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AGE-DEPENDENT CHANGES IN mTOR/S6K1/SREBP-1C SIGNAL PATHWAY ACTIVATION IN RAT LIVER TISSUES

THE MASTER OF SCIENCE THESIS

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ABSTRACT

Yalçın, M. (2019) Age-Dependent Changes in mTOR/S6K1/SREBP-1c Signal Pathway Activation in Rat Liver Tissues, Yeditepe University, Institute of Health Science, Department of Physiology, MSc Thesis, İstanbul.

The liver is one of the most important organs of metabolism, has many different metabolic functions such as bile production, hormone metabolism, blood storage, protein synthesis. The ability of the liver, which can perform different functions in the developmental periods, to perform adult functions through maturation occurs in the postnatal period. The liver has a coordinated set of metabolic functions that are tightly controlled by intracellular signaling pathways. Mechanical target of rapamycin (mTOR); is a serine/tyrosine kinase that regulates protein synthesis, cell growth, survival, and metabolism. Studies have concluded that mTOR expression plays a role in functions such as glucose homeostasis, lipogenesis and ketogenesis in the liver. mTOR regulates hepatic de novo lipogenesis (DNL) via Sterol Regulatory Element Binding Protein-1c (SREBP-1c), which controls the expression of genes responsible for the regulation of lipid metabolism. Proper control of lipid levels by the liver is crucial for metabolic homeostasis, since dysfunctions in the hepatic lipogenic pathway modulated by mTOR/S6K1/SREBP-1c signaling contribute to the pathogenesis of many chronic diseases. Although age-related changes in hepatic lipid metabolism are known to contribute to the pathogenesis of many diseases, there is no study of age-related activation changes of hepatic mTOR/S6K1/ SREBP-1c that can control this metabolic process. In this study, it was aimed to determine age-related changes in activation of mTOR/S6K1/SREBP-1c pathway by Western Blot method and to evaluate age-related lipid localization by staining with Oil Red O and Hematoxylin-Eosin in rat liver tissues. As a result, total mTOR and S6K1 levels increased in the 14-day group compared to the 0-day group, decreased significantly in the 28-day, 3-and 6-month period, and also the phosphorylation level of both proteins (p-mTOR, p-S6K1) and total SREBP -1c expression showed a similar pattern. In this study, age-related changes in the mTOR/S6K1/SREBP-1c signal pathway activity which is responsible for hepatic DNL have been observed.

Key words: liver, mTOR, SREBP-1c, ageing

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ÖZET

Yalçın, M. (2019). Sıçan Karaciğer Dokularında mTOR/S6K1/SREBP-1c Sinyal Yolu Aktivasyonunda Yaşa Bağımlı Değişiklikler, Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Fizyoloji Anabilim Dalı, Yüksek Lisans Tezi, İstanbul.

Metabolizmanın en önemli organlarından biri olan karaciğer, safra üretimi, hormon ve vitamin metabolizması, kanın depolanması, protein sentezi gibi birçok farklı metabolik işlevi yerine getirir. Gelişim dönemlerinde farklı işlevler gerçekleştirebilen karaciğerin maturasyonu ile yetişkin fonksiyonlarını gerçekleştirebilmesi postnatal dönemde gerçekleşir. Karaciğerde hücre içi sinyal yolları ile sıkı bir şekilde kontrol edilen koordineli bir dizi metabolik fonksiyon vardır. mTOR (rapamisinin mekanik hedefi); protein sentezi, hücre büyümesi, hayatta kalma, proliferasyon ve metabolizmayı düzenleyen bir serin / tirozin kinazdır. Araştırmalar, mTOR ekspresyonunun karaciğerde glukoz homeostazı, lipogenez ve ketogenez gibi fonksiyonlarda rol oynadığını göstermiştir. mTORC1, lipid metabolizmasının düzenlenmesinden sorumlu genlerin ekspresyonunu kontrol eden sterol düzenleyici element bağlayıcı protein-1c (SREBP-1c) yoluyla hepatik de novo lipogenezi (DNL) organize eder. Karaciğer tarafından lipid seviyelerinin doğru şekilde düzenlenmesi, metabolik homeostaz için çok önemlidir, mTOR/S6K1/SREBP-1c sinyalleşmesi ile modüle edilebilen hepatik lipojenik yoldaki işlev bozuklukları, birçok kronik hastalığın patogenezine katkıda bulunabilir. Her ne kadar hepatik lipid metabolizmasındaki yaşa bağlı değişikliklerin birçok hastalığın patogenezine katkısı bilinmesine rağmen, bu metabolik süreci kontrol edebilen hepatik mTOR/S6K1/SREBP-1c 'nin yaşa bağlı aktivasyon değişiklikleri hakkında bir çalışma yoktur. Bu tez çalışmasında sıçan karaciğer dokularında mTOR/S6K1/SREBP-1c sinyal yolunun aktivasyonundaki yaşa bağlı değişikliklerin Western Blot metodu ile belirlenmesi ve Oil Red O ve Hematoksilen-Eozin boyaması ile yaşa bağlı lipid lokalizasyonunun değerlendirilmesi amaçlanmıştır. Sonuç olarak, total mTOR ve S6K1 seviyeleri 14 günlük grupta, 0 günlük gruba göre artmış, 28 günlük, 3 ve 6 aylık gruplarda anlamlı derecede azalmış ve aynı zamanda her iki proteinin fosforilasyon seviyeleri (p - mTOR, p-S6K1) ve toplam SREBP-1c ifadesi de benzer bir patern izlemiştir. Bu çalışmada, hepatik DNL'den sorumlu mTOR/S6K1/SREBP-1c sinyal yolu aktivitesinde yaşa bağlı değişimler olduğu gözlenmiştir.

Anahtar kelimeler: karaciğer, mTOR, SREBP-1c, yaşlanma

SYMBOLS / ABBREVIATIONS

ACC	Acetyl-CoA carboxylase enzyme
ACLY	ATP-Citrate Lyase
AKT	Protein Kinase B
ChREBP	Carbohydrate Responsive Element Binding Protein
DNL	De Novo Lipogenesis
ELOVL6	Elongation of Very Long Chain Fatty Acids 6
FA	Fatty Acid
FAS	Fatty Acid Synthase
FOXO1	Fork Head Box Protein O1
G6PC	Glucose-6-Phosphatase Catalytic Subunit
GK	Glucokinase
GLUT2	Glucose Transporter-2
GPDH	Glycerol-3-Phosphate Dehydrogenase
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
L-PK	Liver-Pyruvate Kinase
mSin1	Mammalian Stress-Activated Protein Kinase Interacting Protein
mTOR	Mechanistic Target of Rapamycin
mTORC1	Mechanistic Target of Rapamycin Complex 1
mTORC2	Mechanistic Target of Rapamycin Complex 2
NASH	Non-Alcoholic Steatohepatitis
PI3K	Phosphatidylinositol-3 Kinase
Raptor	Regulatory-Associated Protein of mTOR
Rictor	Rapamycin-Insensitive Companion of mTOR

S6K1	Ribosomal Protein S6 Kinase 1
SCAP	SREBP Cleavage Activating Protein
SCD-1	Stearoyl-CoA Desaturase-1
SREBP	Sterol Regulatory-Element Binding Protein
SREBP-1c	Sterol Regulatory Element-Binding Protein-1c
TCA	Tricarboxylic Acid
TG	Triglyceride
VLDL	Very Low-Density Lipoprotein

