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CROSS-TALK OF INSULIN, LEPTIN AND GLUCOCORTICOID SIGNALING IN THE RAT HYPOTHALAMUS AND SKELETAL MUSCLE DURING METABOLIC DISTURBANCES INDUCED BY DIETARY FRUCTOSE AND STRESS

Doctoral Dissertation

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INTERAKCIJE SIGNALNIH PUTEVA INSULINA, LEPTINA I GLUKOKORTIKOIDA U HIPOTALAMUSU I SKELETNOM MIŠIĆU PACOVA TOKOM METABOLIČKIH POREMEĆAJA IZAZVANIH ISHRANOM OBOGAĆENOM FRUKTOZOM I STRESOM

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Cross-talk of insulin, leptin and glucocorticoid signaling in the rat hypothalamus and skeletal muscle during metabolic disturbances induced by dietary fructose and stress

ABSTRACT

Modern way of living includes unpredictable stressful daily events and consumption of caloric fructose-rich food. Increased incidence of insulin resistance and metabolic syndrome raise the necessity to investigate how these two factors present in our everyday lives influence metabolic health.

The aim of this doctoral dissertation is to test the hypothesis that fructose diet combined with chronic unpredictable stress induces more pronounced metabolic derangements in the skeletal muscle and hypothalamus of male Wistar rats than each of the factors applied separately. With that in mind, we analyzed hypothalamic insulin and leptin signaling and their effects on appetite regulation, skeletal muscle insulin signaling, and lipid metabolism, muscle glucocorticoid signaling and inflammation after challenging conditions of excessive fructose consumption (9 weeks) and/or exposure to chronic unpredictable stress (4 weeks).

Results showed that fructose diet combined with chronic stress altered expression of hypothalamic neuropeptides causing increased appetite and energy intake, increased activity of AMPK energy sensor and impaired insulin signaling. In the skeletal muscle, combination of fructose and stress caused muscle lipid overload and disturbed lipid metabolism by increasing lipolysis and β-oxidation. Decreased muscle glucocorticoid signaling enabled rise of uncontrolled lipid-induced inflammation creating a setting for muscle insulin signaling impairment detected after combination of the treatments.

Results of this doctoral dissertation have clearly showed that combination of fructose diet and chronic stress exposure had more detrimental effects on the hypothalamus and skeletal muscle of male rats than each of the treatments applied separately.

Key words: Chronic stress; fructose; skeletal muscle; hypothalamus; glucocorticoid hormones; leptin; lipid metabolism; insulin resistance; inflammation;

Scientific field: Biology

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Interakcije signalnih puteva insulina, leptina i glukokortikoida u hipotalamusu i skeletnom mišiću pacova tokom metaboličkih poremećaja izazvanih ishranom obogaćenom fruktozom i stresom

SAŽETAK

Moderan način života se sastoji od svakodnevnih nepredvidivih stresnih događaja i konzumiranja kalorične hrane bogate fruktozom. Povećana učestalost insulinske rezistencije i metaboličkog sindroma nameće potrebu da se istraži kako ova dva faktora prisutna u našem svakodnevnom životu utiču na metaboličko zdravlje.

Cilj ove doktorske disertacije je da se ispita hipoteza da ishrana bogata fruktozom u kombinaciji sa hroničnim nepredvidivim stresom izaziva izraženije metaboličke poremećaje u skeletnim mišićima i hipotalamusu mužjaka pacova nego svaki od primenjenih tretmana ponaosob. Imajući to na umu, analizirali smo signalizaciju insulina i leptina u hipotalamusu i njihove efekte na regulaciju apetita, signalizaciju insulina u skeletnim mišićima, metabolizam lipida, signalizaciju glukokortikoida i inflamaciju nakon prekomernog unosa fruktoze (9 nedelja) i/ili izloženosti hroničnom nepredvidivom stresu (4 nedelje).

Rezultati su pokazali da ishrana bogata fruktozom u kombinaciji sa hroničnim stresom menja ekspresiju neuropeptida hipotalamusa, izazivajući povećan apetit i unos energije, povećanu aktivnost energetskog senzora AMPK i narušava insulinsku signalizaciju. U skeletnim mišićima, kombinacija fruktoze i stresa izazvala je povećanje unosa lipida i nivoa lipolize i β-oksidacije. Inhibirana glukokortikoidna signalizacija omogućava povećanje nekontrolisane inflamacije izazvane lipidima, doprinoseći poremećaju mišićne insulinske signalizacije nakon kombinacije tretmana.

Rezultati ove doktorske disertacije jasno su pokazali da je kombinacija ishrane bogate fruktozom i hronične izloženosti stresu izazvala štenije efekte na hipotalamus i skeletne mišiće mužjaka pacova nego svaki od tretmana primenjen pojedinačno.

Ključne reči: Hronični stres; fruktoza; skeletni mišići; hipotalamus; glukokortikoidni hormoni; lipidni metabolizam; insulinska rezistencija, inflamacija

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Abbreviations

11βHSD1	11β-hydroxysteroid dehydrogenase type 1
ACTH	Adrenocorticotropic hormone
AgRP	Agouti related peptide
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
ATGL	Adipose triglyceride lipase
CART	Cocaine- and amphetamine-regulated transcript
CNS	Central nervous system
CPT1	Carnitine palmitoyltransferase I
CRH	Corticotropin-releasing hormone
ERK	Extracellular signal–related kinase
FATP1	Fatty acid transport protein 1
FKBP51	FK506 binding protein 51
GLUT	Glucose transporter
GR	Glucocorticoid receptor
Grb-2	Growth factor receptor-bound proteins
GSK3	Glycogen synthase kinase
GSP	Glycogen synthase phosphatase
H6PDH	Hexose-6-phosphate dehydrogenase
HPA	Hypothalamo-pituatary-adrenal axis
HPRT	Hypoxanthine phosphoribosyl transferase 1
Hsp70	Heat shock protein 70

Hsp90	Heat shock protein 90
IKK	IκB kinase
IL1- β	Interleukin 1β
IL-6	Interleukin 6
IR	Insulin receptor
IRS1	Insulin receptor substrate 1
IκB	Inhibitory protein κB
JAK2	Janus kinase type 2
KLF15	Kruppel Like Factor 15
LPL	Lipoprotein lipase
MR	Mineralocorticoid receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor κB
NPY	Neuropeptide Y
ObRb	Obesity receptor
PDK1	3-phosphoinositide-dependent kinase-1
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
PPARα	Peroxisome proliferator-activated receptor α
PPARδ	Peroxisome proliferator-activated receptor δ
PTP1B	Protein tyrosine phosphatase 1B
RT-PCR	Real-time Polymerase chain reaction

SAM	Sympathetic adreno-medullary
SOCS3	Suppressor of cytokine signaling 3
STAT	Signal transducer and activator of transcription
TG	Triglycerides
TNF α	Tumor necrosis factor α
VLDL	Very low-density lipoprotein

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1. Introduction

1.1 Energy balance

Energy homeostasis can be described as the dynamic steady state. It requires a constant communication of different effector organs such as the liver, skeletal muscle, pancreas, and adipose tissue with several integrating centers located within the hypothalamus. During evolution, animals acquired precise and strong mechanisms for the efficient energy sensing in order to maintain homeostasis. Both nutrient deprivation and increased energy intake require activation of regulatory mechanisms in order to maintain sufficient energy available for everyday function and adequately preserve the energy surplus for latter. Insulin, leptin and glucocorticoids represent essential signaling molecules of the regulatory network involved in the maintenance of energy balance (Sánchez-Lasheras et al., 2010).

1.1.1 Insulin signaling pathway

Maintaining energy balance requires tight regulation of plasma glucose levels. One of the most important signaling molecules used to regulate circulating glucose levels is insulin. Following food intake, blood glucose levels rise which is detected in pancreatic β-cells which produce and secrete insulin. Insulin regulates plasma glucose levels by stimulating its transport into the skeletal muscles and adipose tissue and increasing anabolism of glucose inside the cells (Saltiel and Kahn, 2001). Although almost all cells in the body use glucose as energy source, several organs such as liver and skeletal muscle are specialized to take and preserve glucose in the form of glycogen. At the same time, insulin inhibits *de novo* production of glucose in the liver, lipolysis in the adipose tissue and also reduces appetite in the satiety centers in the hypothalamus in order to maintain energy balance (Petersen and Shulman, 2018).

Insulin accomplishes its effects through binding to the insulin receptor (IR). Hormone-receptor interaction activates receptor tyrosine kinase activity initiating autophosphorylation of the IR (White, 1997). Proteins such as insulin receptor substrate 1 (IRS1), growth factor receptor-bound proteins (Grb-2) and SHC-transforming protein, recognize phosphotyrosine pattern on the IR and become activated as well (Youngren, 2007). The main effects of insulin on glucose and lipid metabolism are mediated through the IRS1 (Figure 1.1). Phosphorylated IRS1 interacts with phosphatidylinositol 3-kinase (PI3K) which leads to the transition of PI3K from cytoplasm to the cell membrane and activation of its catalytic subunit p85. Activated PI3K produces lipid signaling molecules phosphatidylinositol 3,4,5-triphosphate (PIP3) from phosphatidylinositol-4,5-bisphosphate (PIP2). PIP3 recruits protein kinase B (Akt) to the membrane where it becomes activated and phosphorylated by 3-phosphoinositide-dependent kinase-1 (PDK1) and mTORC2 kinase (Miranda et al., 2005; Shepherd, 2005). Activated Akt stimulates translocation and insertion of glucose transporter GLUT4 to the plasma membrane through activation of proteins Rab and RAC1, therefore increasing the rate glucose facilitated diffusion into the cell (Chiu et al., 2011). In addition to that, Akt regulates activity of glycogen synthase phosphatase (GSP) and glycogen synthase kinase (GSK3), two main enzymes that control glycogen synthase activity and therefore rate of glycogen production and destiny of glucose in the cell. Therefore, through activated Akt, insulin stimulates glucose intake and subsequent glycogen production, which is one of the main physiological functions of insulin (Figure 1.3). Described insulin signaling pathway and most of its stated functions are present in main insulin effector organs, adipose tissue and skeletal muscles, nevertheless some of the functions such as glycogen storage are not initiated in adipose tissue.

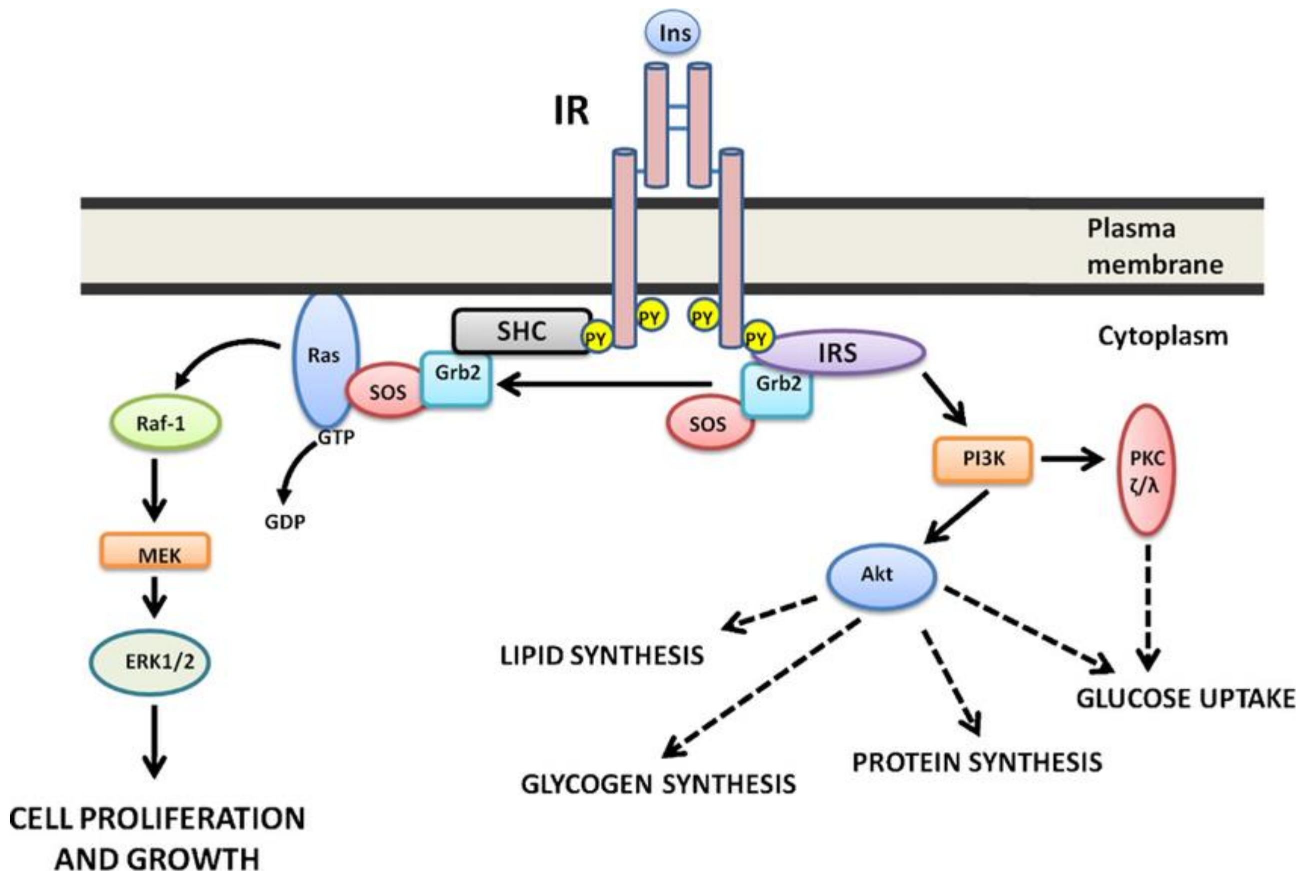

Figure 1.1. Main functions regulated by the insulin signaling pathway. From circulation, hormone insulin recognizes and binds to the insulin receptor (IR), expressed at the cell membrane. Hormone-receptor binding initiates autophosphorylation of the receptor and phosphorylation of the IRS and other proteins that recognize pattern of receptor phosphorylation, such as SHC containing proteins. Phosphorylated IRS will be recognized by the proteins PI3K and Grb2. By binding to IRS, PI3K becomes activated and starts chain of events leading to phosphorylations and activation of PI3K and PKC regulating glucose uptake and synthesis of glycogen, lipids and proteins. IR- insulin receptor; IRS-insulin receptor substrate; PI3K- phosphatidylinositol 3-kinase; Akt- protein kinase B; PKC- protein kinase C. Taken and modified from (Olivares-Reyes et al., 2009)

1.1.2 Insulin resistance and the role of hypothalamus and skeletal muscle

Insulin resistance is a state of decreased sensitivity to insulin or inability of an effector to respond to physiological levels of insulin. Exact mechanisms leading to the insulin resistance are still a subject of research, but it is widely recognized that they involve disruption of insulin signaling pathway most often at the level of IR, IRS1 and Akt (Delibegovic et al., 2007; Taniguchi et al., 2006a). Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of insulin signaling pathway that has the ability to dephosphorylate and thus deactivate both IR and IRS1 (Goldstein et al., 2000).

Insulin has a major role in the central nervous system (CNS), in center for hunger and satiety located in the hypothalamus. Namely, hypothalamus is the part of the brain that plays the essential role in regulation of energy balance. It consists of insulin sensitive neurons that

express orexigenic neuropeptides, such as agouti related peptide (AgRP) and neuropeptide Y (NPY), as well as anorexigenic neuropeptides, like pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). As their names state, orexigenic neuropeptides stimulate food intake, while anorexigenic inhibit consumption of food. Insulin stimulates secretion of anorexigenic neuropeptides and at the same time inhibits secretion of orexigenic, which altogether results in the inhibition of appetite and further energy intake when the level of glucose in the circulation is high. Therefore increase in blood glucose after intake of a meal and subsequent increase in concentration of circulating insulin is important signal to the hypothalamus and to its energy-sensing and appetite-regulating neurons. On the other hand, long term increase in insulin concentration has been shown to cause decrease in insulin sensitivity of the neurons (Benoit et al., 2009; De Souza et al., 2005; Mayer and Belsham, 2010). Central or hypothalamic insulin resistance has been associated with the onset and development of obesity and metabolic disturbances, such as metabolic syndrome, type 2 diabetes and some cognitive impairments (Heni et al., 2015; Mayer and Belsham, 2010) (Figure 1.2). Prolonged consumption of high-caloric and energy-rich food can cause chronic increase in the synthesis and secretion of insulin, exposing the hypothalamus to the pathological levels of this hormone. At first, increase of insulin overstimulates neurons and keep insulin signaling overactive, which ultimately leads to the development of the state of insulin resistance. In this way, regulation of energy homeostasis under insulin regulation is disrupted which is one of the first steps in the onset of obesity and obesity-related metabolic disorders (Belsham and Dalvi, 2021).

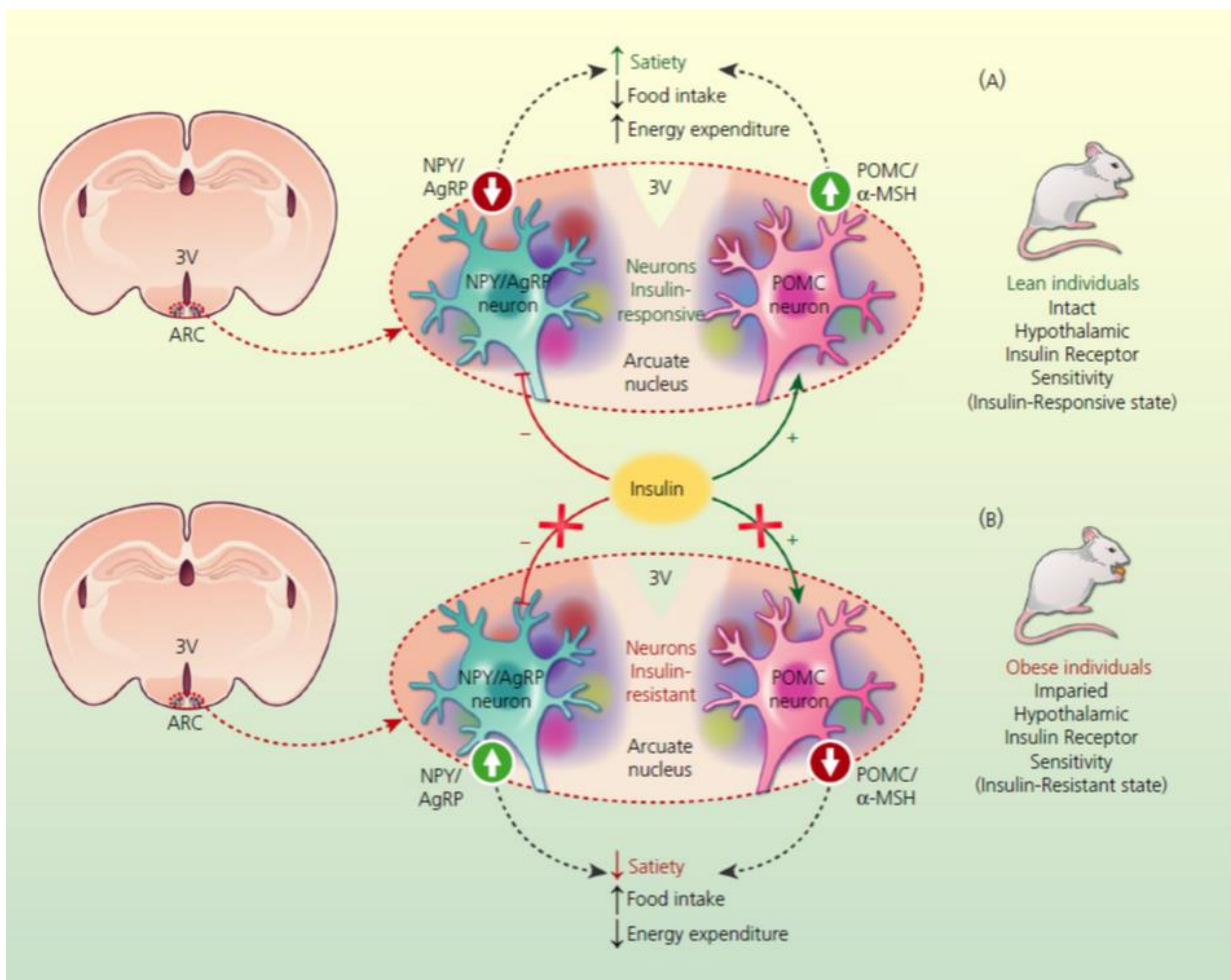

Figure 1.2. Insulin signaling pathway in the hypothalamus in lean and obese individuals. Brain diagram shows coronal section of mediobasal hypothalamus and arcuate nucleus. A) In lean individuals with intact hypothalamic insulin receptor sensitivity, insulin directly acts on neurons that regulate appetite, inhibiting activity of the neurons that express orexigenic neuropeptides NPY/AgRP and activating neurons that express anorexigenic neuropeptide POMC. This results in decrease of food intake, increase of energy expenditure and the filling of satiety. B) In obese individuals with impaired hypothalamic insulin receptor sensitivity, insulin action is compromised due to development of insulin resistance, leading to increase in expression of NPY/AgRP neuropeptides and decrease of expression of POMC. Attenuated neuron responsiveness to the insulin results in delayed satiety, decreased energy expenditure and increased food intake. ARC-arcuate nucleus; 3V-third cerebral ventricle; NPY-Neuropeptide Y; AgRP- agouti related peptide; POMC- pro-opiomelanocortin. Taken and modified from belsham2020 (Belsham and Dalvi, 2021)

Considering that skeletal muscles comprise up to 40% of the total body mass, this tissue represents the most abundant effector for insulin-regulated uptake of glucose (DeFronzo and Tripathy, 2009). Additionally, skeletal muscles are the first to respond to energy disbalance and environmental pressures, changing their carbohydrate, lipid and protein metabolic pathways in order to adjust and balance whole-body metabolism (Stefanaki et al., 2018). This makes muscle an essential peripheral organ for the systemic glucose homeostasis and its metabolic deregulation could lead to systemic insulin resistance and type 2 diabetes (DeFronzo and Tripathy, 2009; Wu and Ballantyne, 2017a). Studies show that skeletal muscle insulin resistance could occur even years before hyperglycemia starts and dysfunction of pancreatic β-cells could even be detected (DeFronzo and Tripathy, 2009). Furthermore, it has been pointed out that the insulin resistance of skeletal muscle is followed by systemic hyperinsulinemia, dyslipidemia, hypertension and cardiovascular abnormalities that are main indicators of metabolic syndrome (Rattanavichit et al., 2016). In animal models, targeted deletions of IR and GLUT4 only in the skeletal muscle, lead to the increased risk of liver steatosis and adiposity (J. K. Kim et al., 2000; Kim et al., 2001b). Overexpression of PTP1B, solely in skeletal muscle, results not only in insulin resistance in this tissue, but in systemic insulin resistance as well (Zabolotny et al., 2004). On the other hand, *in vivo* and *in vitro* studies revealed that lack of PTP1B in muscle increases whole-body insulin sensitivity (Delibegovic et al., 2007; Nieto-Vazquez et al., 2007). That is why it is considered that skeletal muscle insulin resistance could be one of the earliest and most important defects in the sequence of events resulting in development of type 2 diabetes and metabolic syndrome (DeFronzo and Tripathy, 2009; Wu and Ballantyne, 2017a).

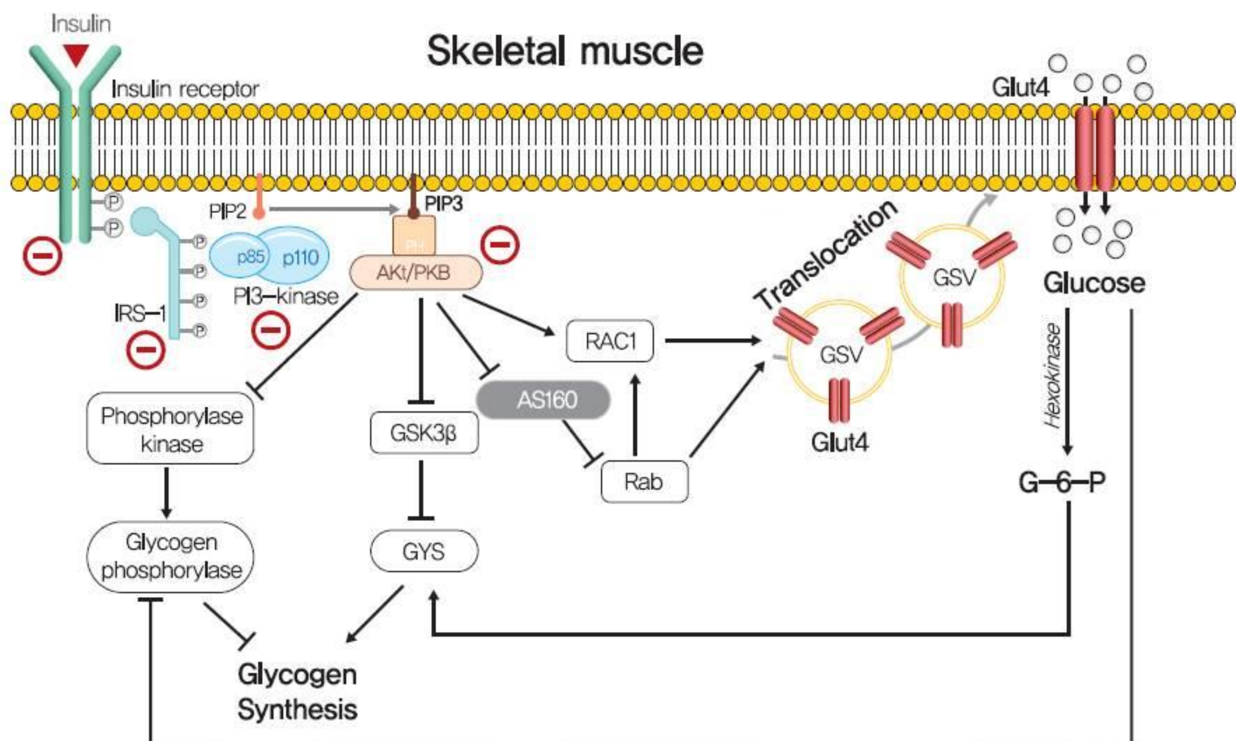

Figure 1.3. Skeletal muscle insulin signaling and insulin resistance. Insulin binds to insulin receptor (IR) which initiates autophosphorylation of the receptor and activation of IRS1. Activated IRS1 will recruit PI3K and through transformation of PIP2 into PIP3, activate Akt. In skeletal muscles, insulin through activated Akt stimulates glucose intake into cell via translocation of vesicle that contain glucose transporter GLUT4 towards plasma membrane. In the process of vesicle translocation and integration with membrane, RAC1 and Rab proteins have a main role. Akt directly stimulates RAC1 and indirectly Rab, through inhibition of Rab inhibitor, AS160. Through Akt, insulin stimulates glycogen synthesis in the skeletal muscle. Glycogen is synthesized by enzyme GYS. Activated Akt blocks GSK3, main inhibitor of GYS. Akt inhibits protein Phosphorylase kinase, direct activator of glycogen phosphorylase which is well known inhibitor of glycogen synthesis. Red marks are set near the members of signaling pathway that are inhibited when skeletal muscle insulin resistance occurs. IRS1 – insulin receptor substrate 1; Akt- protein kinase B; PI3K- phosphatidylinositol 3-kinase; PIP2- phosphatidylinositol-4,5-bisphosphate; PIP3- phosphatidylinositol-3,4,5-trisphosphate; GYS- glycogen synthease; GSK3β- glycogen synthase kinase 3; RAC1- Ras-related C3 botulinum toxin substrate 1; AS160- AKT substrate of 160 kDa. Taken and modified from (Lee et al., 2022).

