTORC2 complex, assessed by Western Blot. Cells were silenced with siRNA(I), siRNA(II) and siRNA(I+II), and then treated for 10' with 3,3'-T_2 1 nM. Gene silencing and Western Blot were performed according to the procedures described in Materials and Methods section.

DISCUSSION

Taken together the results obtained in rat primary hepatocytes (RPH) show that the system we built can be considered a good experimental model to simulate a steatotic and insulin resistant liver, and hence to test the action of potentially therapeutic molecules.

First of all our data demonstrate for the first time in an *in vitro* system that p85α subunit of PI3K is directly involved in the onset of insulin resistance, as previously described *in vivo* in mice (Mauvais-Jarvis F et al., 2002), in this way providing a new useful tool to study this topic and to test the efficacy of new therapeutic molecules. Analogously, we found an increase in PTEN levels, as already reported in endothelial cells and 3T3-L1 cells (Wang XL et al., 2006; Shen YH et al., 2006; Lee SK et al., 2011), further substantiating our model. Interestingly, we also found an increase in the levels of Akt after oleic acid treatment, but actually we are not able to explain this observation, leaving this topic to successive analysis. In this system 3,5-diiodothyronine in the range of physiological concentrations hugely reduces the amount of triglycerides, and induces phosphorylation of Akt at Ser473 residue, possibly activating insulin signaling pathway through a distinct pathway.

For what concerns the role of fructose, these data support the idea that fructose per se, in the range of physiological concentrations, is not able to induce neither triglycerides accumulation nor insulin resistance, and several studies carried out in *in vivo* systems seem to be coherent with our observations. Studies carried out *in vivo* in rodents shown that high doses of fructose are responsible for an increased hepatic inflammation, in this way favouring the transition from a steatotic condition to steatohepatitis. In fact, in mice it has been demonstrated that a high-fructose diet is able to induce a TNFα-

mediated steatosis and insulin resistance together with an increased neutrophil infiltration, suggesting that fructose may be involved in the onset of steatohepatitis as a proinflammatory agent (Kanuri G et al., 2011). Similarly it has been shown that a high fat coupled to a high fructose diet determines an increased hepatic ROS production and a NASH-like phenotype with significant fibrosis. The mechanism of fibrosis may involve an increased hepatic oxidative stress associated with hepatic macrophage aggregation, which in turn induces a TGFβ1-signaled collagen deposition and histologically evident hepatic fibrosis (Kohli R et al., 2010). Interestingly, a recent study carried out in mice suggests that a chronic intake of high doses of fructose is able to induce a huge bacterial overgrowth at the intestine level, that together with an increased permeability, finally results in an endotoxin-dependent activation of hepatic Kupffer cells, which can contribute to the onset of hepatic insulin resistance through a TNFα-mediated mechanism (Spruss A et al., 2009).

These observations are thus consistent with our data, supporting the idea that fructose exerts detrimental effects only at very high doses, and not through a liver specific mechanism, but rather through systemic effects that trigger an increase of oxidative and inflammatory stress at the whole body level. These factors if associated with the other typical risk factors such as calories surplus coupled with high prevalence of fats in the diet and scarce physical activity, might constitute an additional stimulus towards progression to NASH. Quite unexpectedly, a very recent work demonstrates that in humans small doses of fructose ($\leq$10 g/meal), may exert beneficial effects on glycaemic control without adverse effects on body weight, cardiovascular system, TAG levels, insulin and uric acid (Sievenpiper JL et al., 2012 [a]).

For what concerns the data obtained with human primary hepatocytes (HPH), in my opinion they are very important for several reasons. First of all, the experimental

system used, HPH cultured on Matrigel, represents the most reliable reproduction of a real human liver that is actually possible to achieve, as already described in the analysis of other liver specific metabolic pathways (Ellis E et al., 1998; Ellis E et al., 2001; Olsavsky KM et al., 2007). Secondly, in addition to confirm what observed in RPH for 3,5-diiodothyronine effect on triglyceride content and Akt phosphorylation, these results extend the analysis to FoxO1, in this way clarifying better the signal transduction pathway, and even more interestingly to 3,3'-diiodothyronine. Indeed, this diiodothyronine might be of greater therapeutic relevance if compared to 3,5-diiodothyronine, considering that no thyromimetic effects have been found until now for 3,3'-T_2, whereas it has been established that 3,5-T_2 shares some effects with T_3, even if of lesser magnitude (Ball SG et al., 1997). Thirdly, these data demonstrates for the first time that both 3,5-T_2 and 3,3'-T_2 are able to activate the insulin signaling pathway through an mTORC2 mediated mechanism, opening the way to several other very interesting future directions at basic and applied science level. From a basic science point of view, as described elsewhere in this thesis, no information is present until now about signals that could activate mTORC2, with the exception of a recent paper that demonstrate a direct activation mediated by PIP_3 (Gan X et al., 2011). Thus our data might represent an interesting and useful starting point to deeply analyse this topic, trying to clarify which and how many additional pieces this mosaic owns. Two different hypotheses seem to be particularly promising in our opinion, the first being the potential involvement of a membrane receptor, in analogy to what described about the interaction between thyroxin and triiodothyronine and integrin αVβ3, the second the possible participation of a cytoplasmic TRβ1, as already described for triiodothyronine (Verga Falzacappa C et al., 2007; Verga Falzacappa C et al., 2009).

In the perspective of applied, translational research, these results offer an interesting future pharmaceutical tool to try to cope with obesity and type II diabetes, which are now considered new emerging epidemics in the field of metabolic diseases (Pischon T et al., 2008; Zimmet P et al., 2001). Several therapeutic approaches have been suggested to date for NAFLD, taking in account the different steps involved in the progression of this pathological condition. Primarily, several authors described the beneficial effects of lifestyle modifications, weight loss and physical activity, but also underlining the poor sustainability of weight loss and the difficulty to achieve a maintainable rate of physical activity in all patients (Harrison SA, Day CP, 2007; St. George A, et al., 2009; Johnson NA, George J, 2010). Moreover it is now becoming clear that adiposity, fat distribution and body weight regulation are processes that are, at least in part, under genetic control, suggesting that some people could be more predisposed to develop this kind of pathologies (Morton GJ, et al., 2006; Schwimmer JB et al., 2009; Lindgren CM et al., 2009; Willer CJ et al., 2009). Nowadays several molecules are available that can be used to improve NAFLD clinical picture, such as pioglitazone and vitamin E, even if the results are not completely convincing, and some side effects, such as weight gain, have been described (Sanyal AJ et al., 2010).

In this context the possible future use diiodothyronines, and in particular of 3,3'-diiodothyronine, could offer a new tool in the management of NAFLD, to be used together with the lifestyle modifications described above and maybe with other dietary supplements. Diiodothyronines might have a positive role in improving the overall lipid profile of patients, especially reducing abdominal adiposity and intrahepatic fat, two widely recognised triggering factors of NAFLD, as well as of metabolic syndrome (Després JP, Lemieux I, 2006; Gastaldelli A et al., 2009; Fabbrini E et al., 2009; Fabbrini E et al., 2010). Moreover they may contribute to reactivate the insulin

signaling pathway, bypassing the classical IR-IRS1/2 steps, which are typically blocked in type 2 diabetes condition, as described in detail in the introduction. These aspects, in conjunction with the fact that diiodothyronines, but in particular 3,3'-diiodothyronine, do not show the typical adverse effects that can arise after a thyroid hormone supplementation, permit us to be quite confident that in the future these molecules may help clinicians in improving NAFLD and metabolic diseases as well.

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