1. INTRODUCTION and PURPOSE

As one of the most important organs in the metabolism, liver carries out many different metabolic functions such as bile production, hormone metabolism, storage and filtration of blood, protein synthesis. It has a very high metabolic rate and receives 27% of cardiac output per minute. Liver can perform different functions in different stages of the developmental life and its maturation which is necessary for the fulfillment of functions in adults is completed in the after-birth period. From the postnatal period, physiological changes occur in the liver at different age periods and finally reach maturation in the adult (1). There is a coordinated series of metabolic functions in the liver which is tightly regulated by intracellular signaling pathways. mTOR (mechanical target of rapamycin); is a serine/tyrosine kinase that regulates protein metabolism, survival, growth, proliferation, and metabolism (2). It is controlled by the energy level of the cell, growth and different stress factors in the downstream signal transduction pathway of the PI3K/Akt kinase chain. mTOR is a component of two different multiprotein complexes called complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Several studies have concluded that mTOR pathway expression has important effects on the development of hepatic metabolic functions, glucose homeostasis, lipogenesis and ketogenesis (3). mTORC1 organizes hepatic de novo lipogenesis (DNL) via the sterol regulatory element-binding protein-1c (SREBP-1c), which regulates the transcription of the genes involved in lipid metabolism. Proper control of the lipid levels by the liver is crucial for metabolic homeostasis, since the disfunctions in the hepatic lipogenic pathway which can be modulated by mTOR/S6K1/SREBP-1c signaling, contribute to the pathogenesis of many chronic diseases, from hepatic steatosis to neurodegenerative diseases. Although the contribution of age-related changes in hepatic lipid metabolism to the pathogenesis of many diseases such as nonalcoholic steatohepatitis (NASH), insulin resistance as well as cancer is known, there is no study about age-related activation changes of hepatic mTOR/S6K1/SREBP-1c signaling, which can control this metabolic process from the neonatal period to adulthood. In this thesis, it is aimed to evaluate the age-dependent changes in the activation of mTOR/S6K1/SREBP-1c signaling pathway by Western Blot method and to evaluate age-related lipid localization with Oil Red O staining in rat liver tissues of 5 different age groups which are from different developmental stages of life.

2. GENERAL INFORMATION

2.1. Liver Anatomy and Physiology

The liver is one of the largest glands and accounts for 2% of an adult human's weight. It is located under the diaphragm in the right part of the abdominal region and is surrounded by the rib cage.

The anatomy of the liver can be examined morphologically and functionally. Morphologically, the two lobes, the right and the left lobe, are divided by falciform and teres ligaments. The falciform ligament extends vertically between the diaphragm and the liver; the teres ligament widens along the under the falciform ligament starting from the special cleft on the visceral (lower) face of the liver. The ligament extending between the diaphragm and the liver along the frontal plan is called the coronary ligament. There are quadrate and caudate lobes on the visceral side of the right lobe. These lobes are separated by a short and deep fissure called porta hepatis. Porta hepatis is a transverse groove that serves as a gateway for hepatic artery, portal vein, hepatic ducts, lymphatic vessels and nerves. In the functional anatomy, liver parenchyma is divided into 8 segments according to the principles of blood supply and bile drainage. The lobes are covered with a fibrous layer called Glisson's capsule (4).

The basic functional and morphological unit of the liver is called a lobule which is 0.8-2 millimeters in size. There are about 50.000-100.000 lobules in polygonal shape and cylindrical structure in the human liver. In the center of the liver lobule, there is a central vein that empties to the hepatic vein and then empties into the vena cava and at the corner of the lobules, there are portal channels with a portal triad composed of portal vein, hepatic artery and bile duct. Liver cells (hepatocytes) that fill the lobule areas are organized into cell cords extending from a portal area to a central vein in two rows. Among these cords, there are sinusoid areas where blood flow occurs from portal areas to central vein. Sinusoids are surrounded by endothelial cells. The narrow area between the hepatocytes and endothelial cells is called the disse gap (5).

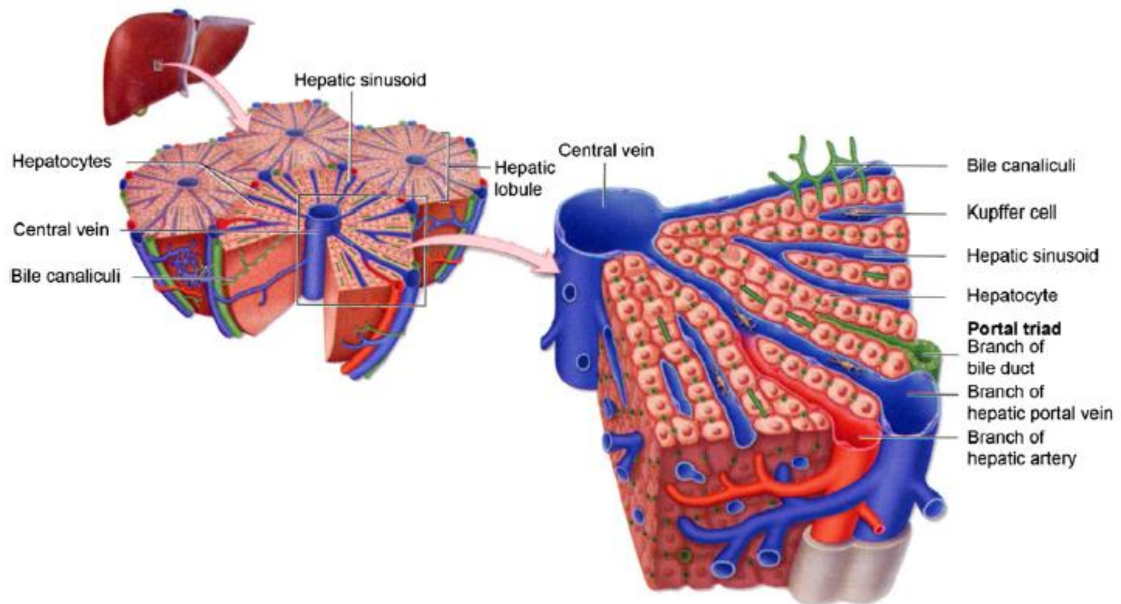

Figure 1. Detailed structure of the liver and hepatic subunits (6).

The liver, which has a very high metabolic rate, receives 27% of the cardiac output per minute (1350 ml / min). While other organs receive blood only from the arterial system, the liver receives it by both arterial (hepatic artery) and venal system (portal vein). Clean blood (300 ml / min) from the hepatic artery provides liver oxygenation. The portal vein is responsible for transferring nutrient-enriched venous blood (1050 ml / min) from the intestine, spleen and pancreas to the liver sinusoids. Thus, nutrients absorbed from the digestive tract are processed by hepatocytes according to the needs of other body systems and stored temporarily. The portal vein transports the nutrients as well as substances that may harm the organism, such as drugs, toxic agents, bacteria from the gastrointestinal tract to the liver. These substances are phagocytosed in the perisinusoidal area by macrophage-type cells called Kupffer cells of the liver to prevent their participation in the systemic circulation. Blood flow from the sinusoids to the central vein reaches the heart via the hepatic veins draining the inferior vena cava (7) . The hepatic sinusoid pores and epithelium being very permeable causes too much lymph formation in the liver. The lymph produced by the liver is collected by the hepatic lymph nodes and transferred to the thoracic canal.