Skeletal muscles are known to have good metabolic flexibility. Term metabolic flexibility was defined as the ability to adjust process of fuel usage and oxidation to the fuel availability (da Silva Rosa et al., 2020). This means that skeletal muscles can use either glucose or fatty acids to produce necessary energy depending of the availability of the substrate. The metabolic flexibility is lost or very impaired in obesity and type 2 diabetes even if availability of the glucose and fatty acid is the same as in lean and healthy patients (Kelley et al., 1999). Studies suggest that loss of flexibility in skeletal muscle could be one of the underlining mechanisms for the onset of insulin resistance (Gilbert, 2021).

Almost sixty years ago, Randle et al. have observed that disorders and abnormalities in carbohydrate metabolism are associated with increase in circulating levels of fatty acids and proposed the term „glucose-fatty acid cycle“, which states that when one type of the fuel is present it inhibits metabolism of the other (Gilbert, 2021; Randle et al., 1963). Randle suggested that glucose transport and phosphorylation are the steps affected by fatty acid

metabolism and that enzymes phosphofructo-1-kinase and pyruvate dehydrogenases are the main targets (Hue and Taegtmeyer, 2009). Fatty acid oxidation initiates change in mitochondrial ratio of acetyl-CoA to CoA and ratio of NADH to NAD+ which inhibits activity of pyruvate dehydrogenase. Described changes also lead to increase in the cytosolic level of citrate which inhibits phosphofructo-1-kinase, leading to the accumulation of glucose 6-phosphate that is known to inhibit hexokinase (Garland et al., 1963). These observations are considered to be the turning point in realizing that fatty acids can play a role in the development of insulin resistance (Gilbert, 2021). Having in mind that obesity is tightly associated with rise in the level of circulating fatty acids, it has been proposed that accumulation of this fatty acid excess into different tissues and organs can lead to development of insulin resistance.

In compliance with this, many researchers using both animal models and clinical studies have shown that accumulation of lipids in skeletal muscle is connected with decrease in muscle sensitivity to insulin and onset of insulin resistance (Krssak et al., 1999a). Having in mind that skeletal muscle is not an organ specialized for lipid storage, overload of fatty acids to the muscles could easily disturb balance between lipogenic and lipolytic pathways and make a setting for the onset of insulin resistance (Gilbert, 2021). Infusion of triglycerides (TGs) and heparin, which provides increase in the level of free fatty acids, have shown development of insulin resistance and increased lipid content in the skeletal muscle cells of healthy subjects (Boden et al., 2001; Boden and Chen, 1995). Similar finding was observed after lipid infusion in euglycemic-hyperinsulinemic clamp revealing decreased IRS1 and Akt phosphorylation levels and activity in the skeletal muscle (J. Y. Kim et al., 2000; Yuzefovych et al., 2010). Interestingly, not only lipid accumulation in the skeletal muscle was associated with insulin signaling impairment. Namely, some studies have shown that excessive fatty acid oxidation in the muscles could as well be detrimental for the sensitivity to insulin and metabolic health. This concept was evaluated when it was noticed that increase in fatty acid oxidation, that would decrease accumulation of lipid in the muscle cells, did not cause expected improvement in insulin signaling (Debard et al., 2006). On the contrary, high-fat diet and the state of obesity *per se* can increase expression of enzymes involved in the process of β-oxidation (Gilde and Van Bilsen, 2003). Inhibition of rate limiting enzyme for fatty acid import into the mitochondria and subsequent β-oxidation, carnitine palmitoyltransferase I (CPT1), protects against development of insulin resistance in spite of increased lipid accumulation in the skeletal muscle (Gilbert, 2021). Many of the aspects in lipid induced insulin resistance are still unknown and exact mechanisms are not yet all discovered. In addition to lipid accumulation and excessive fatty acid oxidation, rise of metabolic inflammation could have a significant role in connecting lipid metabolism and insulin signaling impairment which will be discussed in the following chapters.

1.1.3 Leptin signaling pathway

Leptin is a hormone synthetized in the adipose tissue and represents one of the main signals in brain-adipose tissue communication. When body energy status is positive, adipose tissue synthetizes and secretes leptin in the amount that directly correlates to the amount of the body adipose tissue (Ahima et al., 2000). Leptin travels to the brain through circulation, where it binds to the specific leptin receptor ObR (*Obesity receptor)*, which belongs to the family of cytokine receptors class I. Although there are several isoforms of the ObR (ObRa-f), main isoform through which leptin achieves its effects is long isoform, ObRb (Ribiere and Plut, 2005). After leptin binding, ObRb undergoes a conformational change and activates JAK2

(*Janus kinase type 2*), which after autophosphorylation, phosphorylates tyrosine residues on specific positions of the ObRb (position 985, 1077 and 1138). This phosphorylation pattern of ObRb and JAK2 is then recognized by various signaling molecules which will either activate or inhibit rest of the leptin signaling pathway. Most recognized positive regulators of leptin signaling pathway are STAT3 and STAT5 proteins (*Signal transducer and activator of transcription*), while SOCS3 (*Suppressor of cytokine signaling 3*) and PTP1B have negative regulatory effects. Additionally, activated STAT3 can positively regulate the SOCS3 gene expression and thus contribute to the control and termination of the leptin signal. Therefore, leptin signaling pathway has the ability of autoregulation (Myers et al., 2008) (Figure 1.4).

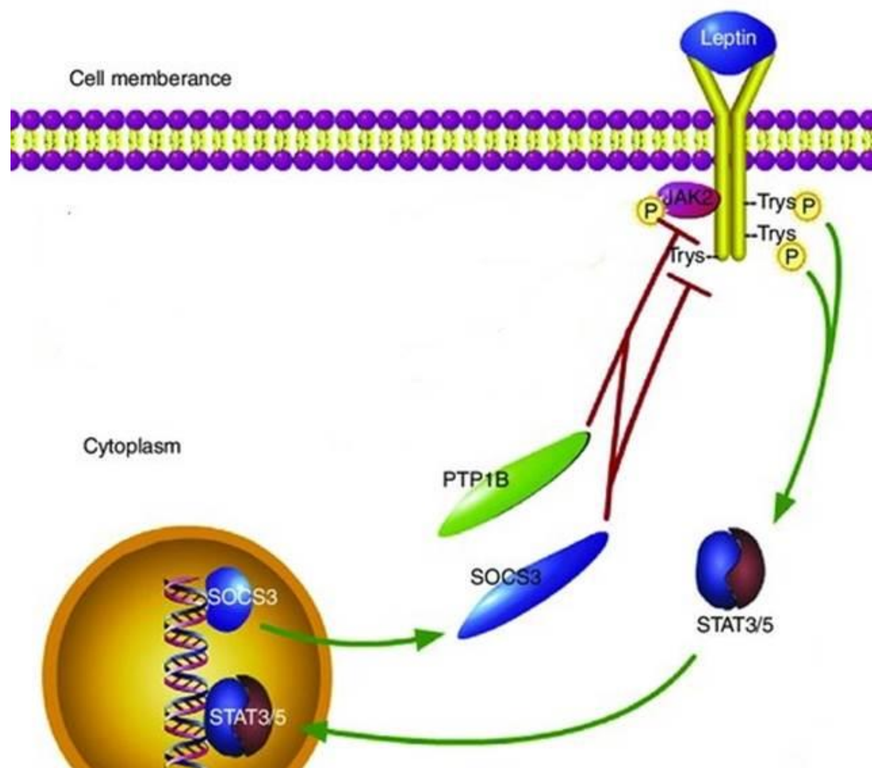

Figure 1.4. Leptin signaling pathway. Leptin binds to leptin receptor which leads to recruitment of JAK2 causing phosphorylation of both, JAK2 and receptor. These phosphorylations are recognized by STAT3 and STAT5, main transcriptional regulators that mediate leptin functions. Activated STAT3 positively regulates expression of the SOCS3, strong inhibitor of leptin signaling pathway. In this way, activated leptin signaling autoregulates itself. JAK2- janus kinase type 2; STAT3/5- signal transducer and activator of transcription 3/5; SOCS3- suppressor of cytokine signaling 3; PTP1B- protein tyrosine phosphatase 1B. Taken and modified from (Xu et al., 2017)

The main role of leptin is related to the maintenance of body energy balance. Namely, when energy status is positive, secretion of leptin ensures that appetite would be inhibited and energy expenditure increased. Therefore it is not surprising that brain regions which express ObRb are the ones involved in appetite regulation, such as nucleus arcuatus and paraventricular nucleus of the hypothalamus, as well as ventromedial and lateral hypothalamus. In this regions leptin inhibits activity of neurons that produce orexigenic neuropeptides, NPY and AgRP, and stimulates production of anorexigenic neuropeptides, POMC and CART (Cowley et al., 2001; Myers et al., 2008). In addition, leptin can activate sympathetic neuronal network therefore increasing energy expenditure. Described roles make leptin a perfect candidate for the treatment of hyperphagia and obesity. Nevertheless, it has been shown that obese patients have increased appetite and adiposity regardless of the high levels of leptin expression in the adipose tissue and increased level of the hormone in the

circulation (Frederich et al., 1995). This is because obese people develop state of decreased sensitivity to leptin, called leptin resistance. Several causes can lead to reduced body reactivity to leptin: inhibited transport of leptin to the brain, decrease in expression or function of leptin receptor, blockade in downstream signaling pathway (Thaler and Schwartz, 2010). Development of leptin resistance is the main reason why leptin cannot be used as a therapeutic once the obesity has been developed. In addition, studies show that development of leptin resistance increases the probability of obesity onset. Decreased sensitivity to leptin prevents leptin to stimulate anorexigenic signals which lead to increase in appetite, especially during the consumption of energy-rich food, creating conditions for the development of obesity (Huang et al., 2004; Patel et al., 2008). Long term clinical trials have shown that high circulating levels of leptin can predict the onset of metabolic syndrome and worsening of the symptoms (Franks et al., 2005). Vasselli et al., have indicated that leptin resistance can be caused by unbalanced nutrition, rich in fats and/or sugars (Vasselli et al., 2013). It is proposed that leptin gene transcription as well as leptin secretion is regulated by the nutrition at least in part through insulin signaling. Namely, it was observed that expression of leptin rises after insulin secretion peak after feeding. In addition, insulin stimulates leptin secretion from adipocyte in culture and when directly injected in rodents (Tsai et al., 2012).

1.1.4 Glucocorticoid signaling pathway

Glucocorticoids are steroid hormones synthesized and secreted by the adrenal glands. These adrenal hormones are named after their ability to promote the conversion of proteins and lipids into glucose, especially during stressful situations (Gross and Cidlowski, 2008). Namely, glucocorticoid secretion during stress exposure is essential in providing enough energy for the survival of the organism, quick recovery and homeostasis maintenance, as well as for the preparation to subsequent stressors and challenges. This is why an alternative name for glucocorticoids is "stress hormones". During stress exposure or in conditions of high energy demands or energy deficit, glucocorticoids stimulate an increase in blood glucose levels. Namely, glucocorticoids stimulate the process of *de novo* synthesis of glucose (gluconeogenesis) and the process of hepatic glycogen breakdown (glycogenolysis) by positively regulating the expression of genes encoding the enzymes of these metabolic pathways. In skeletal muscle, glucocorticoids decrease protein synthesis and increase their breakdown to amino acids that can be used as precursors for gluconeogenesis in the liver (Kuo et al., 2013). In addition, glucocorticoids inhibit glucose transport into the muscle and subsequent glycogen synthesis while stimulating glycogenolysis and glucose diffusion into the blood therefore opposing the effects of insulin. Furthermore, glucocorticoids stimulate lipid mobilization from the adipose tissue into the muscles and regulate muscle lipid metabolism and expression of genes involved in lipogenesis (Morgan et al., 2009). Glucocorticoids act on hypothalamic centers that regulate food intake and energy consumption to acutely inhibit appetite but chronically stimulate food and calorie intake (Sapolsky et al., 2000). This makes glucocorticoids one of the most important regulators of energy balance during stress exposure.

The two basic members of the glucocorticoid family of steroid hormones are active cortisol and inactive cortisone in humans, and active corticosterone and inactive 11-dehydrocorticosterone in rodents. Production and secretion of glucocorticoids is under regulation of the hypothalamo-pituatary-adrenal (HPA) axis. Namely, in response to internal and external stimuli, hypothalamus synthesizes corticotropin-releasing hormone (CRH), which stimulates the secretion of adrenocorticotropic hormone (ACTH) from the anterior lobe

of the pituitary gland. This pituitary hormone achieves a direct positive regulation on the adrenal glands, stimulating the synthesis and release of glucocorticoids. Under normal physiological conditions, the HPA axis is regulated by a negative feedback loop, with each hormone in the cascade inhibiting the secretion of the previous one (Figure1.5). From adrenal glands, glucocorticoids are released into the blood and transported throughout the body.

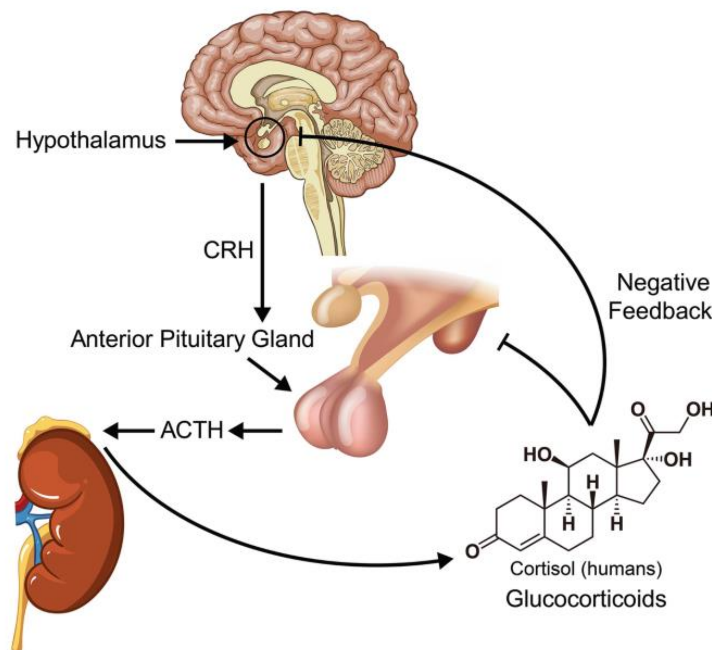

Figure 1.5. Regulation of glucocorticoid synthesis by hypothalamo-pituatary adrenal (HPA) axis. First step in the regulatory cascade of glucocorticoid secretion starts in the hypothalamus. As response to the stress, some other stimuli or simply day/night shift, hypothalamus secretes CRH which stimulate anterior pituitary gland to release ACTH hormone. Secreted ACTH travels through circulation to adrenal glands and binds to its receptor and initiate synthesis and secretion of glucocorticoids. Negative feedback is the main regulation of HPA axis in which every member inhibits the release of the previous one. CRH- corticotropin-releasing hormone; ACTH- adrenocorticotropic hormone. Taken and modified from (Cruz-Topete and Cidlowski, 2018)

Once inside the cell, glucocorticoids become a target to 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) or 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), main enzymes of so called glucocorticoid prereceptor metabolism. Namely, 11βHSD1 converts glucocorticoid hormone from inactive to active form using NADPH (*Nicotinamide adenine dinucleotide phosphate*) as a cofactor, which is generated by hexose-6-phosphate dehydrogenase (H6PDH), an enzyme that is physically coupled to 11βHSD1, on the inner side of the endoplasmic reticulum (Banhegyi et al., 2004). On the other hand, 11βHSD2 uses NAD^+ and catalyzes reaction in opposite direction, thus inactivating the glucocorticoids (Figure1.6). Activity of these two enzymes determines the amount of active glucocorticoid hormone inside the cells, regardless of the circulating concentration. Enzymes are coded by the separate genes and expressed in tissue specific manner. In this way, presence and activity of glucocorticoids is very precisely regulated in each cell.

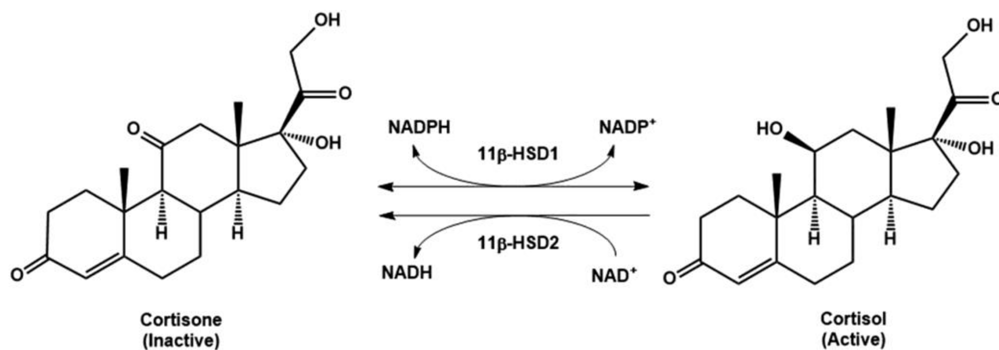

Figure 1.6. Glucocorticoid prereceptor metabolism. Enzyme 11βHSD1 catalyzes reaction of glucocorticoid activation using NADPH for this reaction. Reverse reaction is mediated by the enzyme 11βHSD2. 11βHSD1- 11β-hydroxysteroid dehydrogenase type 1; 11βHSD2- 11β-hydroxysteroid dehydrogenase type 2; NADPH- Nicotinamide adenine dinucleotide phosphate. Taken and modified from (Yang et al., 2018)

Glucocorticoids exert their effects by binding to the glucocorticoid (GR) or mineralocorticoid (MR) receptors that reside into the cytoplasm. Almost all cells in the body express GR, while MR has a dominant role in the secretory organs. MR has higher affinity to glucocorticoid hormone then GR. For that reason, 11βHSD2 has an important role to prevent that active glucocorticoids over activate MR in the kidneys, salivary glands, colon and other organs involved in regulation of salts in the body and blood pressure (Seckl et al., 2004).

When the hormone is not bound to the GR, it is predominantly located in the cytoplasm as part of a dynamic chaperone multiprotein heterocomplex (with heat shock proteins (Hsp70 and Hsp90), FK506 binding protein 51 (FKBP51) and etc.). This complex constantly undergoes cycles of dissociation and reassociation (Hu et al., 1994; Picard and Yamamoto, 1987) (Figure1.7). The chaperone complex ensures proper folding of the receptor leading to its maturation into a conformation that binds the hormone with high affinity (Pratt and Toft, 1997). When activated hormone binds to the GR, receptor undergoes conformational change followed by the change in the chaperones that results in transition of GR from the cytoplasm into the nucleus where it acts as transcriptional regulator of vast majority of genes involved in metabolism of glucose and lipids, inflammation and other processes.

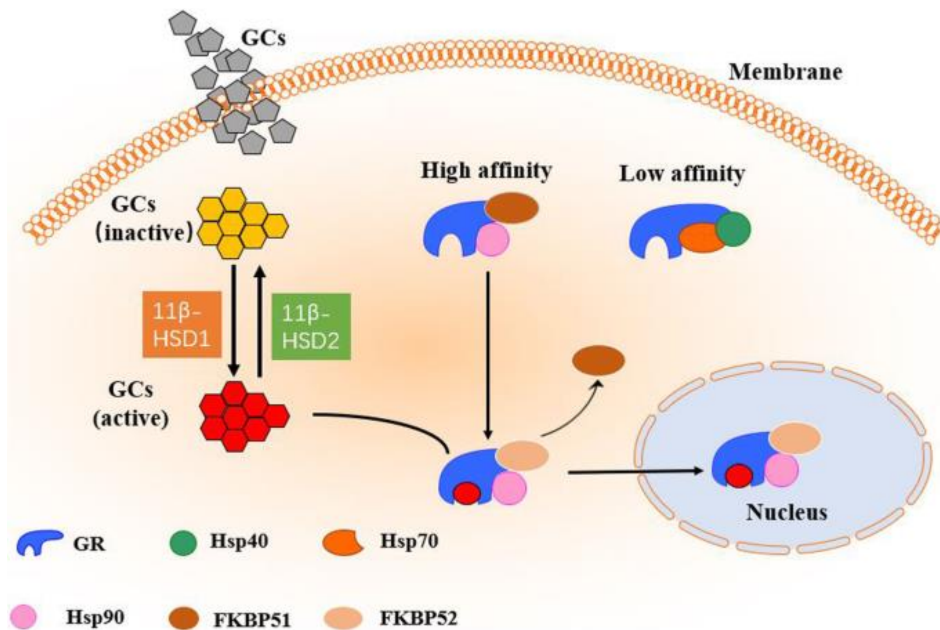

Figure 1.7. Glucocorticoid signaling pathway. Steroid nature of glucocorticoid hormones enables them to enter the cell feely through plasma membrane. Once inside the cytoplasm, glucocorticoid become a substrate for 11βHSD1 or 11βHSD, main enzymes of prereceptor metabolism that will activate or deactivate hormone, respectively. Activated hormone binds to glucocorticoid receptor that is in multiprotein complex with chaperones and heat shock proteins. Binding the hormone to receptor leads to conformational changes and exchange in protein complex, for example FKBP51 will be changed for FKBP52. Activated receptor will enter the nucleus and act as transcriptional regulator. GC- glucocorticoids; 11βHSD1/2- 11β-hydroxysteroid dehydrogenase type1/2; GR- glucocorticoid receptor; Hsp40/70/90- heat shock protein 40/70/90; FKBP51/2- the immunophilin FK506 binding protein 51/2. Taken and modified from (Han et al., 2019).

Having in mind that glucocorticoids play such a profound role in metabolism in almost all organs in the body, it is clear that unregulated levels of glucocorticoids can cause metabolic disorders and disturb energy homeostasis. Indeed, in genetically obese and lipotrophic mice, levels of glucocorticoids in circulation are significantly higher than in lean controls while adrenalectomy improves insulin regulated glucose transport into the muscles (Haluzik et al., 2002). Prolonged activation of glucocorticoid signaling in the skeletal muscle was shown to cause muscle insulin resistance. Morgan et al. have shown that treatment with glucocorticoids cause decrease of activating phosphorylation of IR, protein level of IRS1 and increase its inhibitory phosphorylation on Ser307 in skeletal muscle of mice (Morgan et al., 2009). Interestingly, patients with diabetes not only have increased expression of GR in skeletal muscle, but the level of increase correlates with the degree of insulin resistance while the treatments improving muscle sensitivity to insulin decrease expression of GR to the control level (Vestergaard et al., 2001; Whorwood et al., 2002). Although many studies have investigated effect of glucocorticoid on insulin signaling and glucose metabolism, exact mechanisms and direct targets of glucocorticoids that would affect glucose metabolism in such a fashion in skeletal muscle are still not clear. One interesting aspect could involve glucocorticoid ability to regulate lipid metabolism which has been previously shown to affect glucose homeostasis. Studies show that glucocorticoids promote lipid mobilization from visceral adipose tissue to skeletal muscle and increased level of TG have been detected in muscles after glucocorticoid therapy (Gounarides et al., 2008). The same authors have shown

connection between glucocorticoids and increased level of muscle diacylglycerol, known activator of PKC that was previously involved in onset of insulin resistance. Mice treated with 11βHSD1 inhibitor showed decrease in expression of genes involved in lipogenesis and lipid metabolism in skeletal muscle, indicating that glucocorticoids play significant role in regulation of this process. Another mechanism through which glucocorticoid can regulate energy homeostasis and insulin sensitivity is through control of inflammatory reactions in the cells. Namely, glucocorticoids are well known and widely used as anti-inflammatory agents which is described in the separate chapter of this thesis.

1.2 Energy disbalance

Energy disbalance can be defined as the state in which energy intake and energy expenditure significantly differs. Many regulatory systems have evolved to maintain energy balance and to alarm when disbalance occurs. Insulin, leptin and glucocorticoids are essential part of the wide network of regulatory systems with the role of preserving energy balance. Together, these three hormones regulate glucose and lipid metabolism by tightly balancing their oxidation with formation of energy deposits in skeletal muscles, liver and adipose tissue, as well as appetite, nutrient sensing, energy expenditure, response to stress and inflammation, and body regeneration in every aspect after exposure to stress of different magnitudes.

In everyday life our body is under tremendous amount of challenges. In order to respond to all the needs of modern life, we often put our bodies through stress by skipping regular balanced meals and replacing it with fast, processed and easily available food and soft drinks full of sugar. In addition, even when not hungry, we often crave for “comfort” food and burden our metabolism with unnecessary calories from the sweet and calorie-rich food that tastes good and makes us feel better after a stressful day. Even trying to eat healthier has its traps since many food on the market that is presented as healthy is actually full of hidden fat and sugar, especially fructose which use is extremely rising over the years with not enough caution or limitation because of its fruit origin. All this has led to development of metabolic diseases that follow the modern humans and their way of life.

1.2.1 Metabolic syndrome

The presence of combined metabolic disturbances such as visceral obesity, insulin resistance, hypertension, hyperglycemia and dyslipidemia was first described in 1920s. Almost 60 years later, Revan was the first to use the term "Syndrome X" and emphasized the clinical significance of these disturbances in combination. In the following years, variety of phrases was used to describe the joint appearance of mentioned disorders such as "Deadly Four" and "Insulin Resistance Syndrome". Today, it is considered that the metabolic syndrome is the most adequate term and as such it is most often used in the literature (Grundy et al., 2004; Kaur, 2014). Metabolic syndrome is recognized as a great health treat because it represents a significant predisposition for the development of cardiovascular diseases, type 2 diabetes and kidney disease. The diagnosis of metabolic syndrome is difficult to establish since not all of the described symptoms occur in every patient and usually appearance of one symptom, increases the probability of the following. Therefore, applied therapy is usually only

symptomatic without associating seemingly unrelated symptoms and considering the overall condition of the patient.

The molecular basis of the metabolic syndrome is not fully understood. It is noteworthy that endocrinologists have noticed morphological and metabolic similarities between Cushing's and metabolic syndrome. Namely, Cushing's syndrome is disorder that groups together symptoms and effects caused by chronic exposure to excess glucocorticoids (Nieman, 2018). Additionally, patients treated acutely or chronically with glucocorticoids often develop most of the previously described symptoms, such as visceral obesity, hypertension, insulin resistance, kidney problems etc. Therefore, it can be assumed that increased glucocorticoid signaling could be, at least in part, responsible for the pathogenesis of metabolic syndrome.

It has been proposed that genetic factors can play a significant role in the development of obesity (Rankinen et al., 2002) and it could be assumed that they also contribute to the development of metabolic syndrome. However, given the rate at which obesity and metabolic syndrome have spread around the world and the fact that people with metabolic syndrome are of different origins and backgrounds, it is thought that environmental factors could play a significant role in the development of the syndrome (Bray et al., 2004; Bray and Popkin, 2013). As metabolic syndrome is a disease of the modern age, there are more and more studies that try to connect its origin with the modern lifestyle, diet and habits. Daily exposure to stress and the availability of high-calorie foods rich in sugars, especially fructose, are serious candidates for the causes of metabolic disorders that make up the metabolic syndrome.