The liver has a very rich nerve innervation network. Sympathetic and parasympathetic nerve fibers come from celiac plexus, phrenic nerve, thoracic ganglia, and vagus nerve. Hepatic sympathetic and parasympathetic nerves are effective on regulating a wide range of hepatic functions. Its nervous system involves afferent neurons which can sense of nutrients and metabolites in the postprandial term, and the efferent neurons which are responsible for metabolic regulation, biliary function and other hepatic physiological changes for the homeostasis of the whole-body (8).

The liver has various cell types consists of hepatocytes, Kupffer cells, endothelial cells, and stellate cells. Hepatocytes are the primary group of parenchymal cells that form the basic functional unit and they are 80% of all the cells in the liver (9). These cells are responsible for numerous critical metabolic functions such as macromolecule synthesis, lipid metabolism, detoxification, storage and production of bile. Hepatocytes filling lobule areas are evaluated in 3 different zonal regions according to their proximity to the portal area, their metabolic activities and their degree of perfusion. Zone 1 is the best blooded area since it has portal vein-hepatic artery branches. Hepatocytes in Zone 1 play a more active role in tasks such as beta-oxidation, gluconeogenesis, bile secretion, cholesterol production and amino acid catabolism associated with oxidative metabolism due to their high perfusion rates. Zone 2, a transition area between Zone 1 and Zone 3, is defined as the pericentral region of hepatocytes. Zone 3 represents the central vein circumference and it is the least perfused site due to its distance from the portal triad region. Hepatocytes in this region are mostly responsible for detoxification, ketogenesis, glycolysis, lipogenesis (10).

The liver with its rich vascularization, endothelial cells covers the inner surface of the sinusoids and vessels. Kupffer cells, which are part of the reticuloendothelial system, are macrophage type cells located in the perisinusoidal area. It provides the blood-clearing function of the liver by phagocytizing the foreign substances and bacteria in the venous blood flowing to the liver sinusoids before they enter the systemic circulation. It contributes to the formation of cellular inflammatory response by secreting various cytokines. Another group of cells is the Stellate cells (Ito cells), which are also known as fat cells (lipocytes) and found in the disse space. They contain high amounts of retinol, perform functions such as vitamin A storage and extracellular matrix production.

The liver originates from the hepatic diverticulum developing from the endoderm of the anterior intestine at the 4th week of the embryonic period. Hepatoblasts in the liver bud that develop from hepatic diverticulum differentiate into both hepatocytes and cholangiocytes with bile epithelial cells. While the sinusoids in the liver lobule are formed by angiogenesis, Kupffer cells originate from the mesenchyme (11).

Bile secretion in the liver starts in the 12th week of the gestation period and it takes approximately 2 years after birth to reach full maturity (12). Although mammalian tissues begin to form and function in the embryological period, the adaptation and maturation process which is necessary for to perform adult functions takes place after birth. This process is particularly important in the liver, which acts as a hematopoietic tissue in the embryo, then becomes one of the major metabolic organs in the adulthood with large changes in gene expression (13).

The basic structures of the rat and the human livers are similar; both consist of left lateral, medial; right lateral, medial and caudal lobes. The rat does not have a gall bladder and a long bile duct is poured into the duodenum through the pancreas (5).

2.1.1. Liver Functions

The liver is a vital organ that has numerous roles in many organ systems and enables these systems to communicate with each other. The liver has a central role in the modulation and maintenance of metabolic homeostasis and is known to have more than 500 metabolic functions. Hepatocytes are the liver cells that provide these functions such as bile production, hormone and vitamin metabolism, blood storage and filtration, bilirubin excretion, macromolecule synthesis, biotransformation of drugs, detoxification and so on. Vitamins A, D, E, K, B12 are stored in the liver. The process of metabolic activation of vitamin D starts in the liver. Excess iron in the body is combined with the molecule of apoferritin and stored by the liver in the form of ferritin. Bile production and secretion is one of the most important tasks of the liver. Bile plays a role in the emulsification of lipids as a digestive secretion, as well as the excretion of metabolic end products. Bile fluid has water, plasma electrolytes, bile acids, phospholipids, cholesterol and bilirubin components. Following the destruction of erythrocytes in the organs of the reticuloendothelial system (liver, spleen, bone marrow), bilirubin, which is one of the end products of hemoglobin, is transferred to the liver and conjugated with glucuronic acid. Chemical alteration or detoxification of drugs, hormones and other bioactive substances

is carried out by the liver (9). The liver plays in carbohydrate, lipid and protein metabolism are explained below related to liver metabolic functions.

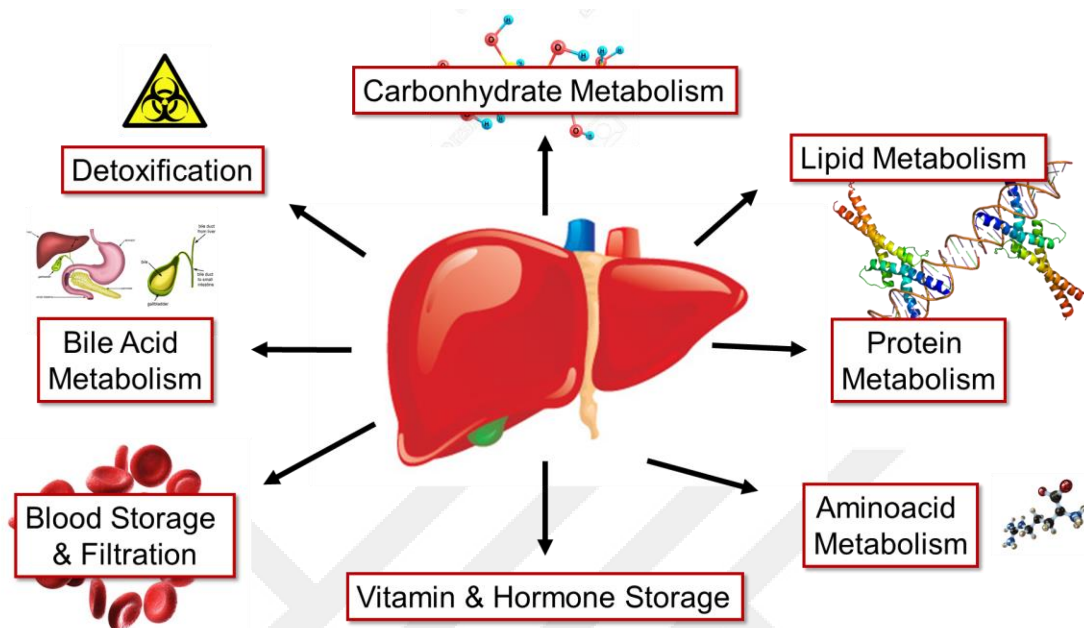

Figure 2. Liver functions.

2.1.1.1. Carbohydrate Metabolism

The liver is highly critical in regulating and maintaining normal blood glucose levels. In fasting, glycogen stores are converted to glucose by hydrolysis with glycogen phosphorylase (glycogenolysis). If the fasting period increases, hepatocytes synthesize glucose (gluconeogenesis) by using substrates such as lactate, pyruvate, amino acids as glycogen stores will be depleted eventually. Gluconeogenesis is stimulated by cortisol and glucagon and inhibited by insulin. In the fed-state, glucose in the blood is taken into hepatocytes via GLUT2 under insulin control, followed by phosphorylation with the enzyme glucokinase and then used to synthesize glycogen (13).

2.1.1.2. Protein Metabolism

In the liver, amino acids are catabolized for energy or used to synthesize of protein and glucose. It may be said that, without the contribution of the liver to protein metabolism, survival is not possible. Except a portion of gamma globulins, plasma proteins, such as albumin, prothrombin, fibrinogen, and lipoproteins, and coagulation

factors are synthesized in hepatocytes. Protein synthesis is stimulated by insulin and growth hormone. In addition, all non-essential amino acids can be synthesized in the liver.

Amino acids need to be converted into α-ketoacids prior to conversion to glucose and lipid molecule. This process requires deamination by separation of an amino group into ammonia. Although deamination occurs in the kidney and other tissues, it occurs primarily in the liver. The ammonia compound produced during amino acid catabolism is toxic to cells and needs to be removed from the body. The liver converts ammonia to urea, and excretes it and prevents the blood pH alterations (14).

2.1.1.3. Lipid Metabolism

The liver has numerous metabolic reactions in lipid metabolism such as lipogenesis, secretion of lipoproteins, fatty acid (FA) oxidation and ketogenesis. These biochemical functions of lipid metabolism are predominantly carried out by hepatocytes. Proper regulation of hepatic lipid metabolism requires a balance between FA synthesis and their oxidation or export to extrahepatic tissues in form of triglycerides (TGs). The energy which is required by the whole-body systems is formed via beta-oxidation of FAs in the mitochondria of hepatocytes. This function does not only provide energy, but also generates ketone bodies (acetone, acetoacetate, ß-hydroxybutyrate) by a process called ketogenesis that provide energy to the extrahepatic organs during starvation (15).

The liver has also anabolic functions and it is one of the main areas of endogenous cholesterol synthesis. Cholesterol is involved in the cell membrane structure, bile acid, and important biological materials such as vitamin D and the precursor of certain steroid hormones. Phospholipid molecules are also highly synthesized in the liver and function as the building blocks of the cell membrane. Lipoproteins, which includes cholesterol esters and apolipoproteins, are macromolecules that transport lipids to cells and are mostly synthesized in the liver (14).

The liver is also able to synthesize fatty acids from excess glycolytic products during the fed state through de novo lipogenesis (DNL). Pyruvate is the main glycolytic product which is a link between glucose and lipid metabolism, is converted to acetyl-CoA molecule in the cytosol of hepatocyte. Acetyl-CoA, as a main substrate of de novo lipogenesis, is a start point of this process. The fatty acids formed by the DNL is then exported in a form of very low-density lipoprotein (VLDL) and stored as triglyceride in

the peripheral tissues. DNL is mostly occurred in the liver and adipose tissue in humans and rodents. The enzyme systems in the DNL process are tightly controlled under post-translational and transcriptional mechanisms by insulin, other metabolic hormones and substrate level, especially glucose. In the fed state, high blood glucose and insulin level activate transcription factors such as Sterol Regulatory Element-Binding Protein-1c (SREBP-1c) and Carbohydrate Responsive Element-Binding protein (ChREBP). These transcription factors promote de novo lipogenesis by enhancement of lipogenic gene expressions in the liver (15, 16).

2.1.1.3.1. Hepatic De Novo Lipogenesis

De novo lipogenesis occurs primarily in the liver and is tightly controlled by hormones and nutritional status. During the fed state after a high-carbohydrate meal, glucose molecules are taken into hepatocytes via Glucose Transporter-2 (GLUT-2) protein. Initially, the ingested glucose is converted to the pyruvate molecule by a series of enzymes through the glycolysis reaction in the cytosol of hepatocytes. The glycolysis step provides a carbon source for fatty acid synthesis via DNL. Following glycolysis, the pyruvate enter into the mitochondria and is converted into the citrate molecule by participating in the tricarboxylic acid (TCA) cycle. The citrate is then exported to cytosol from mitochondria and is used for acetyl-CoA formation via ATP-citrate lyase (ACLY) enzyme. With the formation of acetyl-CoA, de novo lipogenesis starts. A carboxyl group is added to the acetyl-CoA molecule by the acetyl-CoA carboxylase enzyme (ACC) to form the malonyl-CoA subunit. Subsequently, 16-carbon long chain saturated fatty acid Palmitate (C16:0) is synthesized from malonyl-CoA molecule via fatty acid synthase (FAS). The palmitate may be desaturated by stearoyl-CoA desaturase-1 (SCD-1) in order to produce palmitoleic acid or it may provide stearic acid (C18:0) by elongation mechanism. Finally, stearic acid is converted to oleic acid (C18:1) through desaturation reaction, which is the end product of DNL. The fatty acids are then esterified into triglycerides and transported to white adipose tissue via VLDL for storage (17).

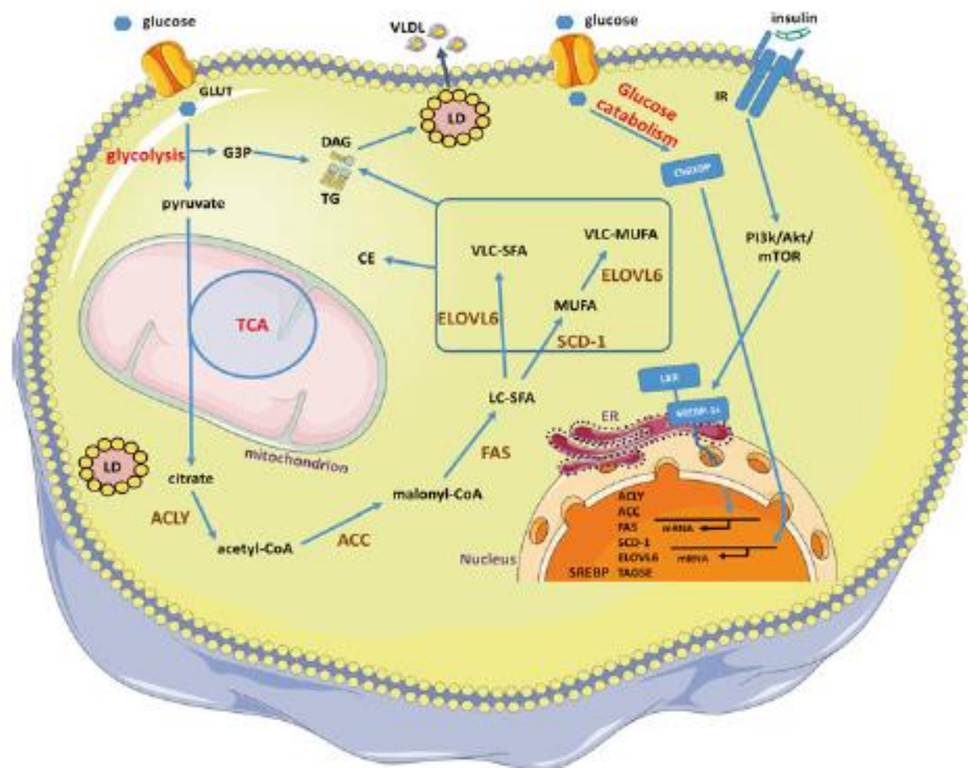

Figure 3. Hepatic De Novo Lipogenesis in the hepatocyte (18).