1.2.2 Fructose diet and metabolic disorders

Fructose, otherwise called fruit sugar, has been present in the human diet from the early begging. The main sources from which humans used to consume fructose were fruits, vegetables and honey. For most of the time during human history, these were the only sources of fructose used by man. This means that fructose was rarely taken as a solitary nutrient, but mainly as a component of sucrose and in combination with fiber and other nutrients from these sources. On the other hand, the diet of modern man contains much larger amount of fructose in vast majority of products. Fructose, being known as fruit sugar, has been considered healthier than glucose and therefore fructose began to replace glucose and to take the lead in the food industry. Thus, in modern society, fructose is not only consumed through fruits and vegetables, but also through canned food, soft drinks, artificial sweeteners and many other processed foods. Unexpectedly, large number of everyday products contains fructose, so the awareness of the amount of fructose intake by the consumer is very low. Regardless of that, the consumption of fructose is still on the rise, so today we are consuming it much more than fifty years ago (Bray and Popkin, 2013).

Many clinical trials, as well as studies on various animal models have shown an association between increased fructose intake and the onset of symptoms that accompany metabolic syndrome, such as obesity, hyperlipidemia and hypertension (Havel, 2005; Tappy and Le, 2010). In fact, increased intake of fructose-rich diet in modern society coincides with the occurrence and prevalence of metabolic syndrome (Bray, 2013). Therefore, it is suggested that fructose overconsumption could be important contributor to the increased incidence of metabolic disorders in modern society.

Observed associations of high fructose intake and onset of metabolic disturbances has raised an important question of what is so different about fructose that can influence metabolic health in such manner. One of the answers could be in distinct way that fructose is metabolized. Namely, although fructose and glucose are very similar hexose sugars their metabolism is quite different. While glucose can be transported into the cells of different organs and tissues through vast majority of transporter proteins called GLUT1-4, fructose is mainly transported through GLUT5 which is mostly located on to top and basolateral membranes of intestinal cells and is not regulated by insulin or energy status of a cell (Aydin et al., 2014). Alternatively, fructose can as well be transported with GLUT2 but it has low affinity for fructose (Douard and Ferraris, 2008). The main site of fructose metabolism is liver. Once inside the hepatocyte, fructose is converted to fructose-1-phosphate by the enzyme fructokinase. The enzyme fructose-1-phosphate aldolase then transforms fructose-1-phosphate into glyceraldehyde and dihydroxyacetone phosphate which can further be included in downstream steps of glycolysis. Dihydroxyacetone phosphate can be converted into glyceraldehyde-3-phosphate by isomerization, while glyceraldehyde will reach that point by phosphorylation. Although fructose metabolism looks similar to metabolism of glucose and eventually, intermediates from fructose degradation will be included into glycolysis, nevertheless fructose bypasses key regulation enzymes, hexokinase and phosphofructokinase (Figure 1.8). The enzymes fructokinase and fructose-1-phosphate aldolase use as a substrate only fructose and fructose-1-phosphate, respectively and their activity is not regulated by insulin or the energy status in the cell.

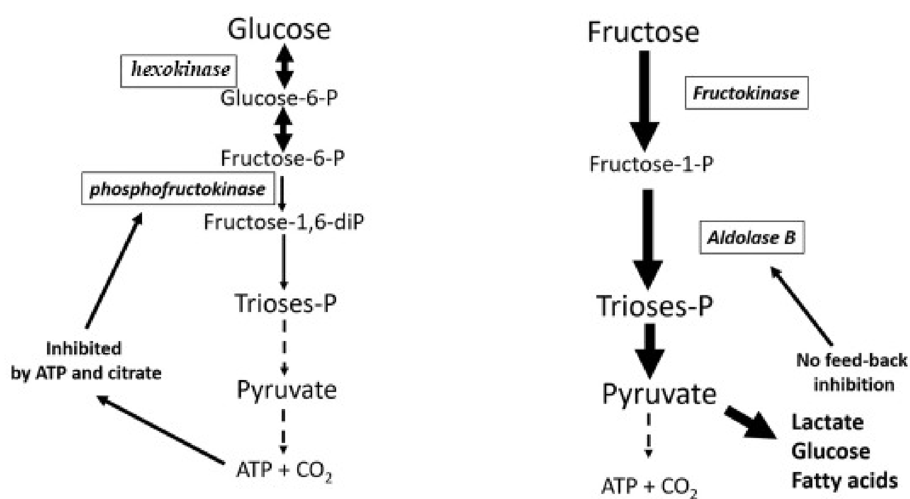

Figure 1.8. Fructose vs glucose metabolism. Fructose metabolism bypasses the key checkpoints of the glycolytic pathway, the enzymes hexokinase and phosphofructokinase, which are regulated by ATP and citrate as well as products of their reactions, so that glucose breakdown is coordinated with the energy status of the cell. As fructose is included in glycolysis downstream of the site of action of these enzymes, the metabolism of fructose continues to provide intermediates for the synthesis of fatty acids and triglycerides independent of energy needs. Taken and modified from (Tappy, 2021)

Therefore, increased fructose intake will continue to give intermediates for glycolysis and furthermore for the synthesis of fatty acids and triglycerides, regardless of energy status in the cell. The resulting triglycerides are packed in the liver cells into VLDL (*very low-density lipoprotein*) particles that are released into the bloodstream. Indeed, research indicates that fructose-enriched diets disrupt lipid homeostasis. Thus, long-term consumption of fructose increases circulating TG levels, increases ectopic fat accumulation in the liver and muscles (Lê

et al., 2009) and contributes to liver steatosis in men (Lê et al., 2006). Increased lipid influx into the skeletal muscle has been connected with onset of insulin resistance (Zhang et al., 2010). Accumulation of TG and lipid metabolites in skeletal muscle have been shown to decrease activating phosphorylations of IR and activate kinases that execute inhibitory phosphorylations of IR and IRS1 (Hegarty et al., 2003; Virkamäki et al., 2001). Clearly, fructose metabolism and its lipogenic potential are very important for the effects of fructose on metabolic health and animal studies have shown that inhibition of fructokinase in mice can protect them from developing metabolic disturbances caused by the excessive fructose intake (Ishimoto et al., 2012).

As indicated previously, essential regulator of glucose blood level and its metabolism is insulin. Nevertheless, insulin has very small to non-effect on fructose blood level and regulation of fructose metabolism. It has been shown that the level of GLUT5 transporter is very low at the cell membrane of pancreatic cells. Therefore insulin secretion is not driven by increase of blood fructose as it naturally increases by the rise of glucose (Aydin et al., 2014). It should be noted that when fructose is taken in combination with other sugars, proteins and fibers which is the case when consuming fruit and vegetables, these additional components will increase blood insulin level and activate appetite regulation signals. This is the main reason why consumption of fructose from these sources does not represent a risk factor for development of metabolic diseases. On the other hand, liquid fructose from the soft beverages will not trigger satiety signals and body will not be informed of the amount of energy it has consumed. Having in mind that insulin is a crucial endocrine regulator of energy balance and long term body weight, inadequate insulin secretion to energy consumption after long term fructose enrich diet can account for the prolonged increase of appetite and development of obesity. In addition to that, several authors have shown that fructose-enriched diet downregulates insulin receptor and decreases activation of IRS1 and Akt in skeletal muscle, one of the most important organs for systemic insulin sensitivity (Benetti et al., 2013; Bezerra et al., 2000; Catena et al., 2003). Several authors have indicated that long term fructose diet can lead to increase in circulating leptin level and development of resistance to leptin, another hormone essential for maintaining energy homeostasis (Alzamendi et al., 2009; Bursać et al., 2014). Shapiro et al have shown that high calorie diet rich in fructose and fat causes leptin resistance in rats and those effects of diet are lost when fructose is excluded (Shapiro et al., 2011). Leptin resistance has been observed after long term fructose diet even in rats that have no apparent metabolic problems and have unchanged body weight and circulating levels of leptin (Shapiro et al., 2008). This indicates that fructose has the ability to somehow exclude leptin from regulatory network that maintains energy balance in the body. In addition, alterations in expression of orexigenic and anorexigenic neuropeptides were detected after fructose diet as well. Because studies consistently suggest that fructose consumption can contribute to disturbances in main signaling pathways involved in maintain energy homeostasis, we choose to investigate and delineate mechanisms of how fructose enriched diet achieves its effects on metabolic health.

1.2.3 Stress and metabolic disorders

Homeostasis represents a dynamic steady state maintained in spite of influence of internal and external forces referred to as stressors. Therefore stress can be defined as a state of threatened homeostasis caused by physical, psychological, infectious or any other environmental factors and accompanied by numerous physiological responses and behavioral

changes with the aim of re-establishing the homeostasis (Kyrou et al., 2006). According to their nature, stressors are divided into: physical (trauma, surgery, intense cold or heat), chemical (reduced oxygen supply, disturbed acid-base balance), physiological (excessive exercise, hemorrhagic shock, intense pain), psychological or emotional (fear, sadness, anxiety) and social (personal conflicts, lifestyle changes). In terms of duration, stressors can be short-term (acute) and long-term (chronic) (Torres and Nowson, 2007). Regardless of their nature stressors trigger the body's general response to stress, which consists of fast (sympathetic adreno-medullary (SAM) axis) and slow (HPA) response. Both components of the response to stress include activation of central regions, predominantly localized in the hypothalamus and brainstem. Namely, the SAM component is activated by stimulation of sympathetic neurons that activate medulla of adrenal glands into secreting catecholamines. As already described, in the HPA component, CRH released from the hypothalamus stimulate the activation of the anterior pituitary and secretion of ACTH and consequently stimulate cortex of adrenal glands and secretion of glucocorticoids. The differences between the two components of the stress response are in the nature of output signals (neurotransmitter vs. hormone), speed of the response and its duration. For example, in response to stress, presynthesized catecholamines are released very quickly from the adrenal medulla upon the sympathetic stimulation. Unlike it, the response mediated by glucocorticoids takes longer to be activated considering that these steroid hormones cannot be stored in the adrenal cortex. Moreover, their synthesis and secretion are controlled by ACTH which takes longer to reach adrenal gland in comparison to electric signals sent via sympathetic neurons. Both components of stress response are responsible for receiving and integrating the diverse central and peripheral signals, which carry information about the action of a stressor (Ulrich-Lai and Herman, 2009).

Catecholamines and glucocorticoids determine the hormonal milieu in the body that will trigger numerous physical, chemical and behavioral changes that occur with the aim of increasing the chance of survival. Behavioral changes are aimed at providing increased cognitive abilities, alertness combined with increased concentration and focus, while inhibiting vegetative functions such as feeding and reproduction. Physical changes take place in the function of redistribution of energy in the organism, by which oxygen and nutrients are sent to the CNS and organs that are most affected by stress (heart, liver and skeletal muscles). Therefore, the increase in heart rate, level of respiration and intermediate metabolism (gluconeogenesis and lipolysis) are aimed at providing all vital substrates while energy-dependent processes such as food digestion, reproduction, growth and general immunity are temporarily suppressed. Also, detoxification processes are intensified both at the cellular and systemic level with the aim of quickly removing toxic substrates and metabolic by-products created during exposure to stress (Ulrich-Lai and Herman, 2009).

Modern lifestyle is characterized by unpredictable chronic stress. Chronic stress can be defined as state in which presence of stressors that threatens homeostasis is prolonged. When exposed to chronic stress, body's response to stress prolongs as well, causing changes in many essential physiological processes, such as immune reponse, energy metabolism, growth and reproduction. Therefore, chronic stress can be considered an important factor that can contribute to onset of metabolic disorders. In line with this are the studies that have shown association of chronic stress with higher body mass index, development of visceral obesity, higher blood pressure and metabolic syndrome (Dallman et al., 2003; Karagiannides et al., 2014; Rosmond, 2005; Spruill, 2010). People exposed to stress usually crave for palatable food, that ordinarily contains lots of sugars and/or fats, which will make them fill better (De Macedo et al., 2016). Kuo et al., (Kuo et al., 2007) have observed that even animals have shown this kind of behavior when under stress. Therefore, it was no surprise when it was discovered that stress can influence appetite and that neural networks in the hypothalamic

centers for stress response and appetite control are closely connected and regulated. It is known that acute stress triggers "fight or flight" stress response, that inhibits functions such as appetite or sense of hunger. On the other hand, chronic stress triggers more complex answer, therefore some people avoid food while others overeat when exposed to prolonged stress (Oliver et al., 2000; Schiffman et al., 2000). That is why exact causality between chronic stress and obesity still remains unclear (Dallman et al., 2003).

There are studies showing that, even when there are no changes in body weight after stress exposure, stress-related disturbances in lipid metabolism can be detected. Namely, increase in blood level of TG and cholesterol in persons that had some major exposure to stress such as suddenly loss of job, financial debt, earthquake etc. (Kelishadi, 2012) have been observed. The same worsening of lipid status has been shown for women working in the police department who were chronically under stress in comparison to the rest of the women population (Yoo and Franke, 2011). It has been shown that stress can stimulate increase of circulating TG and prolong their clearance after a meal (Kiecolt-Glaser, 2010). This so called postprandial lipidemia is a condition tightly associated with type 2 diabetes and metabolic syndrome. Indeed, chronic stress has been connected with development of insulin resistance in both animals (Shen et al., 2017) and humans (Yan et al., 2016). Epidemiological studies show that most of the patients with type 2 diabetes have experienced some major stressful events during life (Siddiqui et al., 2015). Exposure to chronic mild stress was connected with insulin impairment in the hypothalamus of rats (Pan et al., 2013) while diminished insulin signaling was marked in skeletal muscle of mice exposed to noise and psychosocial stressors (Liu et al., 2018; Sanghez et al., 2016). All previously stated, puts stress as important contributor of metabolic health and disease and as such should be taken into consideration when evaluating underlining mechanism of disease that strikes modern society.

1.2.4 Metabolic inflammation

It has been established that metabolic syndrome is often followed by a chronic law grade inflammation (Monteiro and Azevedo, 2010). Generally, inflammation represents physiological response to intracellular or extracellular harming stimuli followed by secretion of proinflammatory molecules in order to eliminate threat and reestablish tissue homeostasis. Proinflammatory mediators include bioactive amines, lipid mediators and cytokines, especially interleukins 1β and 6 (IL-1β and IL-6) and tumor necrosis factor α (TNFα). These signaling molecules lead to vasodilation, increased permeability of the capillary endothelium, and migration of leukocytes and monocytes/macrophages into the damaged tissue (Coutinho and Chapman, 2011). Nuclear factor κB (NFκB) is an inducible transcriptional regulator activated by a large number of inflammatory and environmental factors. In the inactive state, dimers of NFκB are localized in the cytoplasm and associated with inhibitory protein κB (IκB). In the presence of a stimulus, the kinase that phosphorylates IκB (IκB kinase; IKK) is activated, which phosphorylates IκB proteins, making them substrates for ubiquitination and subsequent degradation. Released NFκB dimers move to the nucleus where they bind to specific DNA sequences and regulate the transcription of proinflammatory cytokines and other target genes (Sun and Andersson, 2002).

Interestingly, metabolic inflammation is not caused by particular infection or injury but by disturbed metabolic balance (Hotamisligil, 2006). Although, the intensity of this inflammation is low, it is considered that long term presence of proinflammatory markers such as IL-1β, IL-6 and TNFα can contribute to the onset of various metabolic disorders

(Yudkin et al., 1999). The progression from insulin resistance to type 2 diabetes is accompanied by a state of low grade chronic inflammation (Yusuf et al., 2004), and the level of proinflammatory cytokines IL-6 and TNFα is increased in the serum of type 2 diabetes patients (Cardellini et al., 2007; Mohamed-Ali et al., 1997). Not only systemic but tissue specific inflammation has been detected as well. Presence of proinflammatory markers in the hypothalamus can disrupt insulin and leptin signalization in the neurons that secrete orexigenic and anorexigenic neuropeptides. Namely, increased level of SOCS3, known inhibitor of both leptin and insulin signaling pathway, was detected in neurons after treatment of cell culture with TNFα. In addition, promotor for SOCS3 gene have binding sites for NFκB (Thaler and Schwartz, 2010).

It has been shown that myocytes have the ability to express proinflammatory molecules especially under the lipid and cytokine stimulation (reviewed in (Wu and Ballantyne, 2017b)) (Figure 1.9). *In vivo* and *in vitro* studies revealed that proinflammatory cytokine TNFα can induce inhibitory phosphorylation of IRS1 and decrease stimulatory phosphorylation of Akt therefore causing insulin resistance in the skeletal muscle (Austin et al., 2008; Plomgaard et al., 2005). TNFα knockout mice are protected from insulin resistance even in genetically-derived and diet-induced obese animals (Uysal et al., 1997). Furthermore, high fructose diet has been shown to increase TNFα and proinflammatory transcription regulator NFκB in the skeletal muscle of mice and rats, as well as in cell culture (Benetti et al., 2013; Jaiswal et al., 2015; Togashi et al., 2000). Inflammation is considered an important link between stress exposure and metabolic disease (Liu et al., 2017). Glucocorticoids, as “stress hormones” are interacting with immune system modulating its effects. Glucocorticoids have anti-inflammatory effects and are widely used in medicine in the treatment of vast majority of inflammatory diseases. It has been shown that glucocorticoids inhibit the expression of genes that encode pro-inflammatory cytokines and chemokines, cell adhesion molecules as well as key enzymes involved in the initiation and maintenance of inflammation (Coutinho and Chapman, 2011), while at the same time positively regulating the expression of anti-inflammatory genes (Uhlenhaut et al., 2013).

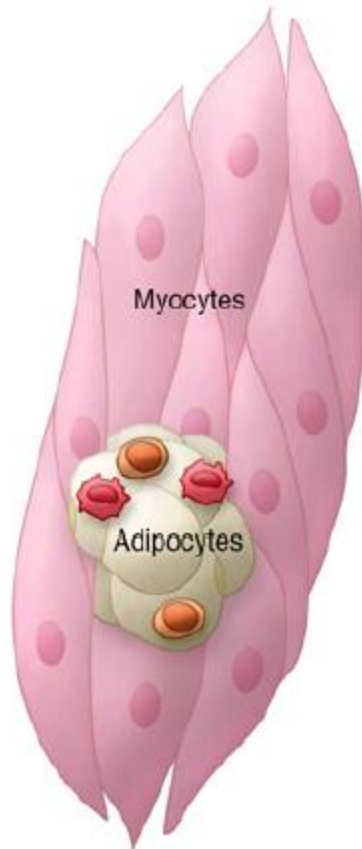

Figure 1.9. Skeletal muscle inflammation in obesity. **A)** In case of a lean subject, skeletal muscle contains few immune cells with anti-inflammatory or resting phenotype, **B)** In case of an obese subject, adipose tissue expands around muscle or even between the fibers of skeletal muscle. Present cells of immune system polarize into proinflammatory phenotype. In addition, myocytes can become inflamed and start secreting proinflammatory cytokines as well. More immune cells become attracted by secreted cytokines and chemokines leading to skeletal muscle inflammation. Taken and modified from (Wu and Ballantyne, 2017a)

Glucocorticoids inhibit NFκB signaling pathway by acting through a number of different mechanisms based on protein-protein interactions. Namely, it was shown that GR directly interacts with the p65 subunit of NFκB in the nucleus itself, thus preventing the transactivation function of NFκB (Liden et al., 1997). Furthermore, GR can block the recruitment of the transcription elongation factor to the promoters to which NFκB is bound and thereby prevent the initiation of transcription of proinflammatory genes regulated by the NFκB (Luecke and Yamamoto, 2005). Recruitment of histone deacetylase to promoters regulated by NFκB (Ito et al., 2006), or preventing the interaction of NFκB with histone acetyltransferases (Mckay and Cidlowski, 2000) are also examples of how glucocorticoids can negatively regulate NFκB activity at the epigenetic level. Although prolonged stress exposure has been connected with high level of inflammatory markers, data on chronic stress enrolment in rise of skeletal muscle inflammation are scarce.

2. Aim

Energy homeostasis requires a constant communication of different effector organs with neural and endocrine integrating centers. Insulin, leptin and glucocorticoids are among the most important hormones in the regulation of energy homeostasis as they ensure control of eating behavior, nutrient processing and energy expenditure within different organs and metabolic pathways. Hypothalamus and skeletal muscle are essential for different aspects of maintaining energy balance since main central regulation of appetite and energy expenditure is maintained in the hypothalamus, while the skeletal muscle use most of the glucose absorbed from the gastrointestinal tract. Signaling disturbances in one organ may affect the maintenance and regulation of energy homeostasis in the other organs, making the study of energy homeostasis and its deregulation highly complex. Though the cross-talk between different organs and signals in preserving the metabolic balance has been intensively studied for many years, we are far from a complete understanding of how this maintenance is achieved.

Overconsumption of caloric food rich in fructose and everyday exposure to unpredictable stressful events has become an inevitable burden of modern lifestyle. It is believed that these aspects of everyday life could be responsible for the growing epidemic of obesity and metabolic disorders almost all over the world. Currently, the biggest health threat is the metabolic syndrome, which includes obesity, dyslipidemia, insulin resistance and hypertension, and poses a high risk for the development of type 2 diabetes, cardiovascular diseases and some types of cancer.

The main aim of this doctoral dissertation is to provide an integrated view of insulin, leptin and glucocorticoid signaling in the skeletal muscle and hypothalamus of male rats under the challenging conditions of excessive fructose consumption and exposure to chronic unpredictable stress. The experiments were designed to test the hypothesis that fructose overconsumption combined with chronic unpredictable stress induces more pronounced metabolic derangements in the examined organs than each of the factors applied separately. The results of the dissertation should help answering an intriguing question whether it is stress, dietary fructose or their combination that is critical for the development of metabolic disturbances in the examined tissues, since this is one of the rare studies focused on the metabolic impact of fructose overconsumption in the stressful environment. In order to achieve the main aim several specific aims have been established:

- ✓ To investigate whether excess fructose consumption, stress and their combination influence energy intake, body weight and adiposity in male rats

- ✓ To determine if and how fructose diet and/or chronic stress affect appetite and expression of orexigenic and anorexigenic neuropeptides in the hypothalamus and to evaluate hypothalamic leptin signaling

- ✓ To investigate if and how applied treatments influence insulin signaling in the hypothalamus

- ✓ To evaluate lipid and glucose metabolism in the skeletal muscle after fructose diet, chronic stress and their combination and if and how these changes affect muscle insulin signaling pathway

- ✓ To analyze glucocorticoid signaling and inflammatory status of the skeletal muscle after fructose enriched diet, chronic stress exposure and their combination

3. Material and methods

3.1 Material

Fructose was purchased from Apipek (Bečej, Serbia) and commercial rodent food from Veterinary Institute Subotica, Serbia. Detailed information about the antibodies used was presented in the Table3.2, while anti-tubulin antibody was WA3 mouse monoclonal antibody raised against bovine brain tubulin (Dr. Ursula Euteneuer). Immobilon-FL polyvinylidene difluoride (PVDF) membrane was a product of Millipore (Burlington, MA, USA). The Amersham ECL Western blotting detection kit was acquired from GE Healthcare Life Sciences (Chicago, IL, USA). X-ray films were obtained from Kodak, Rochester, NY, USA. A Corticosterone EIA kit was obtained from Immunodiagnostic Systems Ltd. (Boldon, UK) while Rat Leptin ELISA kit from Merck Millipore, Burlington, MA, USA. TRIzol® Reagent was obtained from Ambion (Austin, TX, USA), RNase-free DNase I from Ferments (Waltham, MA, USA), while RNase-DNase-free water from Eppendorf (Hamburg, Germany). The following products were purchased from Applied Biosystems (Waltham, MA, USA): the high-capacity cDNA reverse-transcription kit, RNase inhibitor, the TaqMan® universal PCR master mix with AmpErase UNG, and the TaqMan® gene expression assay primer-probe mix for: GR (Rn00561369_m1), 11βHSD1 (Rn00567167_m1), Kruppel Like Factor 15 (KLF15) (Rn00585508_m1), lipoprotein lipase (LPL) (Rn00561482_m1), adipose triglyceride lipase (ATGL) (PNPL2, Rn01479969_m1), IL-1β (Rn00580432_m1), IL-6 (Rn01410330_m1), TNFα (Rn01525859_g1), AgRP (Rn01431703_g1), ObRb (Rn00561369_m1), SOCS3 (Rn00585674_s1) and hypoxanthine phosphoribosyl transferase 1 (HPRT) (Rn01527840_m1).

Power SYBR® Green PCR master mix was purchased from Applied Biosystems, and specific primer pairs for genes stated in the Table 3.1, from Invitrogen (Waltham, MA, USA).

Table3.1. Sequence of forward and reverse primers used for SYBR® Green PCR

Gene name	Forward primer sequence	Reverse primer sequence
CPT1b	5’-CCAGGCAAAGAGACAGACTTG-3’	5’-GCCAAACCTTGAAGAAGCGA-3’
Fatty acid transport protein (FATP1)	5’-CCCAAGTGGATACAACAGGCA-3’	5’-GGTCTAGAAAGAAGAGCCGGTC-3’
Peroxisome proliferator-activated receptor α (PPARα)	5’-CGTTTTGGAAGAATGCCAAG-3’	5’-GCCAGAGATTTGAGGTCTGC-3’
POMC	5’-TCCATAGACGTGTGGAGCTG-3’	5’-GACGTACTTCCGGGGATTTT-3’
HPRT	5’-CAGTCCCAGCGTCGTGATTA-3’	5’-AGCAAGTCTTTCAGTCCTGTC-3’

Table3.2. List of antibodies with their specifications used for Western Blot

Antibody	Code	Manufacturer
Anti-11βHSD1	ab109554	Abcam (Cambridge, UK)
Anti-β actin	ab8227	Abcam (Cambridge, UK)
Anti-GLUT1	ab625	Abcam (Cambridge, UK)
Anti-GLUT2	ab95256	Abcam (Cambridge, UK)
Anti-GLUT3	ab41525	Abcam (Cambridge, UK)
Secondary anti-mouse H&L horseradish peroxidase (HRP)-linked antibody	ab97046	Abcam (Cambridge, UK)
Secondary anti-rabbit IgG H&L horseradish peroxidase (HRP)-linked antibody	ab6721	Abcam (Cambridge, UK)
Anti-pNFκB-Ser536 (93H1)	3033	Cell Signaling Technology (Danvers, MA, USA)
Anti-ERK	91025	Cell Signaling Technology (Danvers, MA, USA)
Anti-pERK-Thr202/Tyr204	9101s	Cell Signaling Technology (Danvers, MA, USA)
Anti-pAMPK-Thr172	41885	Cell Signaling Technology (Danvers, MA, USA)
Anti-GR (H-300)	sc-8992	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-FKBP51	sc-13983	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-H6PDH	sc-67394	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-pIRB	sc-25103	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-IRS1 (E-12)	sc-8038	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-pIRS1-Tyr632	sc-17196	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-pIRS1-Ser307	sc-33956	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-Akt	sc-8312	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-pAkt-Thr308	sc-16646-R	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-pAkt-Ser473	sc-7985-R	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-PTP1B (N-19)	sc-1718-R	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-PPARδ (PPARβ, F-10)	sc-74517	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-NFκB/p65 (C-20)	sc-372	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-IκB	sc-371	Santa Cruz Biotechnology (Dallas, TX, USA)

3.2 Animals and treatments

Male Wistar rats were bred in the Animal facility at the Institute for Biological Research "Siniša Stanković"- National Institute of the Republic of Serbia, University of Belgrade and used as animal model in this study. At the age 2.5 months old, the animals were divided into 4 experimental groups (n = 8–9 per group, 3 animals per cage). Animal allocation to the experimental groups was performed by appropriate randomization method in order to ensure blinding. Animal treatment consisted of high fructose diet (9 weeks) and/or chronic stress exposure (last 4 weeks), as explained in the Table 3.3. Fructose fed groups had 20% (w/v) fructose solution instead of drinking water while stressed groups were exposed to chronic unpredictable stress. Precise stress protocol was defined at the beginning of the treatment and included: the randomly chosen type of the stressor, the number of daily stressors to be applied (one or two stressors per day) and the onset of the stressor. Stressors were randomly chosen from the modified stress protocol of Joels et al and included: forced swimming in cold water for 10 min, physical restraint for 60 min, exposure to a cold room (4°C) for 50 min, wet bedding for 4 h, switching cages for 2 h, rocking cages for 1 h, and cage tilt (45°) overnight. Chosen stressor was never repeated twice a day or two days in a row. Treatments were chosen to resemble modern human lifestyle (Joëls et al., 2004; Ventura et al., 2011) in regard to both, concentration of applied fructose solution, as well as the type, sequence and duration of stressors. Experimental animals had *ad libitum* access to the food and drinking fluids. Food composition is shown in Table3.4. Animals were kept under standard conditions at 22°C with a 12-h light/dark cycle, with constant veterinary care during the whole course of the experiment. All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for scientific purposes and approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research Siniša Stanković, University of Belgrade (permit No. 01-1171 obtained on 28.07.2020).

Table3.3. Animal treatment.

Experimental group	Standard chaw	Drinking water	20% fructose solution (9 weeks)	Unpredictable stress (last 4 weeks)
Control -C	+	+	-	-
Fructose -F	+	-	+	-
Stress - S	+	+	-	+
Stress + Fructose - SF	+	-	+	+

Table3.4. Composition of solid food used in our study for all animal groups.

Composition of food	
Ingredients	**per kg of chaw**
Total protein	**200 g**
Total carbohydrate	**626 g**
Total fat	**32 g**
Total ash	**62 g**
Corn starch	**407.1 g**
Cellulose	**63.6 g**
Methionine + cystine	**7.5 g**
Choline	**1.97 g**
Antioxidant	**0.1 g**
Moisture	**81 g**
Vitamin A	**10000 IU**
Vitamin D3	**1600 IU**
Vitamin E	**40 IU**
Vitamin K	**1.72 mg**
Vitamin B12	**57 µg**
D/biotin	**0.58 mg**
Folic acid	**0.91 mg**
Riboflavin	**4.46 mg**
Thiamin	**9.07 mg**
Pyridoxine	**7.93 mg**
Nicotinic acid	**65.60 mg**
Panthotenate	**24.47 mg**
Zinc	**100 mg**
Copper	**20 mg**
Iodine	**0.50 mg**
Manganese	**30 mg**
Magnesium	**2400 mg**
Chloride	**2000 mg**
Calcium	**10000 mg**
Phosphorus	**3500 mg**
Potassium	**9100 mg**
Sulfur	**2300 mg**
Sodium	**1500 mg**
Molybdenium	**0.06 mg**
Selenium	**0.36 mg**
Flourine	**0.20 mg**
Irone	**120 mg**

3.3 Animal model characterization

Food and liquid intake were measured daily during the whole 9 weeks of the treatment. Energy intake from food and fructose was calculated using measured data and information that 1 g of chow contains 11 KJ and 1 ml of fructose contains 1.72 KJ. The total energy intake was obtained from their sum. Body mass was measured weekly, while the mass of adipose tissue was measured immediately after sacrifice. The ratio of mass of adipose

tissue to body mass on the last day of the experiment was calculated based on these measurements.

Animals were sacrificed by rapid decapitation with a guillotine (Harvard Apparatus, Holliston, MA, USA). The night before sacrifice, food was removed from all animals, and fructose solution was replaced by drinking water for groups on the fructose regime. Trunk blood from individual animals was collected in separate tubes with EDTA and centrifuged at 1600 x g for 10 min, after which the blood plasma was separated as the supernatant. Plasmas were stored at -20°C until use.

Visceral adipose tissue (retroperitoneal and perirenal) was excised and measured. Skeletal muscle, *musculus gastrocnemius*, was isolated, washed with saline, dried, immediately frozen in liquid nitrogen and stored on -80°C until further use. The hypothalamus was isolated from ventral side of the brain between optic chiasm (rostral limit), thalamus (dorsal limit) and mammillary bodies (caudal limit). Isolated hypothalamus was rapidly frozen and kept in liquid nitrogen until needed.

3.4 Measurement of corticosterone, leptin and insulin

Corticosterone levels were measured in blood plasma and skeletal muscle using Corticosterone EIA kit fallowing the manufacturer's instructions. In order to calculate corticosterone concentrations 4PL curve fitting method (Prism 5.0, GraphPad Software, Inc., La Jolla, CA, USA) was used that included spectrophotometric measurements of sample absorbance at 450 nm and reference absorbance at 650 nm. Assay sensitivity was 0.17 ng/ml while intra-assay and inter-assay coefficient of variation (CV) were 5.9% and 8.9%, respectively.

Leptin was measured in plasma using Rat Leptin ELISA kit according to the instructions from the manufacturer. The assay sensitivity was 0.04 ng/ml, while intra-assay CV was 1.88% and inter-assay CV was 3.31%.

Insulin concentrations were measured in blood plasma by RIA method by using rat insulin standards (INEP, Belgrade, Serbia). Sensitivity of this insulin assay was 0.6 mIU/L, while an intra-assay CV was 5.24%. Quantitative insulin sensitivity check index (QUICKI) and Revised quantitative insulin sensitivity check index (R-QUICKI) were used to determine insulin sensitivity of experimental animals. Indexes were calculated using formulas:

$$QUICKI = \frac{1}{\log insulin\left(\mu\frac{U}{ml}\right) + \log glucose(\frac{mg}{dL})}$$

$$R\text{-}QUICKI = \frac{1}{\log insulin\left(\mu\frac{U}{ml}\right) + \log glucose\left(\frac{mg}{dL}\right) + \log NEFA(\frac{mmol}{L})}$$

Circulating glucose levels were measured from the whole blood after decapitation by MultiCare strips (Biochemical Systems International, Italia). The level of non-esterified fatty acids (NEFA) in plasma was determined colorimetrically (Semi-auto Chemistry Analyzer, Rayito 1904C) using a kit for NEFA measurement (Randox Laboratories Ltd., Crumlin, UK).

3.5 RNA isolation

Total RNA from skeletal muscle and hypothalamus was isolated using TRIzol® reagent according to the manufacturer's instructions. Briefly, the tissues were homogenized in TRIzol® reagent 1:10 (m/z) and the homogenates were centrifuged (10 min, 12,000 g, 4°C), after which the upper lipid layer was discarded, and the supernatant was incubated for 5 min at 30°C. Chloroform was then added and by centrifugation (10 min, 12,000 g, 4°C) the surface, aqueous phase containing the RNA was separated, which is then precipitated overnight at -20°C by adding 3M Na-acetate, pH 5.0 and isopropanol. Precipitated RNA was washed with 75% ethanol (10 min, 14,000 g, 4°C) and dissolved in RNase- and DNase-free water. An aliquot of RNA was set aside for concentration measurement and determination of RNA purity. RNase inhibitor was added to the rest of the isolated RNA samples. RNA samples were frozen and stored at -80°C until use.

3.6 DNase treatment of RNA and reverse transcription

Possible contamination of the prepared RNA samples with DNA was removed by enzymatic treatment. The enzyme deoxyribonuclease 1 (DNase 1) is an endonuclease that digests single- and double-stranded DNA. The enzyme was added to the RNA samples and after 30 min incubation at 37°C, the reaction was stopped by heating to 65°C in the presence of EDTA. The "High capacity cDNA reverse transcription kit" was used to obtain cDNA according to the manufacturer's instructions. Reverse transcription was performed in a 20 μl reaction. Reaction was a mixture, which in addition to the RNA sample, contained the enzyme reverse transcriptase (MultiScribeTM), a mixture of deoxyribonucleotide triphosphates (dNTPs) and randomly constructed primers. The reaction took place according to the following temperature regime: 10 min at 25°C, then 2 hours at 37°C and was stopped by heating to 85°C. With each individual reverse transcription reaction, an additional reaction without the presence of the reverse transcriptase enzyme took place, which served as a negative control. Synthesized cDNA was stored at -80°C until further use.

3.7 Real-time Polymerase chain reaction (RT- PCR)

The expression of genes encoding GR, KLF15, 11βHSD1, LPL, ATGL, CPT1, FATP1, PPARα, IL-1β, IL-6 and TNFα in skeletal muscle and genes encoding POMC, AgRP, SOCS3 and ObRb in the hypothalamus was examined. All real-time polymerase chain reactions (RT-PCR), also known as quantitative PCR, were performed on Quant Studio™ 3 Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The total volume of the reaction mixture was 20-25 μl. We used TaqMan (for GR, KLF15,11βHSD1, LPL, ATGL, AgRP and SOCS3) and SYBR® Green (for CPT1b, FATP1, PPARα and POMC) based RT-PCR methods.

SYBR® Green reaction mix consisted of: 1 x power SYBR® Green PCR master mix, specific primer sets, and 20 ng of cDNA synthesized by reverse transcription. Thermal cycling conditions were: 2-min incubation at 50°C for UNG activation, 10 min at 95°C followed by 40 cycles at 95°C for 15s and 60°C for 60 s. The specificity of the SYBR® Green reaction was verified by melting curve analyses. To detect possible reagent contamination no template control was included for each target gene. Relative quantification of gene expression was performed using the comparative $2^{-\Delta\Delta Ct}$ method.

TaqMan reaction mix 1x contained: "TaqMan Universal PCR Master Mix", 1x "TaqMan Gene Expression Assay" and as a matrix 20 ng of cDNA. "TaqMan Universal Master Mix" contains AmpliTaq Gold DNA polymerase, dNTP with dUTP, AmpErase uracil N glycosylase and as a passive reference ROX dye "TaqMan Gene Expression Assay" for the selected gene contains a pair of unlabeled primers and TaqMan MGB (Minor groove binder) probe marked with the reporter FAM fluorescent dye at the 5' end, and at the 3' end there is a quencher that serves to suppress the fluorescence of the reporter dye when it is in its vicinity, i.e. when the sample is intact. In order to avoid contamination with genomic DNA, primers spanning the exon-exon junction were used. The initiation phase of the RT-PCR reaction was achieved by incubation for 2 min at 50°C during which uracil-N-glycosylase is activated and 10 min at 95°C during which AmpliTaq Gold DNA polymerase is activated, followed by 40 cycles of 15 s of denaturation at 95°C followed by 60 s at 60°C during which primer hybridization and elongation occur.

Each RT-PCR measurement included a blank, as a negative control in which the cDNA was omitted. All reactions were performed in triplicate in a 96-well microtiter plate. To reduce variation, the same cDNA mixture was used to amplify all genes in one sample, while each individual gene was tested in different samples on a single PCR plate. HPRT was used as reference gene.

3.8 Preparation of protein extracts from hypothalamus

Hypothalamic total protein was isolated using TRIzol®. As previously described, after homogenization in TRIzol® reagent (1:10, m/z), centrifugation (10 min, 12,000 g, 4°C) and incubation of supernatant at 30°C for 5 min, chloroform was used to separate different phases that will contain RNA, DNA and proteins. For preparation of total proteins from hypothalamus, acetone was added to phenol-ethanol supernatant obtained from organic phase. After centrifugation (10 min at 12 000×g, 4°C), 0.3 M guanidine hydrochloride in 95% ethanol and 2.5% glycerol were added to pellets and sonicated on ice. This part was repeated three times for thoroughly washing. After final centrifugation (5 min at 8 000×g, 4°C), pellet was resuspended in the lysis buffer (2.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 50 mM DTT) and used as total protein from hypothalamus. Samples were stored at –80°C until further use.

3.9 Preparation of protein extracts from skeletal muscle

In order to prepare tissue protein extracts skeletal muscles were thaw, and homogenized in ice-cold RIPA buffer 1:4 (w/v) (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 10 mM EDTA-Na2, 10 mM EGTA-Na2, 0.5% Triton X, 1% NP40, 0.1% SDS, 2 mM dithiothreitol, and protease and phosphatase inhibitors) using Janke-Kunkel Ultra Turrax T25

(IKA®, Staufen, Germany). After sonification of formed homogenates (3 x 5 s, 1A, 50/60 Hz on ice) and incubation on ice for 30 min with frequent vortexing, finale centrifugation (20 min on 14,000 x g, 4°C) lead to formation of supernatants that were saved and used as the muscles tissue extracts. Obtained whole cell protein extracts were stored at –80°C until further use.

3.10 Western blot

Separation of proteins according to molecular masses was performed by electrophoresis on polyacrylamide gels in a Mini-Protean II Electrophoresis Cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Each gel was composed of two functionally different parts, an upper gel for concentrating (4% acrylamide / 0.14% bisacrylamide, 0.1% SDS, 125 mM Tris-HCl, pH 6.8) and a lower gel for separating proteins of molecular mass between 50 and 100 kDa (7.5% acrylamide / 0.27% bisacrylamide, 0.1% SDS, 375 mM Tris-HCl, pH 8.8) or a gel for separating proteins of smaller molecular masses (12% acrylamide / 0 .43% bisacrylamide, 0.1% SDS, 375 mM Tris-HCl, pH 8.8). 0.05% ammonium persulfate and 0.033% TEMED were used as gel polymerization catalysts.

Samples were prepared by boiling for 5 min at 100°C in an equal volume of reducing 2-fold concentrated sample buffer (125 mM Tris, pH 6.8 containing 20% v/v glycerol, 4% v/v SDS, and 10% β-mercaptoethanol), after which they were applied to a gel and separated at a constant voltage of 120 V at 4°C for about 90 min (reservoir buffer: 192 mM glycine, 0.1% SDS and 25 mM Tris-HCl, pH 8.3).

Protein transfer from gels to PVDF membranes was performed in a Mini Trans-Blot Electrophoretic Transfer Cell apparatus (Bio-Rad Laboratories, Hercules, CA), overnight at a constant current of 135 mA per gel at 4°C, in 25 mM Tris buffer, pH 8.3, containing 192 mM glycine and 20% (v/z) methanol. After the transfer, the PVDF membranes were stained with a 1% solution of Ponceau S in 5% acetic acid, in order to check the efficiency of the transfer. Membrane sites not occupied by proteins were blocked by incubation for 1 h at room temperature in PBS buffer (1.5 mM KH2PO4, 6.5 mM Na2HPO4, 2.7 mM KCl, 0.14 M NaCl, pH 7.2) which contains 1% skimmed milk powder. Primary antibodies were used to detect GR, 11βHSD1, H6PDH, FKBP51, CPT1, PPARγ, NFκB, pNFκB-Ser536, IκB, IRB, pIRB-Tyr1162/1163, IRS1, pIRS1-Ser307, pIRS1-Tyr632, Akt, pAkt-Thr308, pAkt-Ser473, extracellular signal–related kinase (ERK), pERK-Thr202/Tyr204, pAMPK-Thr172, PTP1B, GLUT1, GLUT2, GLUT3, β tubulin and β actin. Membranes were incubated with primary antibodies overnight at 4°C with stirring, then washed 4 times for 5 min each with PBS buffer containing 0.1% Tween 20 and incubated with the appropriate secondary antibody conjugated with alkaline phosphatase for 1 h at room temperature. As a control for equal loading of samples on the gel β tubulin and β actin were used for each sample from skeletal muscle and hypothalamus, respectively. Before performing the enzymatic reaction, the membranes were washed with PBS buffer containing 0.1% Tween 20 and then with pure PBS buffer. On the membranes, (HRP-)Immunoreactive bands were established by Amersham ECL Western Blotting Detection Kit and developed using X-ray films or detected using iBright FL1500 Imaging System (Thermo Fisher Scientific, MA, USA). Quantitative analysis of relative integrated optical densities of immunopositive bands was performed with ImageQuant software. After detection of each protein bound antibodies were removed with 0.2 M NaOH, membranes blocked and further incubated with the next antibody.

3.11 Measurement of RNA and protein concentrations

RNA concentration was determined by measuring the absorbance of the sample at 260 nm with a BioPhotometer spectrophotometer (Eppendorf). Since the absorbance of one unit, measured at 260 nm, corresponds to 40 μg/ml of RNA, the concentration of RNA was automatically calculated by the formula: Concentration of RNA = 40 x A260 x dilution factor.

To check the purity of RNA, the ratio of absorbance at 260, 280 and 230 nm was used. Absorption at 280 nm indicates the presence of proteins in the sample, while absorption at 230 nm indicates contamination of the sample with carbohydrates, peptides, phenols and aromatic compounds. The ratios A260/A280 > 1.8 and A260/A230 > 1.8 were considered satisfactory.

Protein concentrations were determined by the Lawry method. Samples were prepared with Reagent C (2% Na-K tartrate, 2% sodium carbonate in 0.1M NaOH and 1% copper sulfate) and incubated 15 min at room temperature. After adding 5 x diluted Folin-Ciocalteau reagent all samples were vortexed and let 30 min on room temperature with occasional vortexing. A standard curve was constructed based on absorbance measurements of bovine serum albumin solutions of known concentrations. Series of dilutions of 1 mg/ml bovine serum albumin in the same buffer containing the samples of unknown concentrations, to give concentrations of 0.1 to 1 mg/ml. Absorbances were measured spectrophotometrically at 650 nm and protein concentrations were calculated from standard curve.

3.12 Statistics

Effects of fructose, stress and their interaction were evaluated by two-way ANOVA followed by the post hoc Tukey test. Shapiro-Wilk test was used to test distribution normality of the obtained data. A probability level less than 0.05 was considered to be statistically significant. Data are presented as mean ± SEM. All analyses were performed using STATISTICA 8.0 software (Stat Soft Inc., USA).

4. Results

4.1 Physiological and biochemical parameters

In order to evaluate energy status of the animals on long-term fructose rich diet, exposed to chronic stress or their combination, we measured daily food and liquid intake and calculated energy intake. Two Way ANOVA showed that fructose had marked effect on solid food intake ($F(1,32)=147.29$, $p<0.001$), liquid intake ($F(1,32)=82.25$, $p<0.001$) and total energy intake ($F(1,32)=130.89$, $p<0.001$). Post-hoc results have shown that animals on fructose diet had higher energy intake in comparison to control animals ($p<0.001$, F, SF vs C) and stressed animals on standard diet ($p<0.001$, SF vs S). Fructose fed animals had increased liquid intake ($p<0.001$, F, SF vs C; SF vs S) and decreased solid food intake ($p<0.001$, F, SF vs C; SF vs S) (Fig 4.1).

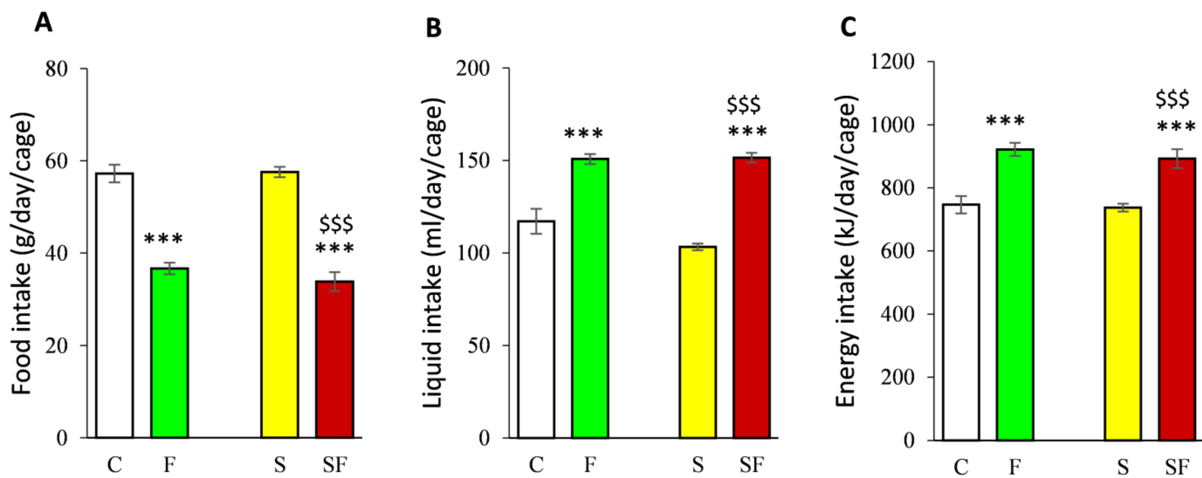

Figure 4.1. Food, liquid and energy intake of rats exposed to long term fructose diet, chronic stress and/or their combination. **A)** Food intake recorded daily and expressed per day per cage, **B)** Liquid intake recorded daily and expressed per day per cage, **C)** Energy intake calculated as sum from the calories originated from food and liquid consumed by male Wistar rats of control (C), fructose (F), stress (S) and stress+fructose (SF) experimental groups (n=6-8 animals per group). Two Way ANOVA was used to determine effects of fructose diet, stress exposure and their interaction on rats while post-hoc Tukey test was used to evaluate intergroup differences. Values of $p<0.05$ were considered statistically significant. Presented data are means ± S.E.M. Statistical significant differences: $^{***}p<0.001$, treated animals vs. C; $^{\$\$\$}p<0.001$, SF vs. S.

Nevertheless, the body mass of fructose fed animals remained unchanged (Fig 4.2A). Adiposity index was calculated as ratio of visceral adipose tissue (VAT) mass to total body mass and Two Way ANOVA revealed that stress ($F(1,20)=4.80$, $p<0.05$) and fructose diet ($F(1,20)=13.64$, $p<0.01$) had significant effect on its value. Namely, according to *post hoc* test stressed group had significant decrease in adiposity index compared to the control group ($^{*}p<0.05$, S vs. C) while stressed rats on fructose diet increased adiposity index in comparison to stressed rats on standard diet ($^{\$\$}p<0.01$, SF vs. S) (Fig 4.2B).

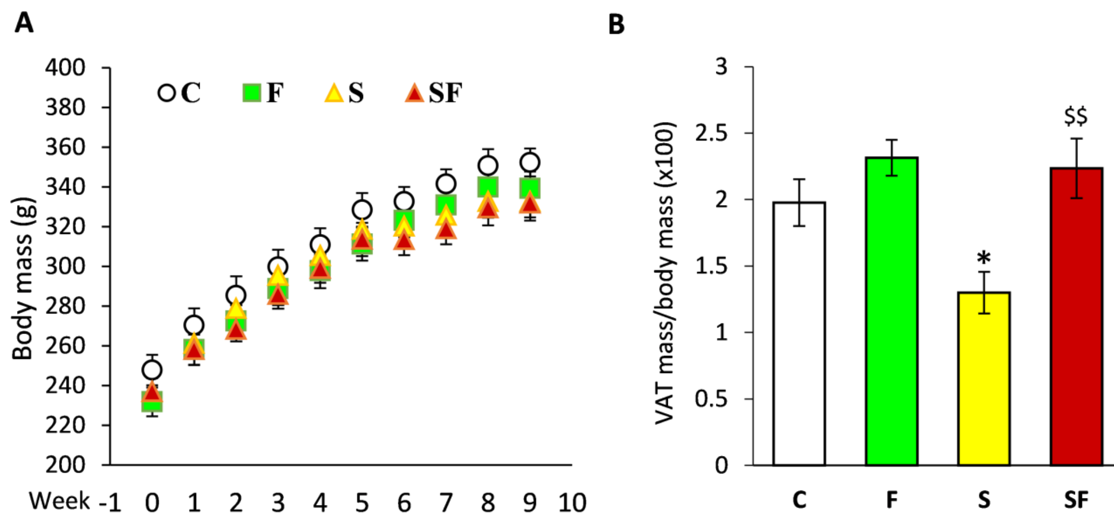

Figure 4.2. Physiological parameters of rats exposed to long term fructose diet, chronic stress and/or their combination. **A)** Weekly recorded body mass, **B)** Ratio of VAT mass to body mass, calculated from the measurement taken at the end of the treatments from male Wistar rats of control (C), fructose (F), stress (S) and stress+fructose (SF) experimental groups (n=6-8 animals per group). Two Way ANOVA was used to determine effects of fructose diet, stress exposure and their interaction on rats while post-hoc Tukey test was used to evaluate intergroup differences. Values of $p<0.05$ were considered statistically significant. Presented data are means ± S.E.M. Statistical significant differences: *$p<0.05$, treated animals vs. C; $^{\$\$}p<0.01$, SF vs. S. VAT- visceral adipose tissue.

Two Way ANOVA detected main effect of fructose ($F(1,20)=42.16$, $p<0.001$) on plasma insulin levels. Nevertheless, only fructose fed rats exposed to stress had increased plasma insulin levels compered to untreated animals (*$p<0.05$, SF vs. C) and stressed animals on standard diet ($^{\$\$}p<0.01$, SF vs. S), as shown by *post hoc* test (Fig 4.3A). QUICKI and R-QUICKI are indexes that are often used to estimate insulin sensitivity. Two Way ANOVA showed significant effect of fructose diet on R-QUICKI ($F(1,28)=5.98$, $p<0.05$) leading to decrease of this index in stressed rats on fructose diet comparing to stress rats on standard diet ($^{\$}p<0.05$, SF vs. S). Value of QUICKI index remained unaltered by either of the applied treatments (Fig 4.3B and C).

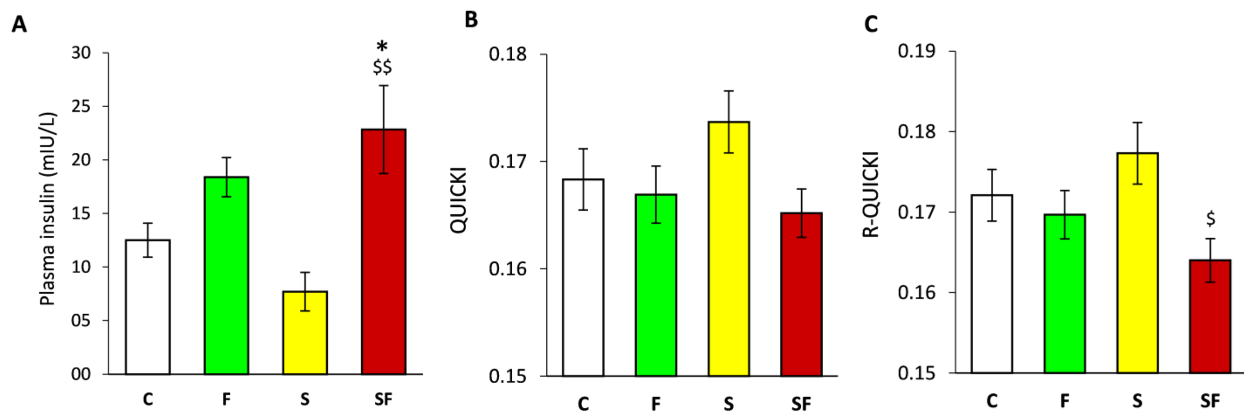

Figure 4.3. Insulin concentration and indexes of insulin sensitivity. **A)** Concentration of insulin in circulation (mIU/L), **B)** QUICKI index calculated as 1/log[insulin]+log[glucose] and **C)** R-QUICKI, calculated as 1/ log[insulin]+log[glucose]+log[NEFA] in male Wistar rats of control (C), fructose (F), stress (S) and stress+fructose (SF) experimental groups (n=6-8 animals per group). Data are presented as mean ± S.E.M. Two Way ANOVA was used to determine effects of fructose diet, stress exposure and their interaction on rats while post-hoc Tukey test was used to evaluate intergroup differences. Values of $p<0.05$ were considered statistically significant. Statistical significant changes were marked: *$p<0.05$, treated animals vs. C; $^{\$}p<0.05$ and $^{\$\$}p<0.01$, SF vs S. QUICKI- Quantitative insulin sensitivity check index, R-QUICKI- Revised quantitative insulin sensitivity check index.