2.1.1.3.2. Signaling Pathway in the Hepatic De Novo Lipogenesis

As stated above, there is a coordinated series of enzyme reactions in the hepatic DNL process which is tightly regulated by intracellular signaling pathways. Activities of the enzymes in this process are controlled by post-translational and transcriptional mechanisms induced mainly by insulin and glucose (19). In the post-translational level, high blood glucose and insulin levels activate two key transcription factors Carbohydrate Responsive Element-Binding protein (ChREBP) and Sterol Regulatory Element-Binding Protein-1c (SREBP-1c) respectively to promote de novo lipogenesis by enhancement of lipogenic genes expression in the liver. ChREBP is induced by increased blood glucose and/or fructose level and does not only promotes the expression of the enzymes in glucose metabolism such as liver-pyruvate kinase (L-PK), glucose-6-phosphatase catalytic subunit (G6PC), GLUT4, and glycerol-3-phosphate dehydrogenase (GPDH) but also lipogenic enzymes like ACLY, ACC, FAS and SCD1. As blood glucose levels increase, insulin production and secretion are enhanced in the pancreas. High blood insulin level then activates SREBP-1c, which is the main regulator of de novo lipogenesis in the

translation of the hepatic lipid metabolism enzymes such as glucokinase (GK), ACC, FAS, SCD1, ELOVL fatty acid elongase 6 (ELOVL6).

As mentioned, insulin is the primary hormone which stimulates hepatic lipogenesis in the fed state. It binds the insulin receptor (IR) on the cell membrane which is found mainly on the cell surface of metabolically active tissues like skeletal muscles, liver, and adipose tissue. Subsequently, insulin-binding to IR results in autophosphorylation of the receptor followed by phosphorylation of insulin receptor substrate (IRS) on tyrosine residues. The phosphorylated IRS triggers protein kinase B (AKT) phosphorylation through phosphatidylinositol 3 kinase (PI3K) pathway. AKT increases the expression of two transcription factors under insulin stimulation such as forkhead box protein O1 (FOXO1) which is responsible for the regulation of gluconeogenic genes expression and SREBP-1c, which is the main regulator of lipogenic gene expression. FOXO1 is directly phosphorylated and inhibited by AKT, while SREBP-1c is regulated by the mammalian / mechanistic target of rapamycin complex 1 (mTORC1) in the downstream of AKT (20, 21).

2.2. Sterol Regulatory Element-Binding Protein-1c (SREBP-1c)

Sterol regulatory-element binding proteins (SREBPs) are transcription factors that control the expression of genes involved in lipid synthesis and they are synthesized in the endoplasmic reticulum as inactive precursors. These precursors bind to SREBP cleavage-activating protein (SCAP) which mediates their transport from the ER to the Golgi apparatus, then proteolytically cleaved to release transcriptionally-active SREBPs (16).

There are three isoforms of SREBPs (SREBP-1a, -1c and -2) with distinctive physiological roles. SREBP1a has functions in the lipid synthesis and growth; SREBP1-c activates the genes that control fatty acid and triglyceride synthesis for energy storage; SREBP2 is responsible for the genes that control cholesterol synthesis. SREBP-1c and SREBP-2 are expressed in the liver in abundance (15).

2.3. Mammalian / mechanistic target of rapamycin (mTOR)

mTOR is a serine / tyrosine kinase (molecular weight = ~289 kDa) that regulates protein synthesis, cell growth, survival, proliferation, and metabolism (2). It is controlled by the energy level of the cell, growth and different stress factors in the downstream signal transduction pathway of the PI3K/Akt kinase chain. mTOR is a component of two different multiprotein complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Some protein components of these mTOR complexes are in common, but they also have unique components which differs their activities from each other: regulatory-associated protein of mTOR (Raptor), rapamycin-insensitive companion of mTOR (Rictor), and mammalian stress-activated protein kinase interacting protein (mSin1). Rapamycin inhibits mTORC1 activation, whereas mTORC2 is insensitive to acute rapamycin treatment, although prolonged rapamycin treatment may disrupt mTORC2 signalization.

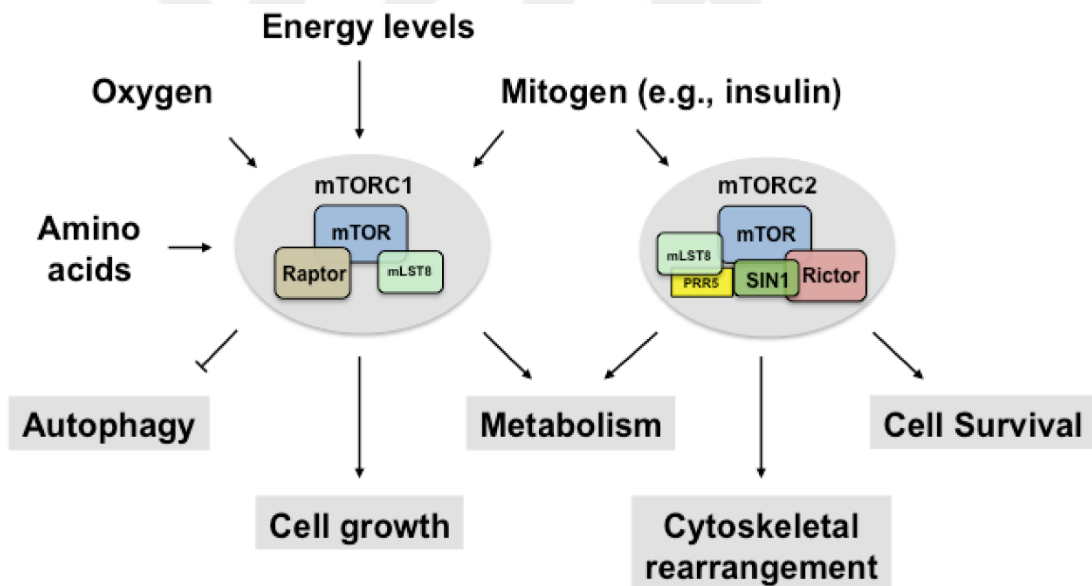

Figure 4. mTORC1 and mTORC2 (22).

mTORC1 is responsive to amino acids, stress, oxygen, energy levels and growth factors. In eukaryotic cells, it plays a central role as a signaling node that receives and integrates a wide range of intracellular and extracellular inputs and links these inputs with cellular metabolism. When these signals activate mTORC1 via growth-factor and hormone signaling pathways with available energy level in the cell, anabolism is promoted whereas catabolic reactions like autophagy is attenuated, thereby cell growth

and proliferation are enhanced by increased net macromolecular synthesis. mTORC2 is only sensitive for growth factors and plays key roles in cell survival, metabolism and cytoskeleton organization (23).

The mTOR signaling is needed in almost all tissues, but particularly important in metabolic ones such as liver, which is one of the most metabolically active organs. Research studies have concluded that the mTOR signaling pathway plays important roles in the development of hepatic metabolic functions, glucose homeostasis, lipogenesis and ketogenesis. These studies showed that genetically modified mouse models with defective hepatic mTOR signaling are glucose intolerant, hyperinsulinemic, hyperglycemic and hyperlipidemic (hepatic insulin resistance), indicating that hepatic mTOR plays a major role in both glucose and lipid homeostasis in the liver (22).

As mentioned before, mTORC1 organizes de novo lipogenesis via SREBP-1c, which controls the expression of genes responsible for regulating lipid metabolism. Porstmann et al. reported for the first time that the mTORC1 activation is necessary to initiate lipogenesis via activation of SREBP-1c in their in vitro studies (24). In another study, researchers investigated the mechanism by which insulin activates SREBP-1c transcription factor in the transgenic mouse model and initiates hepatic lipogenesis and concluded that this mechanism is mediated through mTORC1 (20). SREBP-1c can be activated by mTORC1 signaling through the S6K1, which is a downstream target protein of mTORC1 or in response to the low sterol level. It was determined that mTORC1 activated by AKT was reported to initiate DNL function by stimulating SREBP isoforms via Ribosomal Protein S6 Kinase 1 (S6K1) (25). This finding was supported by another study which explains the insulin-induced posttranscriptional processing of SREBP1c is dependent upon S6K1 by using a chemical inhibitor of S6K (26). In addition, mTORC1 can phosphorylate another substrate called Lipin1, a phosphatidic acid phosphatase, and leads its nuclear translocation to provide SREBP-1c activation in hepatocytes. Lipin1 inhibits SREBP-1c in the absence of the mTORC1 signal and mTORC1-Lipin1 signaling contributes to diet-induced hepatic steatosis in mice (27).