4.2 Leptin signaling in the hypothalamus

Stress had significant effect on plasma leptin level ($F(1,24)=36.24$, $p<0.001$) as revealed by Two Way ANOVA. *Post-hoc* test showed that plasma leptin levels were decreased in stressed group in comparison to control group (**$p<0.01$, S vs. C). On the other hand, trend in increase in plasma leptin levels was detected in stressed rats on fructose diet compared to stressed rats on standard diet ($p=0.07$) (Fig 4.4A).

Similarly, *post-hoc* test showed decreased expression of ObRb in stressed rats in comparison to control group (*$p<0.05$, S vs. C), and marked increase in receptor expression in fructose fed stressed rats compared to stressed rats on standard diet (Fig. 4.4B, $^{\$}p<0.05$, SF vs. S), as a consequence of interaction of fructose and stress ($F(1,24)=13.78$, $p<0.01$) detected by Two Way ANOVA (Fig 4.4B).

Fructose ($F(1,24)=6.59$, $p<0.05$) and stress ($F(1,24)=14.16$, $p<0.001$) had significant effect on expression of SOCS3, one of the most important inhibitors of leptin signaling pathway, as shown by Two Way ANOVA. Decrease in mRNA level of SOCS3 was detected in stressed group of rats in comparison to control group (Fig 2b, *$p<0.05$, S vs. C), after *post-hoc* test (Fig4.4B).

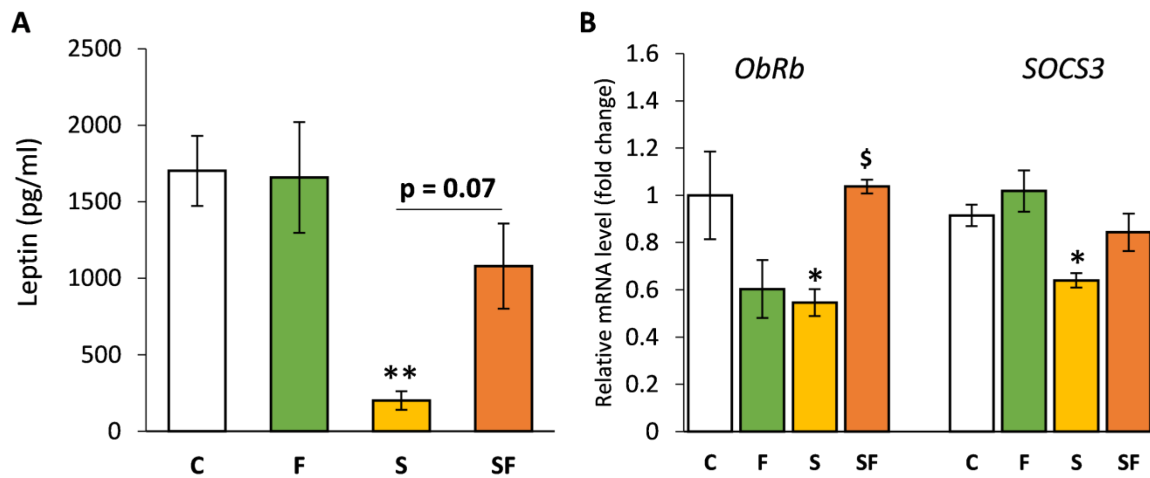

Figure 4.4.Leptin signaling pathway. **A)** Concentration of leptin in circulation (pg/ml), **B)** Relative mRNA levels of ObRb and SOCS3 in hypothalamus of control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8 animals per group). Leptin concentration was measured by ELISA method while RT-PCR was used to determine the levels of mRNA of selected genes. Data are presented as mean ± S.E.M and expression of genes was normalized to HPRT mRNA level. Two Way ANOVA was used to determine effects of fructose diet, stress exposure and their interaction on rats while post-hoc Tukey test was used to evaluate intergroup differences. Values of $p<0.05$ were considered statistically significant. Statistical significant changes were marked: *$p<0.05$ and **$p<0.01$, treated animals vs. C; $^{\$}p<0.05$, SF vs S. ObRb- Obesity receptor b; SOCS3- Suppressor of cytokine signaling 3, HPRT- hypoxanthine phosphoribosyl transferase 1.

4.3 Appetite-regulating neuropeptides in the hypothalamus

Two Way ANOVA showed significant effect of fructose ($F(1,27)=5.01$, $p<0.05$) and interaction of fructose and stress ($F(1,27)=4.75$, $p<0.05$) on expression of orexigenic neuropeptide AgRP in hypothalamus. *Post-hoc* test revealed significant increase in the expression of AgRP in animals exposed to both, fructose diet and unpredictable stress, compared to control group (*$p<0.05$, SF vs. C), fructose group ($^{\#}p<0.05$, SF vs. F), and stress group ($^{\$}p<0.05$, SF vs. S) (Fig 4.5).

On the other hand, stress had significant effect on the expression of anorexigenic neuropeptide POMC ($F(1,24)=30.47$, $p<0.001$), shown by Two Way ANOVA. Marked reduction in mRNA for POMC was detected in both groups of animals exposed to stress compared to control group (*$p<0.05$, S vs. C; *$p<0.05$, SF vs. C) as well as in stressed rats on fructose diet compared to the fructose-fed unstressed rats ($^{\#}p<0.05$, SF vs. F) (Fig 4.5).

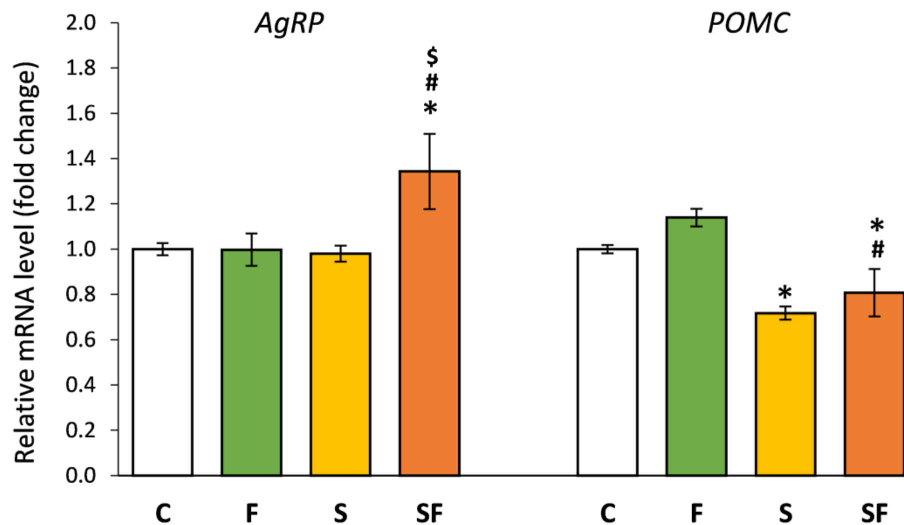

Figure 4.5. Expression of orexigenic and anorexigenic neuropeptides in the hypothalamus. Relative mRNA level of orexigenic neuropeptide AgRP and anorexigenic neuropeptide POMC were measured by RT-PCR (normalized to HPRT mRNA level) in hypothalamus from control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8 animals per group). In order to evaluate effects of the fructose diet, chronic stress and their interaction Two Way ANOVA was used fallowed by the post-hoc Tukey test for determination of intergroup differences. Data are presented as mean ± S.E.M. Values of $p<0.05$ were considered statistically significant. Statistical significant changes were marked: *$p<0.05$, treated animals vs. C; $^{\#}p<0.05$*, SF vs. F; $^{\$}p<0.05$, SF vs S. AgRP- agouti-related protein; POMC- proopiomelanocortin; HPRT- hypoxanthine phosphoribosyl transferase 1.

4.4 Insulin signaling in the hypothalamus

Neither fructose diet, stress exposure nor their combination had significant effect on protein levels of glucose transporters (GLUT1, GLUT2, GLUT3), pIRS1-Ser307, IRB, pIR-Tyr1162/1163, or its ratio (pIRTyr1162/1163/IRB) (Fig 4.6).

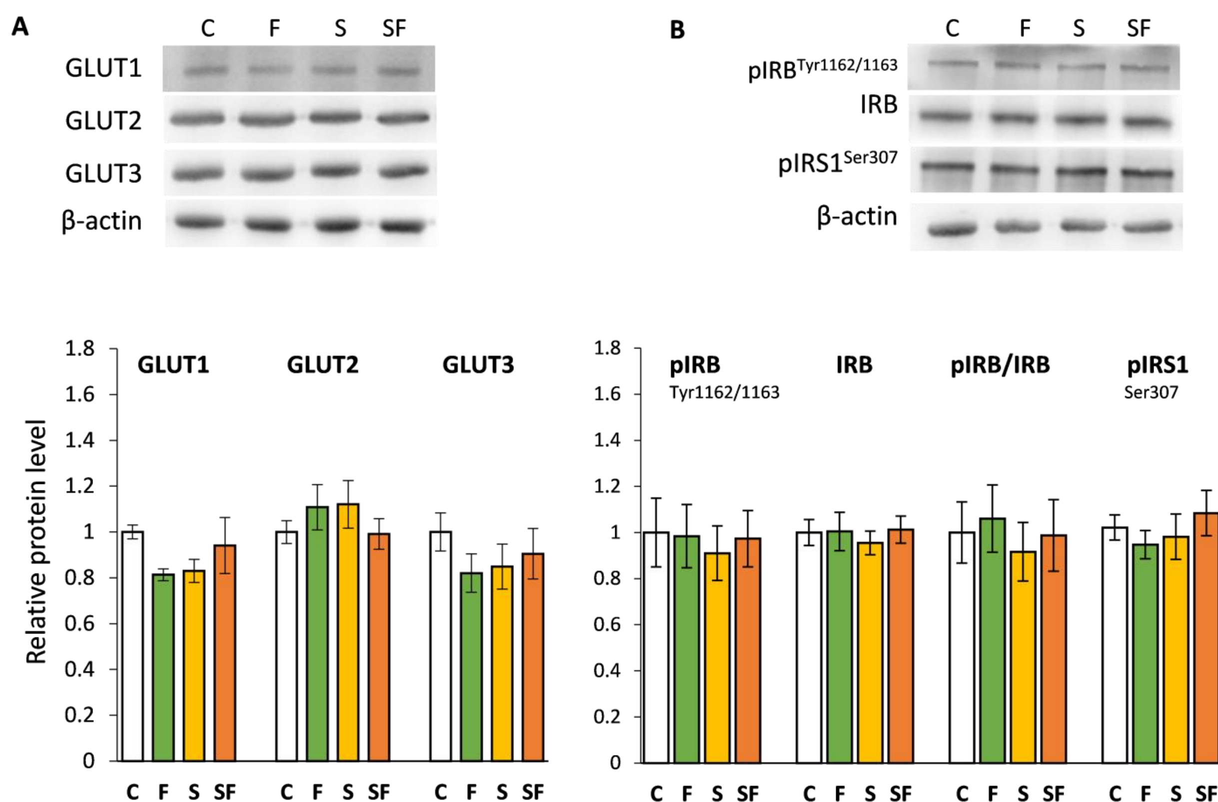

Figure 4.6. Protein level of glucose transporters and insulin receptor signaling in the hypothalamus. **A)** Relative protein level and representative Western blots of glucose transporters GLUT1, GLUT2 and GLUT3, **B)** Relative protein level and representative Western blots of IR, pIR-Tyr1162/1163 and pIRS1-Ser307 in the hypothalamus from control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8 animals per group). Relative protein levels were measured by Western blot and normalized to β-actin. In order to evaluate effects of the fructose diet, chronic stress and their interaction Two Way ANOVA was used fallowed by the post-hoc Tukey test for determination of intergroup differences. Data are presented as mean ± S.E.M. Values of $p<0.05$ were considered statistically significant. GLUT1/2/3- glucose transporter1/2/3; IRB- insulin receptor B; IRS1- insulin receptor substrate1.

While protein level of total Akt was unaltered by any of the treatments, fructose and interaction of fructose and stress had significant effect on pAkt-Ser473 ($F(1,32)=13.83$, $p<0.001$; $F(1,32)=11.99$, $p<0.01$, respectively) and on ratio of phosphorylated to total Akt (effect of fructose: $F(1,28)=18.61$, $p<0.001$; effect of interaction: $F(1,28)=15.04$, $p<0.001$) according to Two Way ANOVA. Post hoc test showed increase of pAkt-Ser473/Akt in stressed rat on standard diet compered to control group (*$p<0.05$, S vs. C) while decrease in protein level of both, pAkt-Ser473 and pAkt-Ser473/Akt was detected in stressed rats on fructose diet compared to control (*$p<0.05$, SF vs. C) and stressed group ($^{\$\$\$}p<0.001$, SF vs. S) (Fig 4.7B).

Two Way ANOVA showed that fructose ($F(1,31)=17.89$, $p<0.001$), stress ($F(1,31)=9.31$, $p<0.01$) and their interaction ($F(1,31)=4.05$, $p<0.05$) had significant effect on protein level of pERK-Thr202/Tyr204 in the hypothalamus as well as on the ratio of phosphorylated ERK to total ERK (fructose effect: $F(1,31)=28.09$, $p<0.001$; stress effect: $F(1,31)=19.64$, $p<0.001$; interaction of factors: $F(1,31)=4.53$, $p<0.05$). Increase in protein level of pERK-Thr202/Tyr204 and ratio of pERK-Thr202/Tyr204 to total ERK was marked after *post hoc* test in the hypothalamus of animals exposed to both, stress and fructose diet compared to

control (***p<0.001, SF vs. C) and stress ($$$p<0.001, SF vs. S) group as well as in comparison to fructose fed unstressed rats (for pERK-Thr202/Tyr204: ##p<0.01; SF vs. F; for ratio pERK-Thr202/Tyr204 and total ERK ###p<0.001; SF vs. F). Applied treatments did not had any effects on the total ERK protein level (Fig 4.7C).

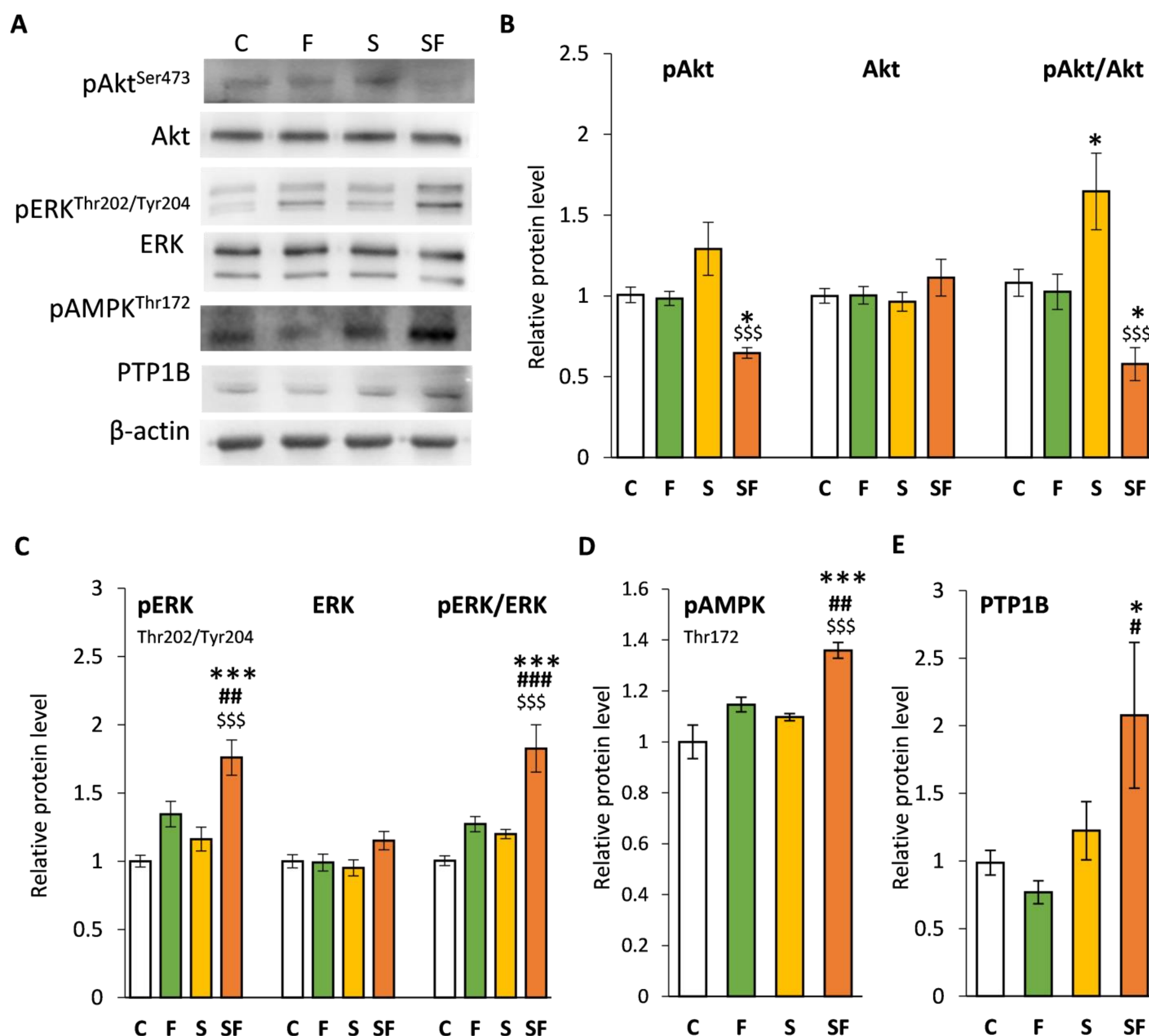

Figure 4.7. Protein level of Akt, ERK, PTP1B and AMPK in the hypothalamus. Representative Western blots **(A)** and relative protein levels of Akt and pAkt-Ser473 **(B)**, ERK and pERK-Thr202/Tyr204 **(C)**, pAMPK-Thr172 **(D)** and PTP1B **(E)** in the hypothalamus from control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8 animals per group). Relative protein levels were measured by Western blot and normalized to β-actin. In order to evaluate effects of the fructose diet, chronic stress and their interaction Two Way ANOVA was used fallowed by the post-hoc Tukey test for determination of intergroup differences. Data are presented as mean ± S.E.M. Values of p<0.05 were considered statistically significant. Statistical significant changes were marked: *p<0.05 and **p<0.01 treated animals vs. C; #p<0.05, ##p<0.01, ###p<0.001, SF vs. F; $p<0.05 and $$$p<0.001, SF vs S. Akt-protein kinase B; AMPK- AMP-activated protein kinase; PTP1B- protein tyrosine phosphatase 1B; ERK-extracellular signal–related kinase.

As shown in Figure 4.7D upregulated pAMPK protein level was detected after *post hoc* test in the hypothalamus of animals exposed to combination of fructose diet and chronic stress in comparison to untreated animals (***p<0.001, SF vs. C), animals exposed to stress on standard diet ($$$p<0.001, SF vs. S) and unstressed fructose fed animals (##p<0.01; SF vs. F).

Two Way ANOVA showed that both, chronic stress exposure ($F(1,20)=23.75$, $p<0.001$) and fructose diet ($F(1,20)=41.26$, $p<0.001$) had significant effect on the changes in protein level of pAMPK.

Stress ($F(1,31)=7.79$, $p<0.01$) and interaction of stress and fructose ($F(1,31)=3.74$, $p<0.05$), as shown by Two Way ANOVA, had main effect on protein level of PTP1B, main inhibitor of insulin signaling pathway. Marked increase of PTP1B protein level was detected after post hoc test in stressed rats on fructose diet in comparison to control (*p<0.05, SF vs. C) and fructose group (#p<0.05, SF vs. F) (Fig 4.7E).

4.5 Lipid Metabolism in the skeletal muscle

Lipid metabolism in skeletal muscle was assessed through analyzing the levels of expression of genes whose protein products are involved in lipid transport into the cell, lipolysis and fatty acid β-oxidation. Main effect of stress ($F(1,26)=4.640$, $p<0.05$) and interaction of stress and fructose diet ($F(1,26)=9.523$, $p<0.05$) on LPL, main protein for fatty acid transport through plasma membrane, was detected by Two Way ANOVA. Expression of one of the main lipolysis enzymes ATGL was under significant effect of fructose ($F(1,28)=7.581$, $p<0.01$) while expression of fatty acid transporter FATP1 was affected by fructose diet ($F(1,22)=10.793$, $p<0.01$), stress ($F(1,22)=10.967$, $p<0.01$) and combination of the treatments ($F(1,22)=11.209$, $p<0.01$). *Post hoc* test revealed that transcription of analyzed genes was upregulated in stressed animals on fructose diet in comparison to control group (for LPL and ATGL- *p<0.05, SF vs. C; for FATP1- ***p<0.001, SF vs. C) fructose group (for LPL- #p<0.05, SF vs. F; for FATP1- ###p<0.001, SF vs. F) and stress group (for LPL- \$\$p<0.01, SF vs. S; for FATP1- \$\$\$p<0.001, SF vs. S) (Fig 4.8).

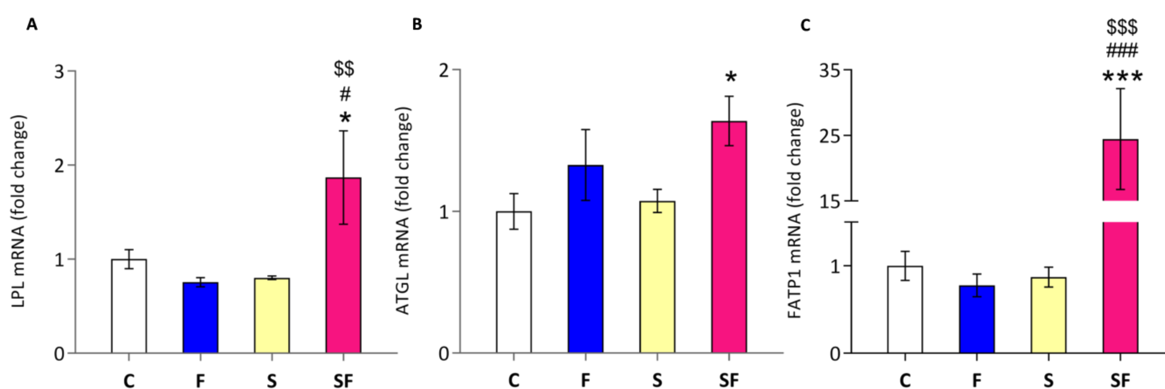

Figure 4.8. Skeletal muscle expression of LPL, ATGL and FATP1. Relative mRNA levels of LPL **(A)**, ATGL **(B)** and FATP1 **(C)** were measured by RT-PCR in skeletal muscle from control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8-9 animals per group). The values are normalized to HPRT1 mRNA level and presented as the mean ± S.E.M. In order to evaluate effects of the fructose diet, chronic stress and their interaction Two Way ANOVA was used fallowed by the post-hoc Tukey test for determination of intergroup differences. Values of p<0.05 were considered statistically significant. Statistical significant changes were marked: *p<0.05 and ***p<0.001 treated animals vs. C; #p<0.05, and ###p<0.001, SF vs. F; \$\$p<0.01 and \$\$\$p<0.001, SF vs S. LPL- lipoprotein lipase; ATGL- adipose triglyceride lipase; FATP1- fatty acid transport protein 1; HPRT—hypoxanthine phosphoribosyltransferase 1.

Expression of rate limiting enzyme for fatty acid transport into the mitochondria and subsequent β-oxidation, CPT1b was under significant effect of stress ($F(1,22)=4.518$, $p<0.05$) according to Two Way ANOVA while stress had main effect on the expression of PPARα ($F(1,17)=8.743$, $p<0.01$) and PPARδ ($F(1,12)=9.502$, $p<0.01$), lipid sensing transcriptional regulators. As shown in Figure 4.9, *post hoc* test revealed increased mRNA of CPT1b and PPARα and increased protein level of PPARδ in rats exposed to stress in combination with fructose diet compared to untreated animals (for all three- *$p<0.05$, SF vs. C) and to fructose fed unstressed animals (for PPARα - #$p<0.05$, SF vs. F).

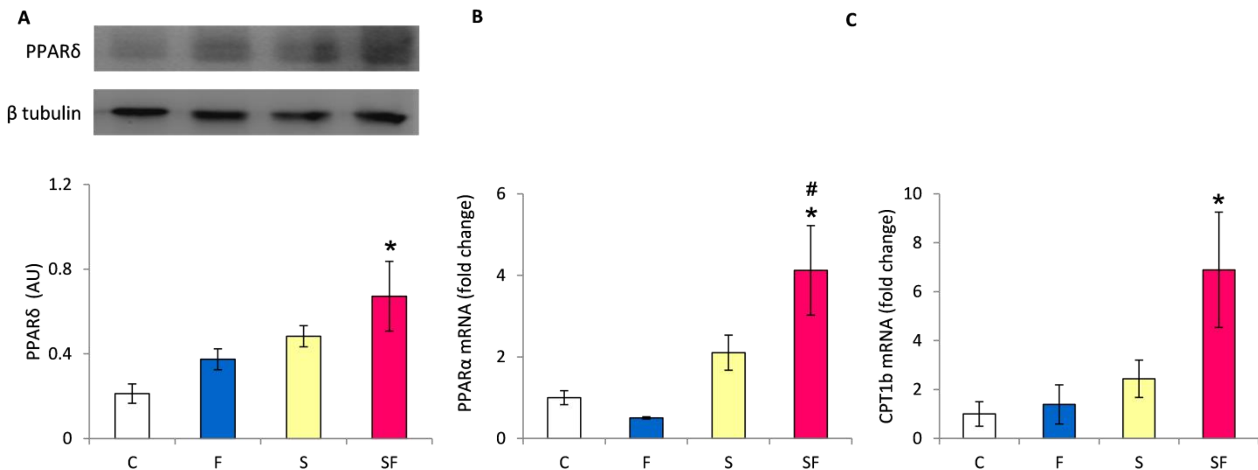

Figure 4.9. Levels of PPARα, PPARδ and CPT1b. **A)** Representative Western blot and protein level of PPARδ, **B)** Relative mRNA level of PPARα and **C)** Relative mRNA level of CPT1b in skeletal muscle from control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8-9 animals per group). Protein level was determined by Western blot (normalized to protein level of β tubulin) while mRNA level were measured by RT-PCR (normalized to mRNA level of HPRT). In order to evaluate effects of the fructose diet, chronic stress and their interaction Two Way ANOVA was used fallowed by the post-hoc Tukey test for determination of intergroup differences. Values of $p<0.05$ were considered statistically significant. Statistical significant changes were marked: *$p<0.05$ treated animals vs. C; #$p<0.05$, SF vs. F. PPARα/δ- peroxisome proliferator-activated receptor α/δ; CPT1b- carnitine palmitoyltransferase 1b. HPRT- hypoxanthine phosphoribosyltransferase 1.

4.6 Inflammation in the skeletal muscle

Skeletal muscle inflammatory status was analyzed by determining protein level of proinflammatory transcription regulator NFκB and protein level of its phosphorylated form on serine 536. Main inhibitor of NFκB is IκB, protein that interacts with NFκB in cytoplasm. Protein levels of IκB have been evaluated as well as expression levels IL-1β, IL-6 and TNFα, main proinflammatory cytokines under NFκB regulation. Two Way ANOVA showed that stress had main effect on the protein level NFκB ($F(1,12)=17.08$, $p<0.001$) and ratio of pNFκB-Ser536 to total NFκB ($F(1,12)=13.24$, $p<0.01$). After *post hoc* test significant increase in protein level of NFκB and pNFκB-Ser536 to NFκB ratio was detected in both stressed groups regardless of diet compared to untreated rats (*$p<0.05$, S vs. C and SF vs. C) (Fig 4.10A). Expression of TNFα was under effect of stress ($F(1,25)=32.29$, $p<0.0001$) while IL-1β expression was affected by fructose diet ($F(1,28)=4.776$, $p<0.05$) and combination of fructose and stress ($F(1,28)=10.366$, $p<0.01$), according to Two Way ANOVA. Skeletal muscle mRNA levels of IL-1β (Fig 4.10C) and TNFα (Fig 4.10D) were increased in stress group in comparison

to untreated animals (*p<0.05, S vs. C). Stressed rats on fructose diet had increased transcription of TNFα compared to control (*p<0.05, SF vs. C) and fructose group (###p<0.001, SF vs. F), while transcription of IL-1β was decreased compared to stress group ($$p<0.01, SF vs. S). Expression of IL-6 remained unaltered by either fructose, stress or their combination (Fig 4.10E).