In a summary, mTORC1 regulates hepatic lipogenesis through the phosphorylation of different substrates which both regulate the level and activity of SREBP-1c. Therefore, hepatic DNL function might be mediated by the activation of mTORC1 by insulin-induced Akt, followed by translation of enzymes involved in

lipogenesis by mTORC1 activating SREBP-1c via S6K1 and here it is thought that mTOR/S6K1/SREBP-1c pathway activation in the liver might change with age in the different stages of the life in which the 0 days - 6 months.

3. MATERIAL & METHOD

3.1. Experimental Groups

In this study, Spraque Dawley male rats were used from different age groups, which were provided by Yeditepe University Experimental Research Center. All animals were kept in the standard laboratory conditions (22 ± 1 ˚C, 12 hours light / dark cycle) and fed ad libitum. Experimental procedures were approved by Yeditepe University Local Ethic Committee.

In the rat experimental model, 5 experimental groups were determined according to the physiologically important developmental periods (neonatal, infantile, weaning, prepubertal, young adult) from 0-6 months. There was 5 Sprague Dawley male rats in each group (n=5/group). The offspring of different mothers was used to create groups to minimize the possible effects of genetic factors.

Table 1. Experimental groups from different age groups.

Group 1	Newborn	Male (n=5)
Group 2	14-day-old	Male (n=5)
Group 3	28-days-old	Male (n=5)
Group 4	3-month-old	Male (n=5)
Group 5	6-month-old	Male (n=5)

3.2. The Sacrification of Animals, Collection and Storage of Tissue Samples

Rats that have completed follow-up in standard conditions were euthanized by decapitation after the weights are measured, and liver tissues were removed. The extracted organ was placed directly on the dry ice and frozen for a few minutes, then transferred into the labeled falcon tubes indicating that the age group, the mother of the offspring and the organ name. The liver tissues were stored at -80°C to be used in morphological analysis from frozen sections which were obtained using cryostat and some portion of the tissues were homogenized with specific homogenization buffer for the molecular studies.

3.3. Western Blot

3.3.1. Protein Extraction from the Tissue Samples

After weighing the tissue sample, 50 mg tissue was lysed with RIPA lysis buffer (Santa Cruz, #sc-24948) prepared by adding fresh protease-phosphatase inhibitor cocktail and homogenized with a homogenizer (28). After 14000 RPM at + 4 ° C for 15 minutes, the supernatants were transferred into new ependorfs and protein concentrations were determined by Bradford Assay Kit (ThermoFisher Scientific, #23236). Loading samples containing 60 µg/30 µl of protein was generated by adding; supernatant at the specified ratio, 10X reducing agent (ThermoFisher Scientific, #B0009), 4X LDS buffer (ThermoFisher Scientific, #NP0007) and then distilled water to complete the remainder of the samples to 30 µl of total volume. After preperation of the loading samples was done, they were boiled at 70 ° C for 10 minutes before gel electrophoresis.

3.3.2. Gel Electrophoresis

The running chamber of the electrophoresis device was filled with 1X MOPS Running Buffer (ThermoFisher Scientific, #B0002). In order to separate proteins based upon their molecular size, the samples containing the same amount of protein were loaded onto the Bolt 4-12% Bis-Tris gel (ThermoFisher Scientific, #NW04125BOX) with a molecular weight marker for reference (ThermoFisher Scientific, #26625) and separated electrophoretically for approximately 1.5 hours to the end of the gel at 120 V 30 mA.

3.3.3. Immunoblotting

Following gel electrophoresis, immunoblotting was performed to transfer proteins from the gel to the membrane. The PVDF membrane (ThermoFisher Scientific, #88518) was cut according to the size of the gel and treated with 1X Transfer Buffer (20% v/v methanol, 0.19M Glycine and 0.05M Tris) followed by shaking with 100% methanol for 10 min. Transfer cassettes contained the following layers: one sponge, one extra thick filter paper (ThermoFisher Scientific, #88615), gel, PVDF membrane, one extra thick filter paper, and one sponge. All equipment was pre-immersed in the transfer buffer. The transfer cassette was then placed in a transfer tank filled with transfer buffer and wet-transfer was performed for overnight at 15V 90 mA at + 4 ° C.

The following day, the membrane was blocked in the blocking buffer which was 5% skimmed milk powder in Tris Buffered Saline with Tween-20 (TBS-T; 1% Tween-

20, 0.15M NaCl, 50mM Tris, HCl pH 7.4) for 1 hour at room temperature on the shaker in order to prevent non-specific binding sites. After blocking, the membrane was incubated overnight at + 4 ° C with the relevant protein-specific primer antibody. The primary antibodies were used in this experiment are shown in Table 2. All antibodies were diluted in the blocking buffer at the dilution rate recommended by the manufacturer.

Following incubation with primary antibodies, the membrane was washed in TBS-T, 3 times for 10 minutes each time, then incubated with secondary antibody (Table 2) conjugated to horse-radish peroxidase (HRP) in 5% skimmed dried milk in TBST for 1.5 hours at room temperature. After secondary incubation, the membrane was washed again in TBS-T 5 times for 10 minutes each time. The membrane was visualized on the imaging device after treatment with enhanced chemiluminescence (ECL) reagent (ThermoFisher Scientific, #1856146, #1856145). All experiments were performed in duplicate, and the intensity of the signals was analyzed using Image Lab 6.0.1. GAPDH was used as an internal control and ratio of expression level of individual protein to that of GAPDH from the same samples was used to determine the final expression levels of each protein (29).

Table 2. Antibodies used in this study.

Primary Antibodies	Molecular Weight	Company, Catalog no
mTOR	289 kD	#2972S, CST
p-mTOR	289 kD	#2971S, CST
S6K1	70 kD	#9202S, CST
p-S6K1	70 kD	#9205S, CST
SREBP-1c	68 kD	# PA1-46142, Invitrogen
GAPDH	38 kD	#5174, CST
Anti-rabbit IgG, HRP-linked Antibody		#7074S, CST

3.4. Morphological Analyses

3.4.1. Oil Red O Staining

Frozen liver tissue sections of 3 microns was prepared by using cryostat. Sections were fixed in the %10 formalin and briefly washed with distilled water. After fixation, sections were rinsed with %60 isopropanol, and then stained with freshly prepared Oil Red O working solution for 15 mins. Afterwards, they were rinsed with %60 isopropanol again and stained with Hematoxylin for 10 secs. The sections were washed tap water and then in distilled water. Finally, sections were coverslipped using an aqueous mounting medium and displayed by a light microscope (Zeiss Axio Zoom Microscope, Germany).