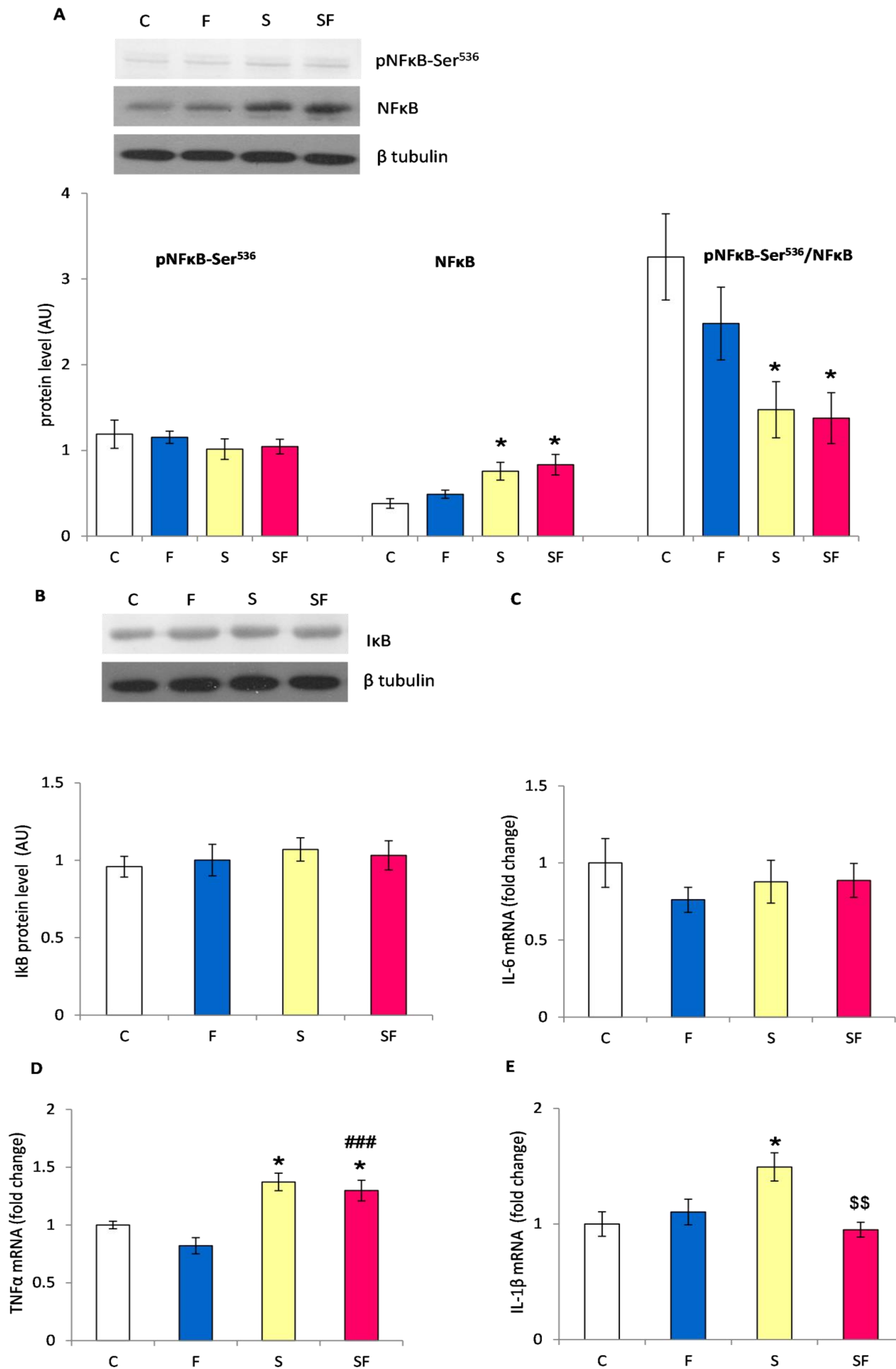

Figure 4.10. Inflammatory status of skeletal muscle. Protein levels and representative Western blots of NFκB and pNFκB-Ser536 **(A)** and IκB **(B)**, relative mRNA levels of IL-6 **(C)**, TNFα **(D)** and IL-1β **(E)** in skeletal muscle of control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8-9 animals per group). Protein level was determined by Western blot (normalized to protein level of β tubulin) while mRNA level were measured by RT-PCR (normalized to mRNA level of HPRT). In order to evaluate effects of the fructose diet, chronic stress and their interaction Two Way ANOVA was used fallowed by the post-hoc Tukey test for determination of intergroup differences. Values of p<0.05 were considered statistically significant. Statistical significant changes were marked: *p<0.05 treated animals vs. C; $^{###}$p<0.001, SF vs. F; $^{\$\$}$p<0.01, SF vs. S. NFκB —nuclear factor κB; IκB - inhibitor of NFκB; IL1β/6—interleukin 1β/6; TNFα—tumor necrosis factor α; HPRT—hypoxanthine phosphoribosyltransferase1.

4.7 Glucocorticoid signaling pathway in the skeletal muscle

Plasma corticosterone level was affected by fructose diet ($F(1,28)=16.536$, $p<0.001$), chronic stress ($F(1,28)=32.256$, $p<0.0001$) and their combination ($F(1,28)=15.748$, $p<0.001$) while significant effect of stress ($F(1,28)=9.689$, $p<0.01$) was detected on corticosterone level in skeletal muscle, according to the Two Way ANOVA. *Post hoc* test showed that corticosterone level in plasma was increased after stress exposure while significance was reached only in stressed rats on standard diet compared to control (***p<0.001, S vs. C) while addition of fructose diet to stressed rats decreased plasma corticosterone level in comparison to stressed animals on standard diet ($^{\$\$\$}$p<0.001, SF vs. S) (Fig 4.11A). On the other hand, *post hoc* test showed increased level of skeletal muscle corticosterone after stress exposure regardless of the diet (*p<0.05, S vs. C; **p<0.01, SF vs. C) (Fig 4.11B).

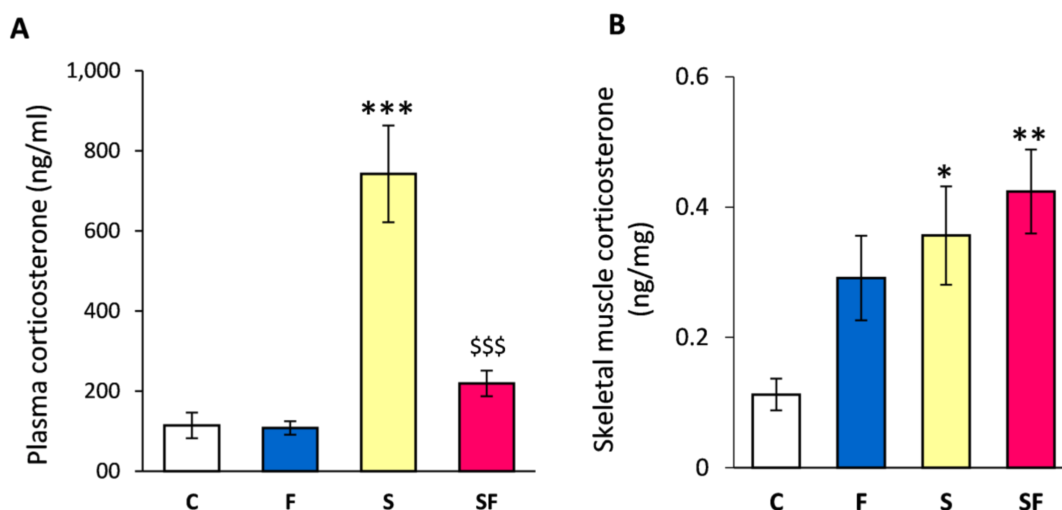

Figure 4.11. Concentration of corticosterone in plasma and skeletal muscle. Concentration of corticosterone in plasma **(A)** and skeletal muscle **(B)** of control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8-9 animals per group). For concentration measurement ELISA test was used. In order to evaluate effects of the fructose diet, chronic stress and their interaction Two Way ANOVA was used fallowed by the post-hoc Tukey test for determination of intergroup differences. Values of p<0.05 were considered statistically significant. Statistical significant changes were marked: *p<0.05, **p<0.01 and ***p<0.001, treated animals vs. C; $^{\$\$\$}$p<0.001, SF vs. S;

As prereceptor metabolism of glucocorticoids is one the main determinates of intracellular level of active glucocorticoids, we examined expression of two main enzymes involved in this process, 11βHSD1 and H6PDH. Although, protein levels of both enzymes were not significantly changed by either of applied treatments (Fig 4.12A,B,C), main effect of stress ($F(1,25)=34.933$, $p<0.0001$) was detected by Two Way ANOVA on skeletal muscle mRNA level of 11βHSD1. *Post hoc* test showed that transcription levels of gene for 11βHSD1 was decreased in stressed rats on standard (**$p<0.01$, S vs. C) and fructose diet (**$p<0.01$, SF vs. C; ##$p<0.01$, SF vs. F) (Fig 4.12D).

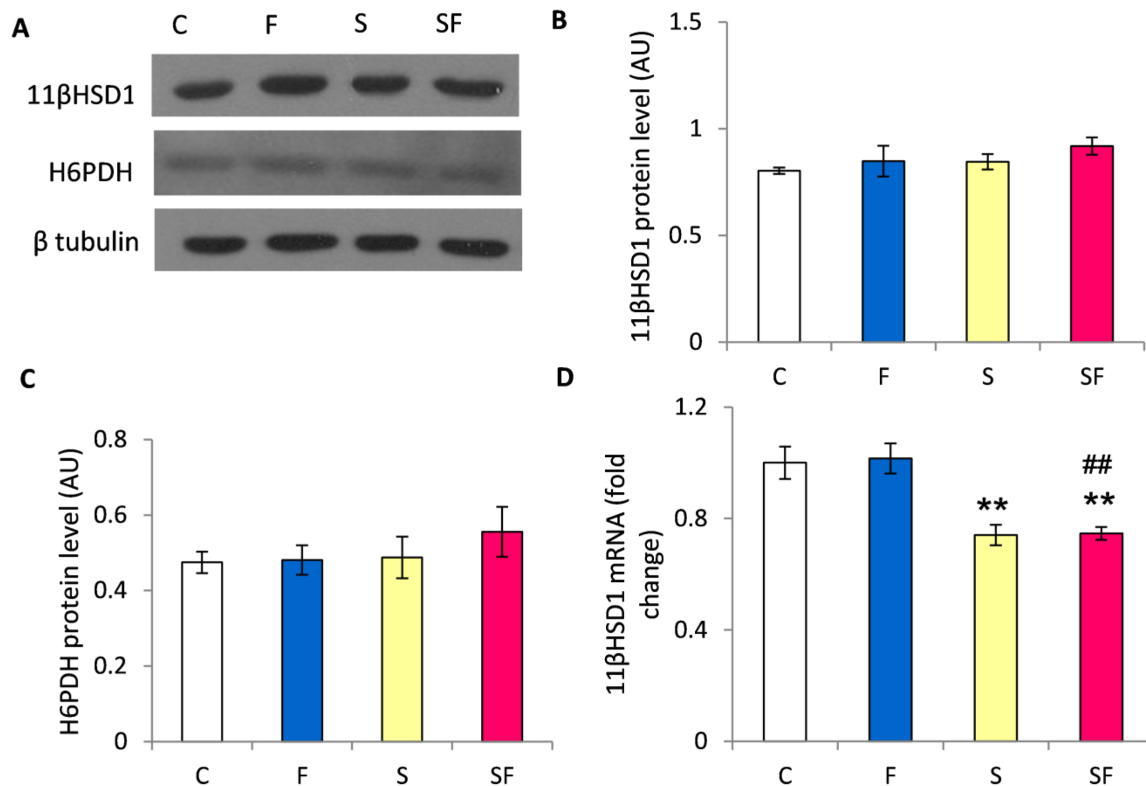

Figure 4.12. Prereceptor metabolism of glucocorticoids in skeletal muscle. Representative Western blots **(A)**, relative protein levels of 11βHSD1 **(B)** and H6PDH **(C)**, relative mRNA level of 11βHSD1 **(D)** in skeletal muscle of control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8-9 animals per group). Protein level was determined by Western blot (normalized to protein level of β tubulin) while mRNA level were measured by RT-PCR (normalized to mRNA level of HPRT). In order to evaluate effects of the fructose diet, chronic stress and their interaction Two Way ANOVA was used fallowed by the post-hoc Tukey test for determination of intergroup differences. Values of $p<0.05$ were considered statistically significant. Statistical significant changes were marked: **$p<0.01$ treated animals vs. C; ##$p<0.01$, SF vs. F. 11βHSD1- 11β hydroxysteroid dehydrogenase type 1; H6PDH- hexose 6 phosphate dehydrogenase; HPRT- hypoxanthine phosphoribosyltransferase 1.

Fructose diet had main effect on GR mRNA ($F(1,28)=16.220$, $p<0.001$) and protein ($F(1,12)=9.518$, $p<0.01$) levels causing significant decrease of the GR transcription (*$p<0.05$, F vs. C; **$p<0.01$, SF vs. C) and protein (*$p<0.05$, F vs. C; SF vs. C) expression in fructose fed unstressed as well as fructose fed stressed rats (Fig 4.13A and C).

Two Way ANOVA showed main effect of fructose diet ($F(1,32)=11.641$, $p<0.001$) and combination of fructose and stress ($F(1,32)=28.513$, $p<0.000001$) on the level of mRNA for

KLF15 while stress affected protein level of FKBP15 ($F(1,12)=9.403$, $p<0.01$). *Post hoc* test revealed that KLF15 transcription was upregulated in fructose, stress and stress+fructose group compared to control (***p<0.001, F vs. C; S vs. C; **p<0.01, SF vs. C) (Fig 4.13B). Protein level of FKBP51 was increased in stressed rats on fructose diet compared to untreated rats (*p<0.05, SF vs. C) and fructose fed unstressed rats (##p<0.01, SF vs. F) (Fig 4.13C).

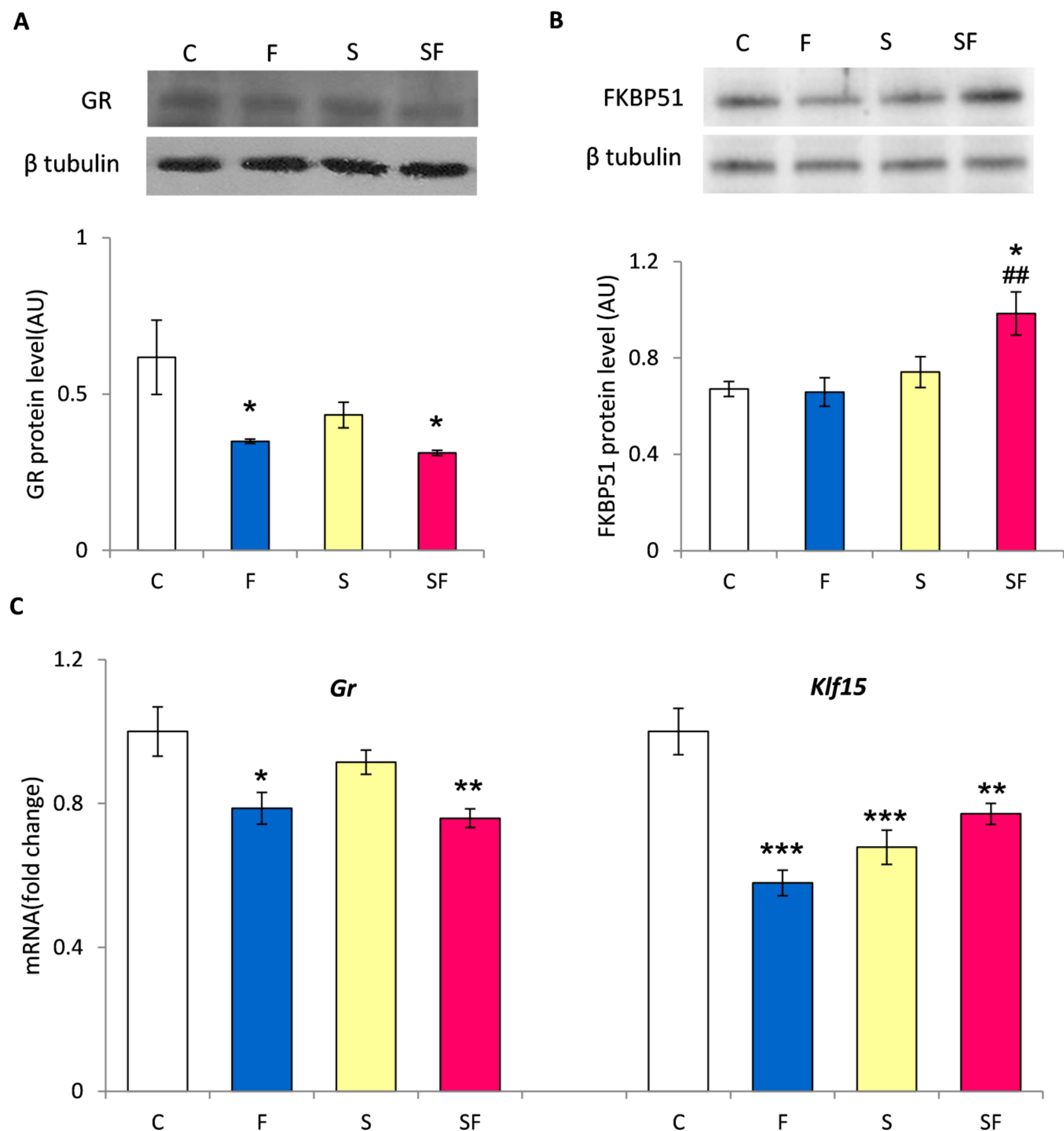

Figure 4.13. Skeletal muscle glucocorticoid signaling pathway. Representative Western blots and protein levels of GR (A) and FKBP51 (B), relative mRNA levels genes for GR and KLF15 (C) in skeletal muscle of control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8-9 animals per group). Protein level was determined by Western blot (normalized to protein level of β tubulin) while mRNA level were measured by RT-PCR (normalized to mRNA level of HPRT). In order to evaluate effects of the fructose diet, chronic stress and their interaction Two Way ANOVA was used fallowed by the post-hoc Tukey test for determination of intergroup differences. Values of p<0.05 were considered statistically significant. Statistical significant changes were marked: *p<0.05, **p<0.01 and ***p<0.001 treated animals vs. C; ##p<0.01, SF vs. F. GR- glucocorticoid receptor; FKBP51- FK506 binding protein 51; KLF15- Kruppel Like Factor 15; HPRT1- hypoxanthine phosphoribosyltransferase 1.

4.8 Insulin Signaling Pathway in the skeletal muscle

Stress had main effect on protein level of total IRS1 ($F(1,12)=10.738$, $p<0.01$), level of IRS1 activating phosphorylation on Tyr632 ($F(1,12)=6.536$, $p<0.05$), ratio of IRS1 inhibitory phosphorylation on Ser307 to total IRS1 ($F(1,12)=13.040$, $p<0.01$) and ratio of inhibitory to activatory IRS1 phosphorylations ($F(1,12)=6.951$, $p<0.05$). Two Way ANOVA also showed effect of interaction of stress and fructose diet on protein level of pIRS1-Tyr632 ($F(1,12)=5.449$, $p<0.05$). *Post hoc* test revealed decreased protein level of total IRS1 (Fig 4.14C) and the level of its activating phosphorylation (Fig 4.14D) in stressed rats on fructose diet compared to untreated animals ($^{**}p<0.01$, SF vs. C; and $^{*}p<0.05$, SF vs. C, respectively) and to fructose fed unstressed rats ($^{\#\#}p<0.01$, SF vs. F; $^{\#}p<0.05$, SF vs. F, respectively), as well as to stressed rats on standard diet (only for the pIRS1-Tyr632: $^{\$}p<0.05$, SF vs. S). In addition, stressed rats on fructose diet had increased ration of pIRS1-Ser307 to total IRS1 compared to control group ($^{*}p<0.05$, SF vs. C), and to fructose fed unstressed rats ($^{\#\#}p<0.01$, SF vs. F) (Fig 4.14G). Interestingly, decreasing trend of pIRS1-Tyr632 to pIRS1-Ser307 ratio was detected in fructose fed stressed rats ($p=0.08$) (Fig 4.14H).

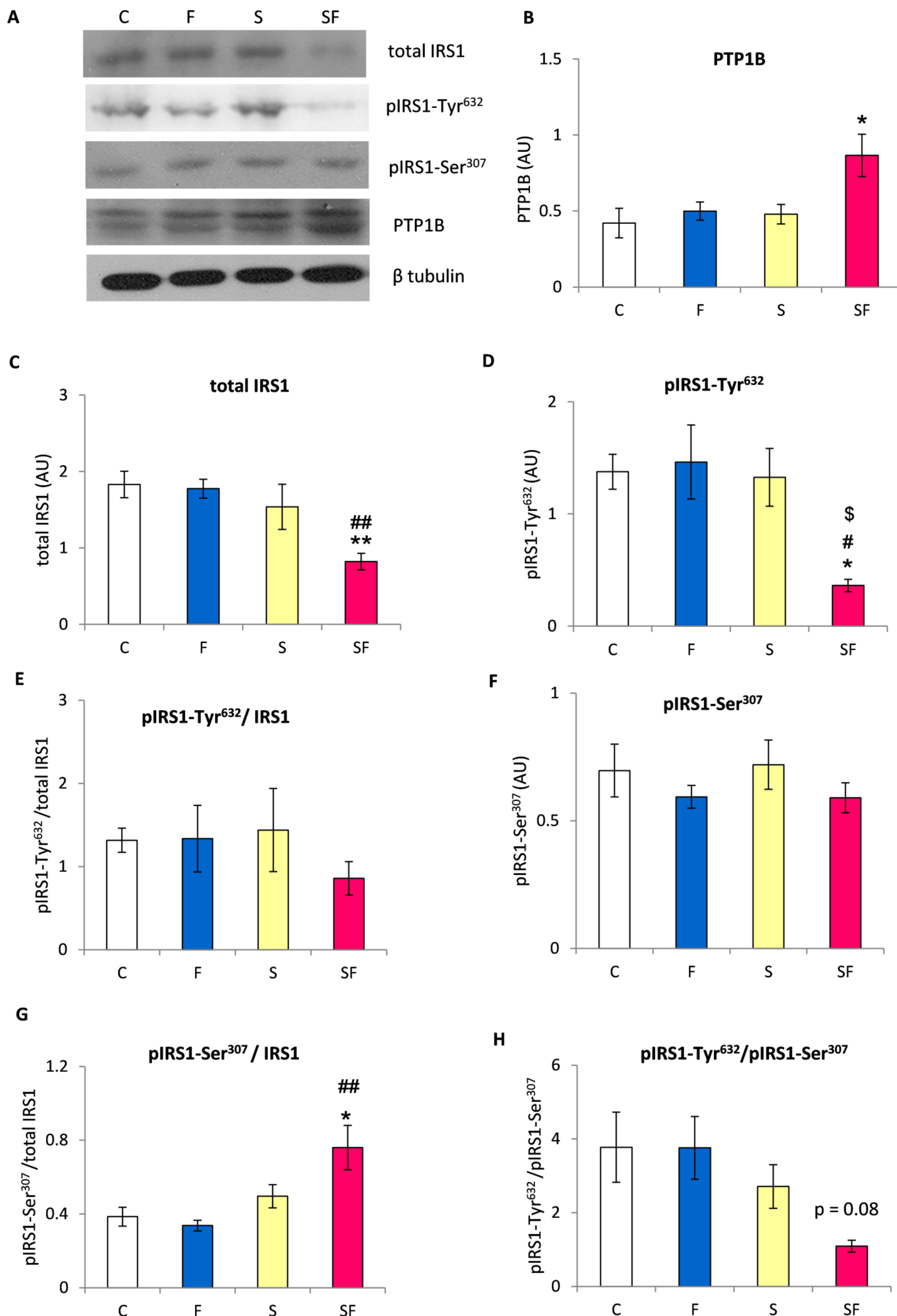

Figure 4.14. Skeletal muscle insulin receptor signaling and the level of PTP1B. Representative Western blots **(A)** and relative protein levels of PTP1B **(B)**, total IRS1 **(C)**, pIRS1-Tyr632 **(D)**, pIRS1-Ser307 **(E)** and ratio of pIRS1-Tyr632 to total IRS1 **(F)**, pIRS1-Ser307 to total IRS1 **(G)** and pIRS1-Tyr632 to pIRS1-Ser307 **(H)** in skeletal muscle of control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8-9 animals per group). Protein level was determined by Western blot (normalized to protein level of β tubulin). In order to evaluate effects of the fructose diet,

chronic stress and their interaction Two Way ANOVA was used fallowed by the post-hoc Tukey test for determination of intergroup differences. Values of $p<0.05$ were considered statistically significant. Statistical significant changes were marked: *$p<0.05$ and **$p<0.01$, treated animals vs. C; #$p<0.05$ and ##$p<0.01$, SF vs. F; $p<0.05, SF vs. S. IRS1- insulin receptor substrate 1; PTP1B- protein tyrosine phosphatase 1B.

Regarding the downstream component of insulin signaling, Akt and its activity, Two Way ANOVA showed significant effect of fructose on protein level of total Akt ($F(1,12)=5.065$, $p<0.05$) and its activating phosphorylation on Thr308 ($F(1,12)=10.036$, $p<0.01$) and effect of combination of fructose and stress on total Akt ($F(1,12)=6.360$, $p<0.05$). As shown in Figure 4.15, post hoc test revealed decrease in total Akt and pAkt-Thr308 protein levels in stressed rats on fructose diet (for Akt: *$p<0.05$, SF vs. C, #$p<0.05$, SF vs. F and $p<0.05, SF vs. S; for pAkt-Thr308: *$p<0.05$, SF vs. C, #$p<0.05$, SF vs. F) while its ratio remained unaltered.

Protein level of PTP1B, main inhibitor of insulin signaling pathway, was under effects of fructose diet ($F(1,12)=5.926$, $p<0.05$) and stress ($F(1,12)=4.944$, $p<0.05$) which led to significant increase in its level in stressed rats on fructose diet compared to control group (*$p<0.05$, SF vs. C) (Fig 4.14B).