3.4.2. Hematoxylin and Eosin Staining

Frozen liver tissue sections of 3 microns were fixed in the %10 formalin. The sections were washed with distilled water and nucleus was stained with Hematoxylin. After Hematoxylin staining, sections washed with tap water and then stained with Eosin staining. After washing with distilled water, the sections were incubated in the increasing concentrations of alcohol series (%70, %80, %90). After waiting for 2 minutes in %100 alcohol, sections were cleared through changes of Xylene. The slides were covered with coverslip using xylene-based mounting medium (30). Imaging of the slides was performed with a light microscope (Zeiss Axio Zoom Microscope, Germany).

3.5. Statistical Analysis

Shapiro Wilk test was used to determine whether the groups match the normal distribution. Differences between the groups were investigated by One-way ANOVA followed by Tukey multiple comparison test. All statistical calculations were performed using GraphPad Prism® V.5.00 (GraphPad software Inc.). $P < 0.05$ was considered as statistical difference.

4. RESULTS

4.1. Western Blot Results

In order to investigate whether mTOR/S6K1 pathway activity changes during the liver development and SREBP-1c protein levels which is regulated by the activity of this pathway, we determined the phoshporylated mTOR at Ser2448 and S6K1 at Thr389 and their respective total protein levels in respect to age difference, as well as the total levels of SREBP1 by Western Blot. All experiments were performed twice.

According to the results of our study, mTOR/S6K1 signaling shows age-dependent changes in different developmental processes of day 0 - 6 months in the rat liver tissues. The total mTOR protein expression level was the highest in the 14 day-old group and significantly higher than compared to 0-day old and 6 month-old groups (Fig. 2a, $p<0.05$). Although the quantification of the phosphorylation levels of mTOR (Ser2448) revealed a decrease over time, there were no difference in the relative phosphorylation of them as the total protein levels were also found to be changed in parallel (Fig. 2b).

Total S6K1 level was higher in the 14-day-old group than 0 day-old and 3 and 6-month-old groups significantly (Fig. 2c, $p<0.0001$). Its total expression level showed a decrease from 14-day-old group to 6-month-old group. In a parallel of this result, p-S6K1 (Thr389) level was also found highest in the 14-day-old group, although the results were not significantly different between groups (Fig. 2d).

On the other hand, SREBP-1c levels were found to be changed in parallel with the total and phosphorylated forms of the proteins suggesting that mTOR/S6K1 axis of the pathway regulates the expression of SREBP-1c levels. Its total expression level was the highest in the 14-day-old group and significantly different from other age groups (Fig. 2e, $p<0.05$). There was markedly a decrease in the expression of the SREBP-1c from 14-day-old group to 6-month-old group.

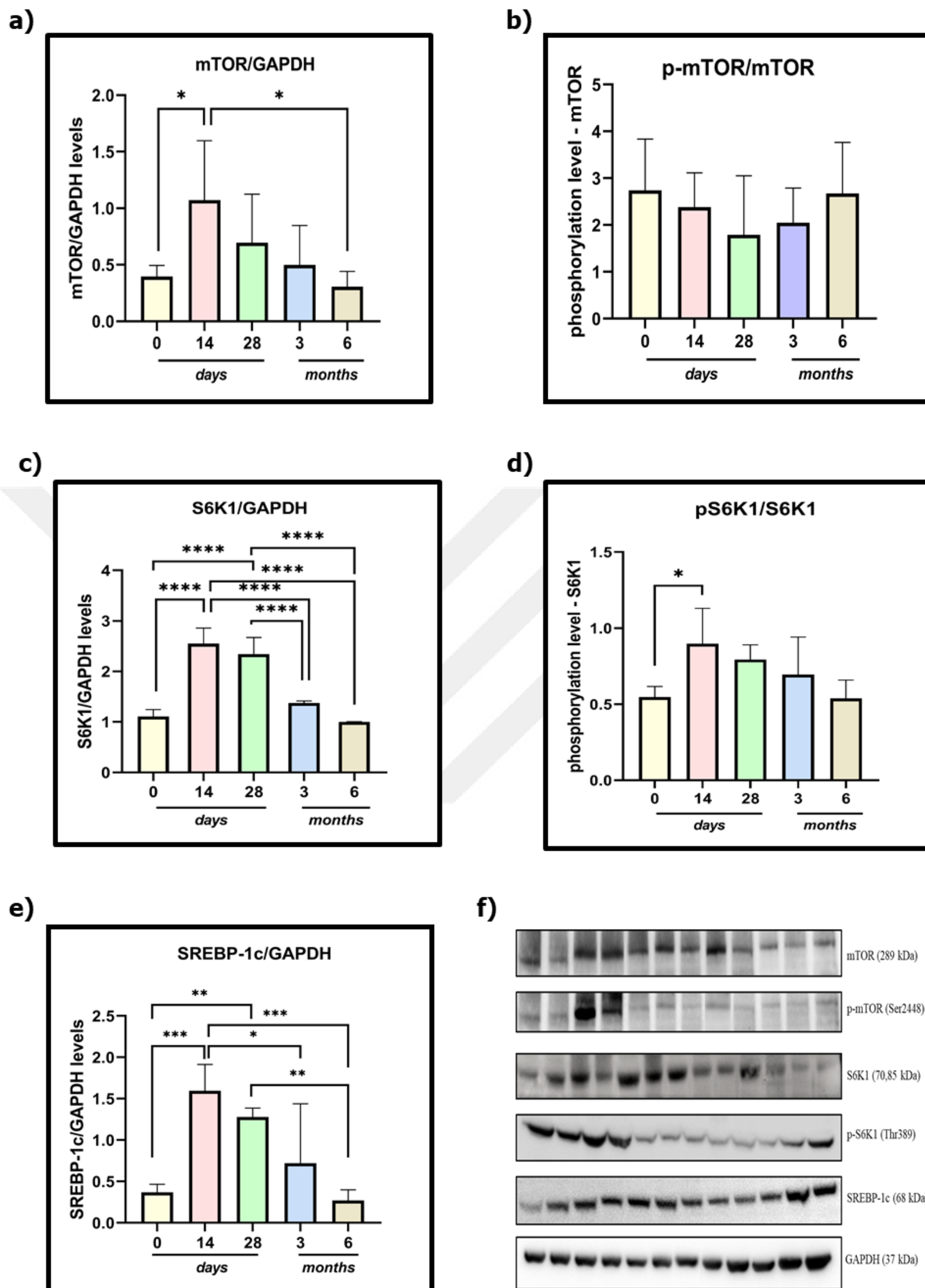

Figure 5. Quantification of the (a) total mTOR, (b) p-mTOR/mTOR, (c) S6K1, (d) p-S6K1/S6K1, (e) SREPB-1c and (f) representative western blot image. Statistical analyses: One-way ANOVA followed by Tukey's multiple comparison test (*p<0.05, **p<0.01 and ***p<0.001 and ****p<0.0001). Data are presented as mean ± SD (n=5 animals in each group).

4.2. Morphological Analyses

In a purpose of morphological evaluation of age-related lipid localizations was conducted with Oil Red O (ORO) staining in liver tissue sections was performed. The frozen liver tissue sections were obtained by using cryostat. In contrast to H&E staining, no specific staining of fat cells was observed in ORO staining. However, H&E staining showed fat cells that differed by groups.