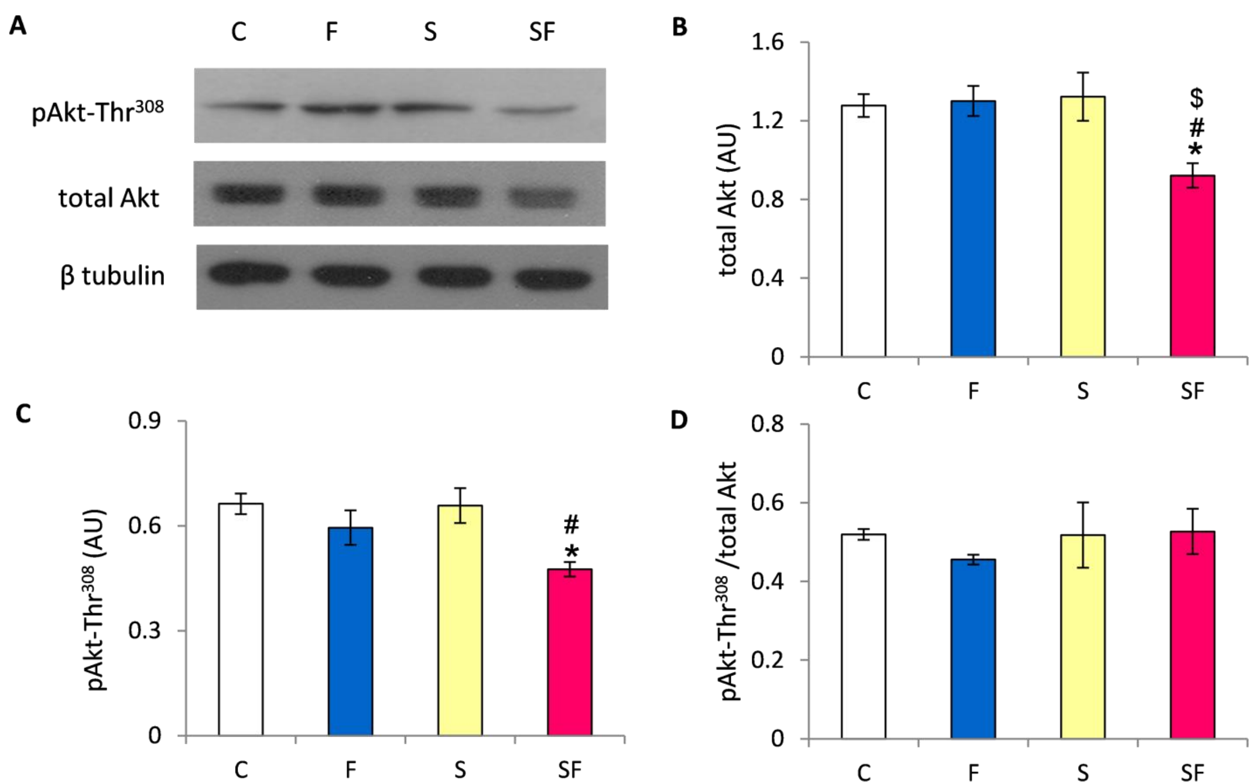

Figure 4.15. Level of Akt in skeletal muscle. Representative Western blots **(A)** and the protein level of total Akt **(B)**, pAkt-Thr308 **(C)** and ration of pAkt-Thr308 to total Akt **(D)** in skeletal muscle of control (C), fructose (F), stress (S) and stress+fructose (SF) male Wistar rats (n=8-9 animals per group). Protein level was determined by Western blot (normalized to protein level of β tubulin). In order to evaluate effects of the fructose diet, chronic stress and their interaction Two Way ANOVA was used fallowed by the post-hoc Tukey test for determination of intergroup differences. Values of $p<0.05$ were considered statistically significant. Statistical significant changes were marked: *$p<0.05$ treated animals vs. C; #$p<0.05$, SF vs.F; $p<0.05, SF vs S. Akt-protein kinase B.

5. Discussion

Maintenance of energy balance is essential for preserving homeostasis. There are molecular sensors capable of detecting energy changes and adequately answering by modulating metabolic processes in needed direction. Insulin, leptin and glucocorticoids are signaling molecules crucial for maintaining energy balance and disturbance in their signaling pathways (especially in hypothalamus and skeletal muscle) can contribute to development of diseases such as diabetes type 2, obesity and its related disorders, heart problems, stroke, and some types of cancer. Metabolic syndrome, which includes visceral obesity, hypertension, dyslipidemia and insulin resistance, has been described as the disease of modern society. Incidence of metabolic syndrome has greatly increased overtime and it correlates with increased consumption of processed and sweetened food and beverages. In the last few decades fructose is widely used as a main sweetener in majority of food that people daily consume. Although initially considered a healthy fruit sugar, it is now known that fructose is differently metabolized from glucose and that it is more lipogenic than glucose. Moreover, humans are not evolutionary equipped for ingesting large amounts of fructose taken independently from fibers, other sugars or proteins. In addition to sugar rich caloric diet, chronic stress has nowadays become an inevitable part of everyday life. Exposure to stress has been connected with not only psychological and cognitive disorders but with metabolic consequences as well. More than a half of physician visits are due to the stress-related health problems.

The research presented in this doctoral thesis contributes to a better understanding of the molecular mechanisms by which dietary fructose and exposure to chronic unpredictable stress, separately or in combination, have on insulin, leptin and glucocorticoid signaling in the hypothalamus and skeletal muscle of male Wistar rats. For that purpose, we investigated main signaling molecules in insulin, leptin and glucocorticoid pathways and evaluated appetite regulation, lipid metabolism and inflammatory status in these organs.

5.1 Regulation of energy intake and leptin signaling in hypothalamus after fructose diet, chronic stress and/or their combination

Hypothalamus is the key region of the CNS for the regulation of energy homeostasis in the body. This part of the brain is specialized to receive, interpret and integrate a wide range of signals that contain information about the nutritional and energy status of the organism, and based on this information to modulate food intake and energy consumption. This makes hypothalamus one of the main centers of energy balance regulation.

Previous research has shown that fructose enriched diet, being palatable and pleasantly sweet, can increase energy intake in animals and humans (Bray, 2013; Bray and Popkin, 2013). On the other hand, exposure to stress has made a less clear effect since some people exposed to stress increase food intake and weight gain while others experience the loss of appetite and weight loss (Dallman et al., 2003). Our results showed that rats that consumed fructose had increased energy intake, regardless of stress exposure. Since both fructose-fed experimental groups had decreased intake of solid food, increased energy intake probably originated from fructose overconsumption. Since increased fluid intake could indicate that animals had a problem with osmolality, it is important to mention that in our pilot experiment, we evaluated blood osmolality in all animals and there were no difference

between experimental groups (data not shown). Changes in energy intake could be due to changed levels of neuropeptides that regulate appetite in the neurons of hypothalamus. Analysis of orexigenic and anorexigenic neuropeptides in the hypothalamus after fructose diet, stress exposure and/or their combination reveled that fructose-fed stressed rats had increased expression of orexigenic neuropeptide AgRP and decreased expression of anorexigenic POMC. This is in line with increased energy intake in the same experimental group, but noteworthy, fructose alone did not change expression of neuropeptides although energy intake was increased in this group as well. Although many studies point out that increased intake of fructose enriched diet can lead to upregulation of orexigenic neuropeptides in the hypothalamus (Beck, 2006; Li et al., 2015), this is not always the case. Namely, intracerebroventricular injection of fructose showed no effects on the level of AgRP in the hypothalamus (Cha et al., 2008). Additionally, functional magnetic resonance imaging of hypothalamic activity after intravenous fructose administration did not reveal significant differences compared to the basal level of activity after saline administration (Purnell et al., 2011). Also, previous studies have indicated that 10% fructose solution did not cause changes in the mRNA level of orexigenic and anorexigenic neuropeptides in young male and female rats (Kovačević et al., 2019; Milutinović et al., 2014), but increasing the percentage of consumed fructose to 60% has led to an increased expression of orexigenic neuropeptide Y (Bursać et al., 2014). Some studies also indicate a decrease in the expression of orexigenic neuropeptides as a result of a compensatory mechanism due to increased energy intake after long term diet with sugar solution, high-fat diet or in genetically obese animals (Beck, 2006; Lindqvist et al., 2008). In the present study, 20% of fructose solution caused increase in orexigenic and decrease in anorexigenic neuropeptides only when combined with chronic stress. In addition, there were no compensatory changes detected in expression of neuropeptides as a response to increased energy intake in both fructose-fed groups which may indicate a problem with some regulatory mechanisms which will be further discussed.

Increased energy intake detected in the present study in rats after long term fructose enriched diet did not lead to increase in body weight nor to higher ratio of VAT weight to body weight in these animals. Animal studies on fructose diet have diverse results regarding the weight outcome of the treatment. While some studies report weight gain others report unchanged or even decreased weight after fructose consumption (Sangüesa et al., 2017; Shapiro et al., 2008; Yoo et al., 2017). Even our previous studies have shown that fructose can cause weight gain, weight loss and unchanged weight depending on the gender of the animals, percent of fructose solution used and age of the animals at the start of the treatment (Bursać et al., 2014, 2013; Kovačević et al., 2017, 2014). In addition, a chosen strain of rats has been shown to have influence on the fructose effects, with Spraque-Dawly rat strain being more sensitive to fructose feeding than Wistar rats used in our research (Bocarsly et al., 2010; Crescenzo et al., 2014; Nyakudya et al., 2018; Zubiría et al., 2013). We could speculate that in our study ingested energy surplus from fructose was probably spent on increased locomotor activity and energy expenditure or activated lipolysis in adipose tissue. Similarly, as shown previously by Bursać et al (Bursać et al., 2018) in the stressed animals on standard diet increased lipolysis in VAT was probable cause of detected decrease in VAT to body ratio although body weight remained unchanged.

Leptin is a very important regulator of appetite and energy expenditure. It is synthesized in adipose tissue and secreted from adipose cells into circulation, which is why leptin level is directly dependent on the size of adipose tissue. Leptin binds to its hypothalamic receptor and activates signaling pathway that leads to inhibition of orexigenic and stimulation of expression of anorexigenic neuropeptides. In this way leptin participates in limiting food intake during periods of energy abundance and stimulating appetite when there

is a drop in energy reserves. That is why we decided to investigate systemic level of leptin hormone, as well as leptin signaling pathway in the hypothalamus of rats after fructose diet, exposure to chronic, unpredictable stress and their combination. Our results showed that circulating level of leptin was markedly decreased in stressed animals on standard diet, while addition of fructose to stress normalized leptin level to almost control value (p=0.07). In accordance with this, hypothalamic mRNA level of leptin receptor ObRb and main negative regulator of leptin signaling pathway SOCS3, were decreased in stressed rats on standard diet. Having in mind that leptin level is tightly regulated by the amount of adipose tissue, measured leptin level in our study is in compliance with detected decreased VAT to body ratio after stress exposure and its increase towards control level when fructose was combined with stress. Interestingly, decreased leptin level did not cause an increase in the level of orexigenic neuropeptides in the hypothalamus of stressed animals on standard diet. Similarly to these results, Harris et al., (Harris et al., 2002) detected a decrease in leptin levels during and after exposure of rats to stress, but did not observe a stimulation of food intake even after the end of the stress action. Also, Jeong et al. (Jeong et al., 2013)observed a decrease in leptin levels in mice chronically exposed to immobilization stress. Even in studies that observed an increase in leptin and leptin receptor levels in the arcuate nucleus after exposure to stress, a change in orexigenic neuropeptides was not necessarily detected (Wang et al., 2012). Although leptin is known to be an important adipokine that regulates food intake and maintenance of energy balance, and although its level changes in stressful situations, our results together with the aforementioned studies suggest that during exposure to stress leptin may not be responsible for the regulation of food intake and changes in expression orexigenic neuropeptides that occur as a result of stress.

It has been previously reported that long term fructose diet can increase leptin level in animals and humans (Lê et al., 2006; Lindqvist et al., 2008) and cause leptin resistance (Shapiro et al., 2011, 2008). Interestingly, in our study this effect of fructose was detected only when applied in combination with stress, while fructose treatment alone did not cause any changes in leptin signaling pathway. Namely, addition of fructose diet to stressed rats, increased expression of ObRb in hypothalamus, leptin in circulation and VAT to body ratio in comparison to stressed rats on standard diet. Fructose has previously shown to have blunting effect on hormones raised after stress exposure, such as glucocorticoids (Corona-Pérez et al., 2017). Effect of fructose on hypothalamus of fructose-fed stressed rats observed in the present study leads to the normalization of leptin signaling pathway after stress exposure, showing neuroprotective character. Mechanism behind this phenomenon could be found in the ability of fructose and its metabolites to restore oxidative changes initiated by stress (Che et al., 2015; Spasojević et al., 2009) since fructose has the ability to influence oxidative stress and can have protective effect depending on the cellular environment.

Another important energy sensor and very potent regulator of energy intake is the AMPK. Studies showed that activated AMPK stimulates orexigenic and inhibits anorexigenic neuropeptides after fasting (Huynh et al., 2016; Kola, 2008). In the present study, level of activating phosphorylation of AMPK was increased in hypothalamus of fructose-fed stressed rats. Therefore, it could be suggested that activated AMPK could directly influence expression of neuropeptides promoting appetite in rats after combination of treatments. Although, increased energy intake should initiate downregulation of AMPK that was not the case in fructose-fed stressed rats. Sustained increase in AMPK activity could be a consequence of stress treatment and exposure to stress hormones, which have been shown previously to have the ability to directly influence AMPK in hypothalamus. Nevertheless, this stress-related influence on activity of AMPK could rather be through endocannabinoid system, since it has been shown that cannabinoid receptor 1 has essential role in the effects that stress has on

AMPK activity in the hypothalamus (Scerif et al., 2013). In addition, cannabinoids have been shown to have orexigenic effects and this cannabinoid function was associated with increase in AMPK activity (Kola et al., 2005; Thuijl et al., 2008). Since insulin is very important regulator of energy balance and can have significant effect on regulation of appetite and hypothalamic response to energy overload after fructose diet, we further investigated sensitivity of hypothalamus to insulin by analyzing main signaling molecules of hypothalamic insulin pathway.

5.2 Hypothalamic insulin signaling in rats exposed to fructose diet, chronic stress and /or their combination

Insulin regulates glucose metabolism and hence contributes to maintaining energy metabolism as a whole. Glucose is the main energy source in the CNS, therefore the sensitivity of the brain to glucose availability and regulation of glucose plasma level is crucial for normal brain function and whole body homeostasis (Carey et al., 2013). Hypothalamus, especially its ventromedial part and *nucleus arcuatus,* is rich in glucose-sensing neurons and represents the part of the brain where nutrient and hormonal signals are being integrated. Information about the energy status of the body that is processed in the hypothalamus determines how this part of the brain will respond and adjust central and peripheral metabolism. Therefore, one of the insulin roles in the CNS is to regulate glucose sensitivity of the brain but also to regulate appetite and whole body weight gain, accordingly. Studies that investigated role of insulin in CNS showed that injection of insulin into the cerebral ventricles (Woods et al., 1979) or insulin sprayed into the nostrils (Kimura et al., 2016) inhibits food intake, while hypothalamic deletion of insulin receptor increases appetite and causes insulin resistance (Belsham and Dalvi, 2021). Namely, insulin directly acts on neurons that synthesize and secrete orexigenic neuropeptides such as AgRP and anorexigenic neuropeptides such as POMC, inhibiting and activating their expression, respectively. Long term intake of food rich in sugar and fats can lead to chronic exposure of hypothalamus to increased insulin concentration which has been shown to contribute to development of resistance to insulin in the neurons of hypothalamus (Belsham and Dalvi, 2021). Research shows that hypothalamic insulin resistance can adversely affect not only central regulation of energy consumption but systemic insulin sensitivity, as well (Vogt and Brüning, 2013). In fact, knock out mice for IR solely in the brain are used as animals models of obesity and whole body insulin resistance (Bruning et al., 2000).

Both fructose consumption and stress exposure have been previously connected with hypothalamic insulin signaling disturbances (Pan et al., 2013; Zhang et al., 2014). Namely, it was reported that long term high fructose diet leads to insulin resistance in hypothalamus of rats which was connected to systemic resistance to insulin (Zhang et al., 2014). Eight weeks of stress exposure caused insulin resistance in the hypothalamus of male rats and even acute stress, such as short exposure to cold, was detrimental for the insulin signaling as well (Pan et al., 2013; Torsoni et al., 2003). It has been long believed that fructose metabolism is not regulated at all by the insulin, since fructolysis bypasses key steps and enzymes that are regulated by insulin and energy status of the cell. Nevertheless, Lindqvist et al., (Lindqvist et al., 2008) have reported that circulating level of insulin rises after fructose-enriched diet. The

results of this thesis have shown increased plasma insulin level after fructose consumption only when it was combined with stress exposure. Fructose-fed stressed rats also had markedly decreased activation of Akt and increased activation of ERK, judged by the decreased protein level of pAkt-Ser473 and its ratio to total Akt and increased protein level of pERK-Thr202/Tyr204 and its ratio to total ERK. In addition, main inhibitor of insulin signaling pathway, PTP1B, was upregulated in the hypothalamus of fructose-fed stressed rats. These results strongly suggest that hypothalamic insulin signaling pathway is disturbed in rats exposed to combination of fructose diet and stress exposure. In line with these findings, mice with dysfunctional brain insulin signaling, as a consequence of knocking out insulin receptor specifically in neurons, have increased insulin level in plasma and higher energy consumption (Bruning et al., 2000), which was also detected in fructose-fed stressed rats in our study. Although blunted insulin signaling often involves disturbed IR or IRS1 and its phosphorylation, our results show no changes in protein levels of IR, pIR, pIRS1 or any of the main transporters for glucose, GLUT1, GLUT2, GLUT3 in the hypothalamus by any of the treatments. Decrease in Akt and increase in ERK activity have been connected with disturbed insulin signaling and development of insulin resistance by several authors (Jiang et al., 2003)(Ozaki et al., 2016). Our previous results on hypothalamic insulin signaling in female rats showed decrease in total Akt, activating phosphorylation of Akt and their ratio, together with unchanged protein level of IRS1 or its phosphorylation after exposure to stress in combination with fructose diet (Kovačević et al., 2019). Similarly to ours, decrease in protein level of pAkt-Ser473 with unaltered pIRS1-Ser307 was reported after 4 months of high-sugar diet (Battú et al., 2012). Zeng et al., (Zhang et al., 2014) showed that high fructose diet can inhibit Akt activity even after 4 weeks of treatment.

Increase in the protein level of PTP1B in fructose-fed stressed rats could lead to dephosphorylation of Akt and could account for decreased protein level of pAkt-Ser473 detected in the same experimental group. The most common mechanism of insulin signaling inhibition by PTP1B involves deactivation of pathway components that are activated through phosphorylation. Akt is a well-known target for PTP1B and PTP1B-deficient mice have increased Akt activity (Sugiyama et al., 2017). In line with this, elevated hypothalamic PTP1B antagonize insulin signaling (Dodd et al., 2019), while hypothalamic specific inhibition of PTP1B leads to restoration of insulin sensitivity, improvement in glucose metabolism and decreased food intake and adiposity in rats (Picardi et al., 2008).

Besides PTP1B, which is considered as the main inhibitor of insulin signaling, the additional mechanism of Akt downregulation in the brain could involve AMPK. Namely, it has been shown that activation of AMPK can trigger Akt dephosphorization in neurons of hippocampus as well as in different cell cultures (Kim et al., 2009; King et al., 2006). In addition, fructose overconsumption was shown to increase AMPK level and activity in the hypothalamus (Zhang et al., 2014). In line with this, in our study increase in AMPK activity was detected in the same experimental group as decrease of activatory phosphorylation of Akt.

It has been reported that long term exposure of hypothalamus to increased plasma levels of insulin can cause insulin resistance (Belsham and Dalvi, 2021) and indeed the results of the present study have shown increased level of circulating insulin and diminished hypothalamic insulin signaling in rats after combination of fructose diet and stress exposure. In addition, increased insulin did not affect appetite neuropeptides as expected, but instead, increased level of orexigenic AgRP and decreased level of anorexigenic POMC were detected in parallel with increase insulin level in fructose-fed stressed rats showing once again that

hypothalamic insulin signaling is impaired in these animals subjected to the combined treatment.

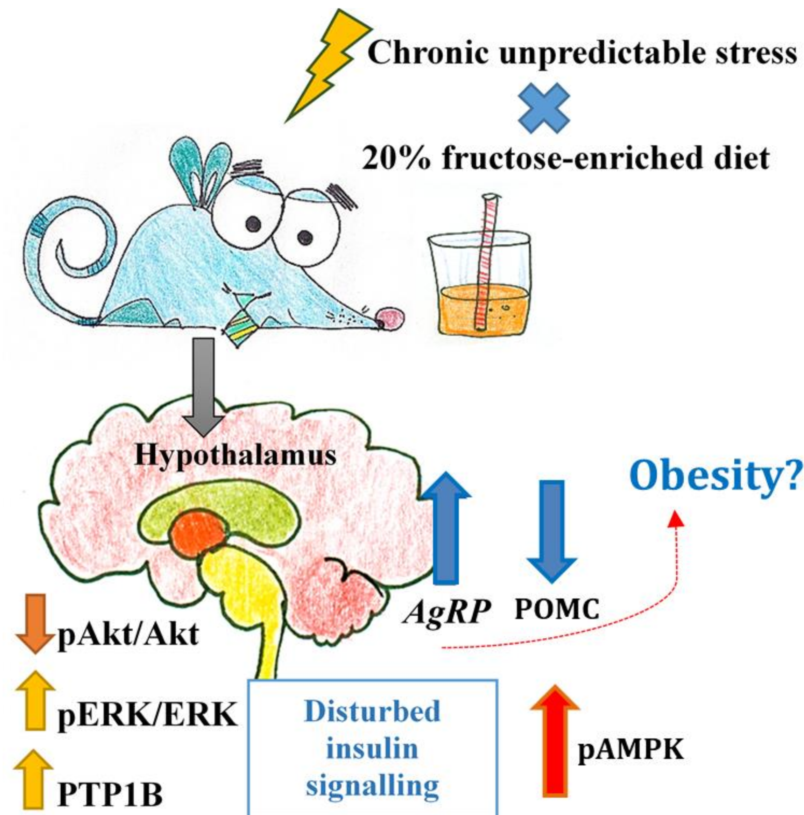

Figure 5.1. Summary of the main metabolic changes induced in rat hypothalamus after 9 weeks treatment of high fructose diet and/or chronic unpredictable stress. Combination of fructose diet and stress exposure lead to decrease in Akt and increase in ERK activity, increase in protein level of PTP1B and upregulation of orexigenic neuropeptide AgRP in hypothalamus of male rats. Together with increase in AMPK activity these results indicate that insulin signaling pathway was disturbed in hypothalamus of fructose-fed stressed rats which makes a setting for deregulation of appetite and increase energy consumption which can lead to obesity and metabolic syndrome.

5.3 Insulin signaling in skeletal muscle of rats exposed to fructose diet, chronic stress and /or their combination

After food ingestion and absorption, rise of glucose in circulation triggers secretion of insulin from the pancreas. Insulin subsequently stimulates facilitated diffusion of glucose from blood into the muscle and adipose cells. Skeletal muscles are the major end point of insulin-stimulated glucose storage. Namely, under physiological conditions, around 80% of the whole body glucose uptake occurs in the skeletal muscles (Bouzakri et al., 2005; DeFronzo and Tripathy, 2009; Lorenzo et al., 2008; Petersen and Shulman, 2002). Inside the muscle cells, part of the glucose is used to produce energy through glycolysis but the most of the

glucose is stored in the form of glycogen (DeFronzo and Tripathy, 2009). Since muscles hold and use majority of energy reserves for the body needs, defects in sensitivity of muscle to insulin can have detrimental effects for whole body insulin signaling and energy homeostasis. In addition, studies suggest that impairment of insulin signaling in the skeletal muscle could be the initial metabolic defect in the sequence of events leading to onset and development of systemic insulin resistance and ultimately type 2 diabetes (DeFronzo and Tripathy, 2009). There is evidence that disturbance in skeletal muscle insulin signaling could appear years before any detectable systemic change in glucose metabolism and sensitivity to insulin (DeFronzo and Tripathy, 2009). Children that have both parents with type 2 diabetes have measurable insulin resistance in skeletal muscle even though they are not obese and do not yet have any other parameter that would suggest upcoming problem with sensitivity to insulin and ability to use glucose (Ferrannini et al., 2003; Perseghin et al., 1997; Tripathy et al., 2003; Vaag et al., 1992). Regardless of the family anamneses, skeletal muscle insulin resistance have been detected in the patients with obesity, dyslipidemia, lipodystrophy, heart failure, polycystic ovary syndrome, kidney failure, glucocorticoid therapy and many others acting as a contributor and/or consequence of the pathological states (Abdul-Ghani and Defronzo, 2010). This shows importance of intact insulin sensitivity and its signaling pathway in skeletal muscles for the whole body energy homeostasis and wellbeing.

Research shows that insulin resistance in skeletal muscles is most frequently followed by the defects in proximal part of the insulin signaling pathway. Indeed, in the present research, decreased muscle protein levels of total IRS1 and Akt, as well as the levels of their activating phosphorylations, pIRS1-Tyr632 and pAkt-Thr308 were detected in rats exposed to combination of fructose diet and stress exposure. Analyzing protein level of main inhibitory IRS1 serine phosphorylation on the 307 position revealed that fructose diet, chronic stress exposure and their combination did not cause any significant changes. Nevertheless, ratio of phosphorylated (pIRS1-Ser307) to total IRS1 was increased in skeletal muscle of fructose-fed stressed rats. Moreover, a marked trend of decrease in the ratio of activating to inhibitory IRS1 phosphorylation (pIRS1-Tyr632/pIRS1-Ser307, $p = 0.08$) was observed in the animals exposed to the combination of treatments. Both findings are considered unfavorable for muscle insulin sensitivity. Studies agree that IRS1 and Akt are critical nodes in insulin signaling pathway (Taniguchi et al., 2006b), and their depletion inhibits insulin response (Bouzakri et al., 2003; Jiang et al., 2003). Preserving IRS1-Akt part of insulin signaling pathway was identified as crucial for maintaining insulin regulated glucose uptake in skeletal muscle (Krook et al., 2000). Therefore, results of our study suggest that insulin signaling pathway in skeletal muscle of stressed rats on fructose diet was reduced.

Studies on skeletal muscle insulin signaling after combined treatment with fructose-enrich diet and chronic stress are still scarce. In our study, impairment of insulin signaling was observed only when both factors were combined. Previous studies investigating individual effects of fructose or stress indicated the role of these factors in development of skeletal muscle insulin impairment. Namely, chronic psychosocial stress has been shown to downregulate insulin signaling pathway in mice skeletal muscle (Sanghez et al., 2016), while noise stress decreased level of pAkt-Thr308 in skeletal muscle and induced tissue and systemic insulin resistance (Liu et al., 2018). Furthermore, decreased level of insulin-stimulated IRS1 and Akt phosphorylations has been shown in skeletal muscle and liver of various rodents after fructose diet (Benetti et al., 2013; Bezerra et al., 2000; Taghibiglou et al., 2002). Previous studies in the same animal model have shown that fructose-enriched diet alone can cause insulin sensitivity impairment in tissues such as liver (Vasiljević et al., 2013; Veličković et al., 2019) and visceral adipose tissue (Kovačević et al., 2021), while in the hypothalamus (Kovačević et al., 2019) and skeletal muscle, presented herein, only

combination of fructose diet and stress exposure was capable to induce such effects. Similarly, recent study on heart, has shown that combination of fructose-enrich diet and chronic stress attenuates IRS1 upregulation and that the first step in heart insulin signaling pathway is affected only when fructose diet is combined with chronic stress (Romic et al., 2020).

The finding of decreased pIRS1-Tyr632 and pAkt-Thr308 in the present study goes in line with highly upregulated PTP1B observed in muscles of fructose-fed stressed rats, since activating phosphorylation of IRS1 and Akt are often targets of this phosphatase. Namely, it has been previously shown that PTP1B inactivates the main members of insulin signaling pathway, such as IRS1 and Akt, causing insulin signaling impairment (Goldstein et al., 2000). Since PTP1B protein level reflects its enzymatic activity (Picardi et al., 2010), studies that measured alterations in PTP1B protein level showed that it could be a major predictor of insulin resistance development in various animal models (Gum et al., 2003; Zinker et al., 2002). Furthermore, muscle cells with overexpressed PTP1B have decreased protein level of pAkt (Stull et al., 2012) that leads to muscle insulin resistance and more importantly to the impairment of systemic insulin sensitivity, as well (Zabolotny et al., 2004). On the other hand, inhibition of muscle PTP1B increases protein level of insulin stimulated pAkt and hyperphosphorylation of IRS1 therefore increasing insulin sensitivity in both, animal and human studies (Nieto-Vazquez et al., 2007; Stull et al., 2012). Therefore, decreased level of pIRS1-Tyr632 and pAkt-Thr308, observed in fructose-fed stressed rats in our study, could be related to the increased PTP1B protein level detected in the same animals. Mechanisms of how fructose enriched diet and exposure to chronic unpredictable stress lead to upregulation of PTP1B in skeletal muscles are still unclear. Rise of metabolic inflammation or disturbances in muscle lipid metabolism could be major contributors, which will be discussed in the following chapters.

5.4 Glucocorticoid signaling and skeletal muscle inflammatory status of rats exposed to fructose diet, chronic stress and /or their combination

Glucocorticoids are adrenal hormones with the main role in activation and regulation of the stress response. Among other things, this includes regulation of the immune response and metabolism during stress , and recovery. Glucocorticoids were even named after their ability to influence and regulate glucose level in the circulation and glucose metabolism in the cells. Their effect on glucose plasma levels is opposite to that of insulin and therefore many studies indicate that glucocorticoids inhibit insulin signaling. Furthermore, prolonged exposure to glucocorticoids can even result in insulin resistance in various tissues including skeletal muscle (revived in (Pivonello et al., 2010)). Our results show that chronic stress exposure increased corticosterone level in circulation of standard-fed rats. On the other hand, stressed rats on fructose-enriched diet had decreased corticosterone level in comparison to the stressed animals group, as hormone value almost reached its control level. While rise in corticosterone level after stress exposure is well established and expected, decreasing effect of fructose consumption on stress-increased corticosterone level is quite intriguing. Studies have shown that palatable food, especially the one that with high content of sugars and fat, can activate parts of the brain responsible for reward behavior and thus ameliorate stress response (Adam and Epel, 2007). Therefore, activation of brain reward system, increased activity of HPA axis or enhanced glucocorticoid negative feedback could be the mechanisms of how fructose consumption influences systemic corticosterone level (Fries et al., 2005;

Hellhammer and Wade, 1993; MacEdo et al., 2012; Sarapultsev et al., 2020) Analysis of corticosterone level in the skeletal muscle revealed increased level of this hormone in both stressed groups regardless of the diet. Higher level of corticosterone in the skeletal muscle of stressed rats on standard diet reflects enhanced hormone level in the circulation. Main regulators of glucocorticoid concentration inside the tissues are two enzymes of prereceptor metabolism of glucocorticoids, 11βHSD1 and H6PDH. Namely, glucocorticoids as steroid hormones freely diffuse through plasma membrane. Once inside the cell, inactive forms of glucocorticoids must be activated in order to successfully bind and activate GR. Enzyme 11βHSD1 is a dehydrogenase that converts inactive 11-dehydrocorticosterone into active corticosterone and H6PDH is the enzyme that provides a cofactor for that reaction. Although protein levels of both enzymes were unchanged by the treatments in the present study, the transcription level of gene for 11βHSD1 was markedly decreased in both stressed groups. Since enzymes of glucocorticoid prereceptor metabolism serve not only to enable the hormones role inside the tissue but also to protect tissue from harmful effects of hormone overexposure, it seems that decrease of 11βHSD1 expression gene could represent a compensatory, protective mechanism from increased systemic corticosterone after stress exposure. Although, fructose-fed stressed rats had no increased level of corticosterone at the end of 9 weeks treatment we could speculate that there were peeks of hormone after each stress exposure that could activate mechanism for protection. Discrepancy between transcription and protein level of 11βHSD1 could be explained by long protein half-life previously reported by several authors in multiple models (Zielinska et al., 2017).

Analyses of GR expression in the skeletal muscle after fructose consumption, exposure to stress and/or their combination, revealed that both mRNA and protein level of muscle GR were decreased after fructose diet, regardless of stress. Furthermore, expression of Klf15, gene under direct glucocorticoid regulation, was decreased in all experimental groups while protein level of FKBP51, a well-known GR inhibitor, was markedly increased in fructose-fed stressed rats. All this suggests that fructose has negative impact on the level and activity of GR and that at least in fructose-fed stressed rats GR transcriptional activity is blunted. Fructose diet has been previously connected with changes in components of glucocorticoid signaling in various organs and tissues (Bursać et al., 2018; Vasiljević et al., 2013). Interestingly, those changes were shown to be both positive and negative, depending on tissue examined or overall molecular context, with fructose showing even dominant effect over stress. This is not surprising having in mind that glucocorticoids have many different roles in the different organs and direction of their action and their regulation depends on the interaction with many other signaling pathways. Thus, these results, as well as the results from other authors, suggest that fructose has a potent influence of glucocorticoid signaling but course of its effects will be markedly determined by the context, and to our knowledge this is the first research on the effects of fructose diet on glucocorticoid signaling in the skeletal muscle.

Glucocorticoids are also essential regulators of inflammation and their anti-inflammatory role is crucial for maintaining systemic and tissue homeostasis. Therefore, decreased glucocorticoid signaling in the skeletal muscle of stressed rats on fructose diet could affect muscle ability to adequately handle inflammatory signals allowing muscle inflammation to persist long enough to cause metabolic disturbances. Indeed, inflammation has been previously connected with the development of muscle insulin resistance (Wu and Ballantyne, 2017b). Skeletal muscle cells have the ability to secrete various cytokines such as IL-6 and IL-8 and studies showed that muscles can become inflamed in the state of obesity (Wu and Ballantyne, 2017b). Myocytes isolated from insulin resistant or diabetic obese patients secrete more cytokines such as TNFα and monocyte chemoattractant protein 1 in comparison to the cells from control lean subjects (Ciaraldi et al., 2016; Green et al., 2011;

Saghizadeh et al., 1996). Furthermore, it has been shown that cells of the immune system could migrate to the skeletal muscles during obesity and even become dominant inflammatory cells in this tissue (Fink et al., 2014; Khan et al., 2015; Lackey and Olefsky, 2016; Olefsky and Glass, 2009; Wei et al., 2008). Even before obesity is evident, overfeeding or even short term intake of high calorie food increases markers of macrophages in the skeletal muscle of healthy patients (Krssak et al., 1999b; Reaven, 1988). Increased skeletal muscle level of macrophages and T cells have been shown in patients with insulin resistance and type 2 diabetes (Ciaraldi et al., 2016; Fink et al., 2014, 2013; Khan et al., 2015; Torres et al., 2004; Varma et al., 2009). Indeed, the results of this doctoral thesis show the presence of inflammation in the skeletal muscle of fructose-fed stressed rats, the same experimental group in which insulin and glucocorticoid signaling were shown to be inhibited. Namely, increased protein level of proinflammatory transcription regulator NFκB, decreased ratio of inhibitory NFκB phosphorylation (pNFκB-Ser536) to total NFκB, and increased transcription of proinflammatory cytokine TNFα in the skeletal muscle of fructose-fed stressed rats was observed. It has been suggested that NFκB activation is a crucial step in the onset of diet- and obesity-induced insulin resistance (Yuan et al., 2001). Furthermore, these two markers form a regulatory cycle as NFκB is positive transcriptional regulator of TNFα (and other proinflammatory cytokines) and TNFα is one of the main activators of the NFκB signaling pathway (reviewed in (Wu and Ballantyne, 2017b)). Increased level of TNFα in the skeletal muscle has previously been reported in the rats on fructose-enriched diet (Togashi et al., 2000) and increased secretion of this cytokine from skeletal muscle can have autocrine effects and cause skeletal muscle insulin resistance (Wu and Ballantyne, 2017a). It has been reported that TNFα has a major role in the development of insulin resistance in obesity and metabolic syndrome, but also in healthy patients in whom TNFα treatment can cause skeletal muscle insulin resistance (Nieto-Vazquez et al., 2007). *In vivo* and *in vitro* studies have shown that treatment with TNFα decreased protein level of activating pIRS1 in skeletal muscle (De Alvaro et al., 2004; Plomgaard et al., 2005). Furthermore, TNFα decreased pAkt-Thr^{308} level in human skeletal muscle cells (Austin et al., 2008). Therefore, it could be proposed that rise of inflammation in skeletal muscle of fructose-fed stressed rats could have an important role in development of insulin signaling impairment observed in these animals. However, it is unclear why this insulin signaling impairment is absent in stressed rats on standard diet despite increased level of inflammatory markers. It is known that muscle activity can cause a rise in inflammatory markers, which have important role in muscle regeneration by facilitating its physiological function after prolonged activation (Yang and Hu, 2018). Chronic stress exposure have been shown to influence skeletal muscle activity, making them often taut and tense, and changing sensitivity of reflexes, therefore putting muscles in prolonged state of guardedness (Alfvén et al., 2017; Schmitz et al., 2011). Although it needs further examination, these results could reflect such situations since rise of inflammatory markers after exposure to stress had no consequences on insulin signaling when stress is applied without fructose-enriched diet. Studies examining fructose-enriched diet showed increased TNFα level and insulin signaling impairment (Togashi et al., 2000), as well as increased level of NFκB and decreased pAkt in the rat skeletal muscle (Benetti et al., 2013). Both groups of authors applied more intense fructose treatment than the one used herein, with either higher percent of fructose (60%) or longer consumption period (30 weeks). This could be the reason why in our study these effects were present only when high fructose diet was combined with chronic stress.

Finally, it has been suggested that inflammation, especially proinflammatory cytokine TNFα, could affect the expression of PTP1B. Namely, TNFα has been shown to stimulate NFκB recruitment to the PTP1B promoter in 3T3-L1 adipocytes *in vitro* and *in vivo* in the mouse liver (Zabolotny et al., 2008). A similar mechanism could be present in skeletal muscle as well,

since TNFα treatment has been shown to increase transcriptional level of PTP1B in this tissue (Zabolotny et al., 2008) and to increase PTP1B activity in cultured myocytes and muscles from adult male mice (Nieto-Vazquez et al., 2007). In addition, Nieto-Vazquez et al., have showed that detrimental TNFα effects on insulin signaling were not detected in the absence of PTP1B, so that TNFα did not cause insulin resistance in PTP1B deficient mice (Nieto-Vazquez et al., 2007). Therefore, PTP1B seems to be involved in TNFα-induced insulin resistance. Taking all these facts into account, it is tempting to speculate that downregulation of glucocorticoid signaling contributes to the increased levels of TNFα and NFκB in the skeletal muscle of fructose-fed stressed rats in our study, initiating a vicious cycle that contributes to the inhibition of insulin pathway directly, or by upregulating insulin signaling inhibitor PTP1B.

5.5 Skeletal muscle lipid metabolism after exposure to fructose diet, chronic stress and/or their combination

Skeletal muscles have metabolic flexibility, in a sense that they can use either glucose or fatty acids for energy source depending on their availability. Although muscles are specialized for storing glucose in the form of glycogen and preserving it for later energy demands, their ability to store lipids is scarce and not without metabolic consequences. Namely, it has been shown that lipid accumulation in the skeletal muscle can decrease muscle sensitivity to insulin and cause insulin resistance. High fructose diet has been shown to cause lipid accumulation in human skeletal muscle after only one week of fructose diet (Lê et al., 2009). Furthermore, presence of excess fatty acids in skeletal muscles correlates with increased markers of inflammation that can contribute to insulin resistance and many other metabolic dysfunctions. Namely, it has been previously shown that fatty acid treatment increases expression of TNFα and NFκB activity and decreases level of activating Akt phosphorylation in skeletal muscle cells (Jové et al., 2006). Therefore the next step in our research was to investigate lipid metabolism in skeletal muscle of rats on fructose enriched diet, exposed to chronic unpredictable stress or their combination.

Studies agree that muscle fatty acid uptake depends on free fatty acid level and the level of lipid transporters (Zhang et al., 2010) Our results have shown increased level of circulating free fatty acids and upregulation of main lipid transporters, LPL and FATP1, in fructose-fed stressed rats, indicating fatty acid overload in the skeletal muscle after combination of treatments. Several studies have connected increased muscle fatty acid intake with the development of insulin resistance and type 2 diabetes. Alterations in supply of fatty acids to the muscle have been recognized as one of the major contributors to these conditions (reviewed in (Zhang et al., 2010)). Upregulation of LPL has been connected with insulin resistance and the onset of type 2 diabetes (Pulinilkunnil and Rodrigues, 2006) and skeletal muscle overexpression of this enzyme can decrease insulin signaling and glucose uptake (Kim et al., 2001a). Increased level of fatty acid transporters in the plasma membrane of skeletal muscle and increased fatty acid uptake was reported in diabetic patients, obese Zucker rats, and high fat-fed insulin resistant (Bonen et al., 2004; Hegarty et al., 2002; Turcotte et al., 2001). Furthermore, FATP1 inactivation restored insulin signaling in skeletal muscle of high fat-fed animals, while FATP1-null mice were protected from systemic insulin desensitization and metabolic syndrome (Kim et al., 2004; Wu et al., 2006).

Increased lipid uptake often changes skeletal muscle lipid metabolism. Vast majority of researches suggest that disturbed lipid metabolism in the direction of skeletal muscle lipid accumulation is the main factor for the onset and rise of insulin resistance (Morino et al., 2006; Ruderman et al., 1999). It has been shown that lipid metabolites, mainly fatty acyl CoAs, diacylglycerols, and ceramides, could activate kinases that interrupt insulin signaling pathway in the skeletal muscle (Hegarty et al., 2003). Nevertheless, studies also showed that athletes that have high triglyceride content because of the nature of the sports they practice, do not develop insulin resistance even though their level of triglycerides was as high as in patients with type 2 diabetes and even exceeded their levels (Gilbert, 2021). In addition, studies showed that not all mechanisms that lead to lipid accumulation lead to insulin signaling impairment. Activation of X receptor leads to the accumulation of lipids without altering insulin signaling pathway in muscle cells from diabetic patients (Cozzone et al., 2006). These observations indicated that lipid accumulation is not a cause of insulin resistance *per se* and that some other disturbances in lipid metabolism in the skeletal muscle could be important contributors to this process.

The results of this doctoral thesis, on the other hand, are in line with the studies showing that disturbances of lipid metabolism in the direction of increased lipolysis and β-oxidation can still contribute to insulin signaling dysfunction. Namely, in addition to upregulated muscle fatty acid intake observed herein, increase in muscle lipolysis was detected after combined treatment, as judged by increased expression of rate limiting lipase ATGL. Muscle ATGL has been recognized not only as an activator of lipolysis, but also as the important factor for prevention of muscle lipid accumulation (Saponaro et al., 2015). Interestingly, products of muscle lipolysis are used as local substrate for β-oxidation and are not excreted into the circulation, as is the case with lipolysis products of adipose tissue (Meex et al., 2015). Therefore we investigated the level of main mitochondrial fatty acid transporter and rate limiting enzyme of β-oxidation, CPT1b, and our results showed significantly increased expression of CPT1b in the skeletal muscle of fructose-fed stressed rats. Having this in mind, it seems that mitochondria in skeletal muscle of fructose-fed stressed rats are under high lipid burden. In line with our results, Koves et al. have previously indicated that metabolic overload of mitochondria in muscle is crucial for the diet-induced glucose intolerance (Koves et al., 2008, 2005). Additionally, authors indicated that fatty acid transport into muscle mitochondria is essential for insulin desensitization, and various methods that inhibit fatty acid mitochondrial intake protected cultured muscle cells and mice from developing insulin resistance (Koves et al., 2008). Genetic manipulation in mice that inhibited only fatty acid transport into mitochondria and not lipid accumulation in muscle cells also showed that insulin sensitivity was not altered regardless of high lipid content (Koves et al., 2008). Increased levels and activity of β-oxidation enzymes and their regulators in skeletal muscles were reported in insulin resistant obese Zucker rats and db/db mice (Turner et al., 2007). The same result was obtained after long term high-fat diet in mice and rats showing significantly higher β-oxidation rate in skeletal muscle and developed insulin resistance (Hancock et al., 2008; Turner et al., 2007). Similarly, studies on heart have shown higher β-oxidation rate in several models of insulin resistance (Buchanan et al., 2005; Carley and Severson, 2005; Mazumder et al., 2004).

The possible explanation as to how increased β-oxidation can contribute to insulin signaling dysfunction is that lipid overload in the skeletal muscle leads to failure of mitochondria to metabolize it properly, so the markers of incomplete β-oxidation start to accumulate (Koves et al., 2008). Increase in these lipid intermediates was reported in skeletal muscle of insulin resistant and type 2 diabetic mice and humans (Adams et al., 2009; Koves et al., 2008). It is still not fully elucidated how accumulation of incomplete β-oxidation products

causes insulin resistance. Adams et al., showed that intermediates can vastly upregulate NFκB activity therefore stimulating inflammation that can cause insulin resistance (Adams et al., 2009). This could be the case in our research since increased β-oxidation and inflammation were both detected in skeletal muscle after fructose overconsumption was applied together with stress. Combination of these factors also increased expression of PPARα and PPARδ, lipid sensing transcription regulators with essential role in activation of fatty acid uptake and β-oxidation in several organs including skeletal muscle (Muoio et al., 2002; Zhang et al., 2010). Having in mind increased expression of main mediators of fatty acid transport, lipolysis and β-oxidation in skeletal muscle together with marked upregulation of both PPARα and PPARδ in our study, it seems that chronic stress and fructose diet exert their effects on muscle lipid metabolism by activating these transcription regulators. Importantly, both PPARα and PPARδ have ability to regulate and shift skeletal muscle fuel usage from glucose to fatty acids (Burri et al., 2010; De Lange et al., 2008; Finck et al., 2005). PPARδ is a main isoform and crucial transcription regulator for prompt metabolic adaptations of skeletal muscle to increased fatty acid availability in muscle cells that favor fatty acid uptake and oxidation, especially under conditions of energy stress (De Lange et al., 2008). Furthermore, Creeser et al have shown that PPARδ agonist, besides increasing protein level of skeletal muscle fatty acid transporters, decreased insulin stimulated protein level of pAkt (Cresser et al., 2010). Similarly, overexpression of PPARα upregulated genes involved in fatty acid influx and β-oxidation together with downregulation of IRS1 and glucose transporters in mice skeletal muscle (Finck et al., 2005). The same result was obtained in mice with heart-restricted PPARα overexpression in which β-oxidation was upregulated and glucose uptake and metabolism downregulated, causing condition similar to diabetic heart (Finck et al., 2002). Activation of muscle PPARα was reported in insulin resistant obese mice, while animals with inhibited PPARα were protected from development of insulin resistance regardless of the diet or hormonal treatment used to initiate insulin desensitization (Finck et al., 2005; Guerre-Millo et al., 2001). Additionally, it has previously been reported that PPARα has ability to decrease fatty acid esterification to triacylglycerol (Muoio et al., 2002), which together with marked roles of both PPAR isoforms in activating β-oxidation can inhibit triglyceride accumulation in skeletal muscle. This emphasizes once again that impairment of insulin signaling in our study was most likely caused by (over)stimulated fatty acid oxidation rather than lipid accumulation. Since fatty acids are one of the main activators of PPARα and PPARδ, it seems that rise in systemic FFA after combination of fructose-enrich diet and stress exposure could be one of the triggers that leads to disturbances in muscle lipid metabolism observed herein.

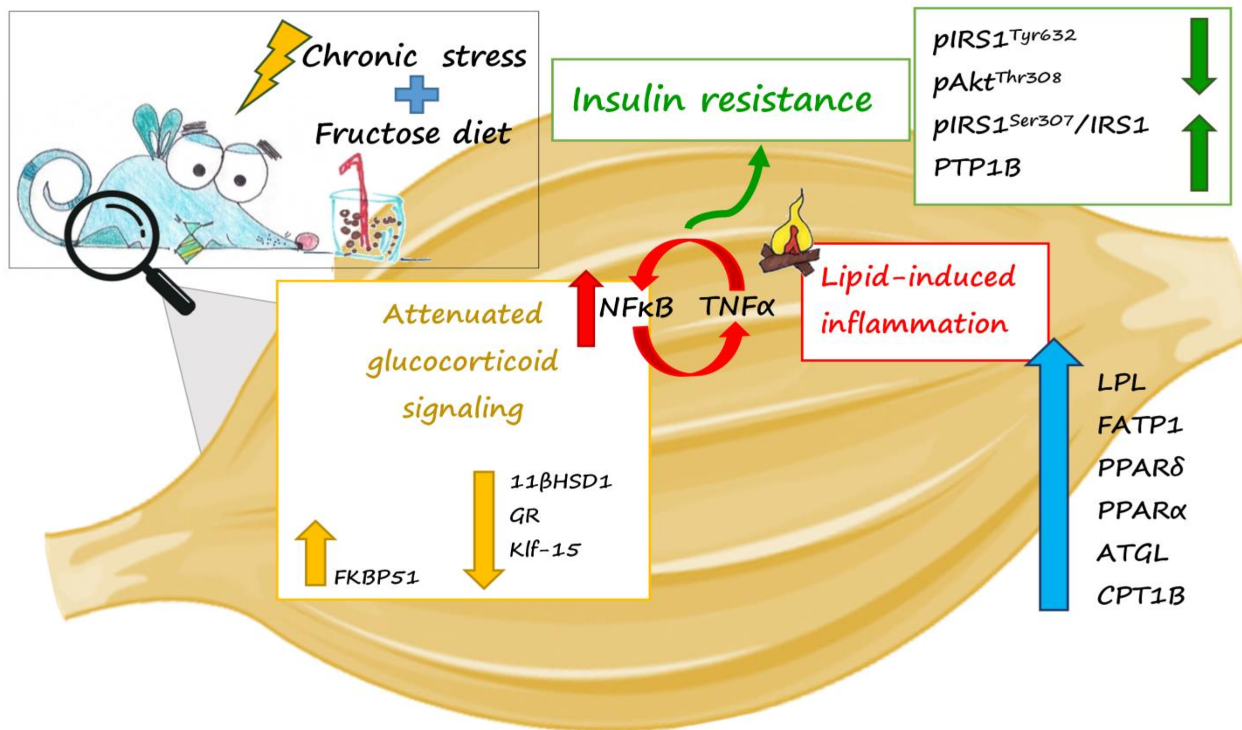

Figure 5.2. Summary on effects of fructose diet, chronic stress and their combination on the skeletal muscle glucose and lipid metabolism and inflammatory status. Combination of fructose and stress attenuates glucocorticoid signaling making a setting for rise of inflammation caused by disturbances in lipid metabolism. Both, inflammation and disturbed lipid metabolism could contribute to impairment of insulin signaling pathway detected in skeletal muscle after combination of treatments. IRS1 – insulin receptor substrate, Akt– protein kinase B, PTP1B– protein tyrosine phosphatase 1B, LPL– lipoprotein lipase, FATP1– fatty acid transport protein 1, PPARδ– peroxisome proliferator activated receptor δ, PPARα– peroxisome proliferator activated receptor α, ATGL– adipose triglyceride lipase, CPT1b– carnitine palmitoyltransferase 1b, FKBP51– FK506 binding protein, GR – glucocorticoid receptor, 11βHSD1– 11β hydroxysteroid dehydrogenase type 1 KLF15– Kruppel Like Factor 15, NFκB – nuclear factor κB, TNFα – tumor necrosis factor α (Shirif et al IJMS)

6. Conclusions

Results of this doctoral dissertation have clearly showed that combination of fructose diet and chronic exposure to stress had more detrimental effects on the certain aspects of hypothalamus and skeletal muscle physiology of male Wistar rats than each of the treatments applied separately. Fructose diet combined with chronic stress altered expression of hypothalamic neuropeptides causing increased appetite and energy intake, increased activity of energy sensor and impairment of insulin signaling. In the skeletal muscle, combination of fructose and stress caused muscle lipid overload and disturbed lipid metabolism by increasing lipolysis and β-oxidation. Decreased skeletal muscle glucocorticoid signaling enabled rise of uncontrolled lipid-induced inflammation therefore making a setting for muscle insulin signaling impairment detected after combination of treatments. This is concluded on the basis of following results:

- ✓ Fructose diet in combination with stress exposure increased energy intake, upregulated hypothalamic expression of orexigenic neuropeptide AgRP and downregulated anorexigenic neuropeptide POMC
- ✓ Combination of fructose diet and chronic stress lead to impairment of hypothalamic insulin signaling, judging by the decreased Akt and increased ERK and AMPK levels of activating protein forms, as well as by the upregulation of PTP1B, the main inhibitor of insulin signaling
- ✓ Fructose diet and chronic stress together increased level of lipid transporters initiating lipid influx into the muscle, upregulated main lipolytic enzyme and rate limiting transporter of fatty acids into mitochondria for subsequent β oxidation
- ✓ Combination of treatments increased level of proinflammatory transcription regulator NFκB and proinflammatory cytokine TNFα causing inflammation in the skeletal muscle
- ✓ Combination of treatments diminished glucocorticoid signaling in the skeletal muscle, judged by decrease in expression of GR and its regulated gene Klf15 and increase in protein level of its inhibitor FKBP51
- ✓ In the skeletal muscle, combination of fructose diet and chronic stress reduced IRS1 and Akt and the protein levels of their activating phospho-forms, increased ratio of negative pIRS1-Ser307 to total IRS1 and increased PTP1B inhibitor, therefore suppressing muscle insulin signaling

7. Literature

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8. Supplements

Author's biography

Abdulbaset Zidane Shirif was born on September 15th, 1967 in Gharian, Libya. In 1989 he graduated from Higher Institute of Animal Production, University of Sirte, Libya. In 2009, Abudlbaset finished master (MSc.) in Agriculture science on Faculty of Agriculture, University of Tripoli, Libya. Abdulbaset enrolled PhD studies at the Faculty of Biology, University of Belgrade, Serbia in the academic year 2015/2016.

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Abdulbaset is author and coauthor of three scientific papers published in the international scientific journals and coauthor in one abstract presented in international congress.

Изјава о ауторству

Име и презиме аутора **Abdulbaset Zidane Shirif**

Број индекса **M3001/2015**

Изјављујем

да је докторска дисертација под насловом

„Интеракције сигналних путева инсулина, лептина и глукокортикоида у хипоталамусу и скелетном мишићу пацова током метаболичких поремећаја изазваних исхраном обогаћеном фруктозом и стресом“

• резултат сопственог истраживачког рада;

• да дисертација у целини ни у деловима није била предложена за стицање друге диплome према студијским програмима других високошколских установа;

• да су резултати коректно наведени и

• да нисам кршио/ла ауторска права и користио/ла интелектуалну својину других лица.

Потпис аутора

У Београду, ________________ ________________________

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора **Abdulbaset Zidane Shirif**

Број индекса **М3001/2015**

Студијски програм **Молекуларна биологија**

Наслов рада „**Интеракције сигналних путева инсулина, лептина и глукокортикоида у хипоталамусу и скелетном мишићу пацова током метаболичких поремећаја изазваних исхраном обогаћеном фруктозом и стресом**“

Ментор **др Сања Ковачевић и др Предраг Вујовић**

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањивања у Дигиталном репозиторијуму Универзитета у Београду.

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

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Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

„**Интеракције сигналних путева инсулина, лептина и глукокортикоида у хипоталамусу и скелетном мишићу пацова током метаболичких поремећаја изазваних исхраном обогаћеном фруктозом и стресом**“

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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