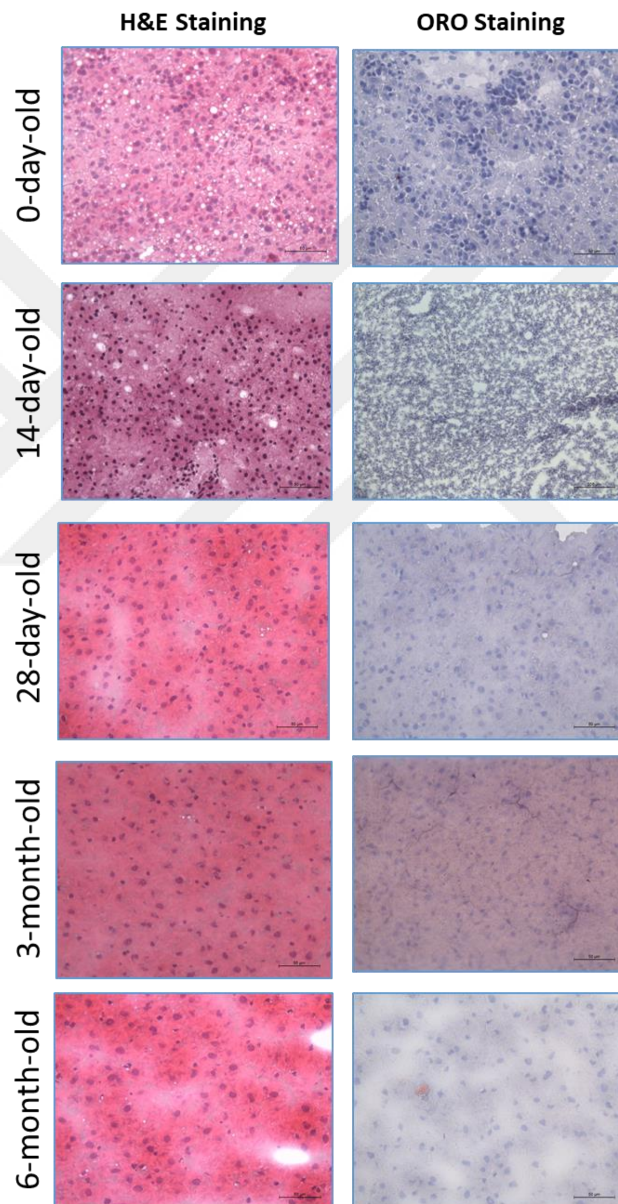

Figure 6. Morphological observation of the frozen liver sections. Frozen liver tissue sections were prepared 3 micron thick and stained with H&E and ORO (H&E: Hematoxylin-Eosin, ORO: Oil Red O).

5. DISCUSSION & CONCLUSION

The liver is one of the most dynamic organs in the body and it can perform various functions in the different stages of the life period. Lipogenesis, as one of the most important functions of the liver, provides high amount of lipids which is required for energy metabolism as well as cell membrane structure. It is highly important anabolic process and it must be controlled by distinct intracellular signaling mechanisms, primarily by mTOR, which can act as a metabolic sensor for nutrients, growth factors and energy. This well-conserved kinase mTOR has a positive role in regulating SREBP-1c activation and lipogenesis, therefore age-related changes in mTOR/SREBP-1c axis might be in high importance for contributing altered metabolic state with age.

Given that, to understand the age-dependent changes of the liver in functionality and morphology and molecular mechanism underlying that are highly critical. In this study, changes in the expression levels and the activities of mTOR and S6K1, as well as the expression levels of SREBP-1c, were investigated in male rats according to the physiologically important time periods from 0-6 months.

In the literature, the results regarding the mTOR pathway activity in different tissues during ageing are inconsistent. In a study examining diet- and age-dependent modulation of mTORC1, it was reported that mTORC1 activity increased with age in mouse liver tissues from four different age groups (two, eight, 20 and 24 months) (31). In another study in which age-related antioxidant system and mTOR signaling changes were investigated in the rat liver tissues belonging to three different age groups, higher mTOR expression was observed in the liver of elderly rats in accordance with other studies (32). On the other hand, Houtkooper et al. (2011) reported that mTORC1 signals in the mouse liver and skeletal muscle of 2 different age groups (six and 24 months) decreased during aging (33). Baar et al. (2016) investigated age, sex (6, 24, 30 months old male mouse and 6, 22, 26 months old female mouse) and tissue-dependent (liver, skeletal muscle, adipose tissue, heart) mTOR signaling changes in the mice liver and reported that elderly mice compared to young group had higher mTOR activation, however, age did not lead to a general increase in mTOR, and this situation changed sex and tissue specifically (34). In a study examining the mechanism of age and diet-related hepatic steatosis, mRNA and protein expression of SREBP-1c and related lipogenic genes was found to increase in age-related increase in wild-type C57BL / 6 J mice at 5, 8, and

12 months (35). On the other hand, Salamanca et al. (2015) reported no changes in the expression of the SREBP-1c of the mice livers with aging (3 vs 24-month-old) in their study which has 3 vs 24-month-old age groups (36).

As above mentioned, although there are studies in the literature examining how mTOR signaling pathway activity changes in liver tissue depending on age, there is no consensus on the results. The groups included in the studies were limited to a maximum of four different age groups and did not include the newborns. At the same time, the number of samples in the studies examining the age-related change of mTOR signaling pathway expression was low and the phosphorylation status of the same proteins (mTOR, S6K1, S6) were investigated. In our study, on the other hand, we found that, total mTOR and S6K1 levels were increased in the 14-day-old group compared to 0-day-old group and markedly decreased in 28-day-old, 3-month-old, 6-month-old groups, gradually. The pattern of phosphorylation of both proteins were also similar over time, as well as of the total SREBP1 levels.

The groups were formed to model "newborn, infant, weaning, pre-puberty, young adult, adult" periods at 2-week periodic intervals from neonatal period to pre-puberty period and 3-month periodic periods after puberty period, taking into consideration the different periods of liver maturation. Although liver begin to form and function in the embryological period, the adaptation and structuring process required to perform adult functions takes place after birth. Enzyme systems which constitute an important step of the metabolic systems in the liver, develop from minimal levels up to postnatal 14th day (1). Based on this information, we think that this might be a reason of why our results showed higher total protein expression levels in the 14-day-old group.

On the other hand, although the mTOR/S6K1/SREBP-1c signaling pathway is often studied in disease models and cell culture studies; activation changes in a physiological development process have not yet been fully elucidated. Although, age-related changes in hepatic lipid metabolism can contribute to the pathogenesis of diseases such as NASH, cancer and diabetes, there is no study on age-related activation changes in the developmental process from neonatal to adulthood under physiological conditions of hepatic mTOR/S6K1/SREBP-1c signaling that can control this metabolic process at the molecular level. Therefore, in this thesis project, age-dependent changes of mTOR expression in liver tissues of Sprague-Dawley male rats were investigated in 5 different

age groups in which the 0 days - 6 months of life period can be examined in different developmental processes. In addition to S6K1 protein from the mTOR signaling pathway, SREBP-1c protein, a transcription factor in hepatic lipogenesis, was also investigated.

Our research project is the first in the literature to make important contributions to the elucidation of the age-dependent changes in the mTOR/S6K1 signaling pathway, the central regulator of metabolism, and SREBP-1c activity which can be regulated by this pathway and its high range of age groups which involves 0-day-old groups. But, for the better understanding of the age-dependent changes of the hepatic lipogenesis, older age groups should be investigated as well as expression levels of the lipogenic enzymes in respect to age which are regulated by SREBP-1c.

Considering the studies suggesting that hepatic lipid metabolism disorders are associated with various diseases such as obesity, insulin resistance, NASH, atherosclerosis and cancer, studies about the molecular mechanisms involved in the regulation of lipid metabolism may contribute to a better understanding of the pathogenesis of diseases and to develop new approaches in prevention, diagnosis and treatment.

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