Role of Brown and White Adipose Tissue in Cold-Induced Thermogenesis

Ph.D. Dissertation

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LIST OF ABBREVIATIONS

ACC	acetyl-CoA carboxylase

AKT	Akt1, also known as "Akt" or protein kinase B (PKB)
BAT	brown adipose tissue
cAMP	adenosine 3’,5’-cyclic monophosphate
CitLy	ATP-citrate lyase
ECL	Chemiluminescent reagent
GLUT4	glucose transporter-4
GSK3	glycogen-synthase kinase 3ß, it is a serine/threonine protein kinase
HSL	hormone sensitive lipase
MALDI	matrix-assisted laser desorption / ionization
MS	mass spectrometry
NE	norepinephrine
P-ACC	phosphorylated-ACC
P-AKT	phosphorylated -AKT
PFK-1	phosphofuctokinase-1
P-GSK3	phosphorylated -GSK3ß
PHD	pyruvate deydrogenase complex
PKA	protein kinase A
TOF	time of flying, mass spectrograph detector
UCP	uncoupling protein
WAT	white adipose tissue

INTRODUCTION

There are different kinds of heat production like shivering and non-shivering thermogenesis (their collecting name are *thermoregulatory thermogenesis*) and the nutrition related: this thermogenesis is called *metaboloregulatory thermogenesis*. Metaboloregulatory thermogenesis connects heat balance with body weight regulation. We focus on thermoregulatory thermogenesis, especially non-shivering thermogenesis in this dissertation.

The first mechanism of heat production activated in the situation of an acute cold exposure is shivering, in which heat is released by skeletal muscle. This process is regulated by the hypothalamus. During extended periods in the cold, mammals switch from shivering to non-shivering thermogenesis. This mammalian-specific mechanism (non-shivering thermogenesis) of heat production does not require muscle contraction under conditions of severe cold stress.

Initially, brown adipose tissue was generally mistaken for a part of the thymus. Only in the early 1960-ies, was evidence provided that brown adipose tissue is a site of thermogenesis.

There are two types of adipose tissues, white and brown adipose tissue (WAT, BAT), and there are some distinctive differences between them. The functions and histological appearance of these tissues are quite different. White adipose tissue store fat as an energy reserve, and release the fat to the blood stream as fatty acids to be used by other organs. WAT has a typical univacoular cells. Upon release of insulin from the pancreas, insulin receptors of white adipose cells cause a dephosphorylation cascade that lead to the inactivation of hormone-sensitive lipase (HSL). Upon release of glucagon from the pancreas, glucagon receptors cause a phosphorylation cascade that activates HSL, causing the breakdown of the stored fat to fatty acids, which are exported into the blood and bound to albumin, and glycerol is exported into the blood freely. Fatty acids are taken up by muscle and cardiac tissue as a fuel source, and the liver takes up glycerol for gluconeogenesis or

producing energy. WAT has a considerable role in the heat conservation also helping to maintain body temperature.

BAT serves another purpose and it separates from environment by its brown-reddish colour. Its colour is a result of large number of mitochondria, thus large amount of red cytochromes. Their major function of BAT comes from the existence of many mitochondria, which produce heat by fatty acid oxidation, and thereby contribute to maintain the body temperature and regulation of energy expenditure. It is possible only in the partially uncoupled mitochondria, because the coupled mitochondria are ADP dependent. Stimulation of brown adipocytes with e.g. norepinephrine (NE) leads to a rapid hydrolysis of stored lipid droplets. The resulting fatty acids are oxidised at high rate in the abundant mitochondria. A series of experiments performed in the 1970-ies indicated that brown fat mitochondria possess “atypical” ion permeability properties, and it was suggested that a specific protein in the mitochondrial inner membrane is responsible for the abnormal ion conductance of these mitochondria. Such a protein was identified and called thermogenin, presently known as uncoupling protein-1 (UCP1).

UCP proteins are responsible for the re-entry of proton gradient bypassing the F_OF_1 ATP-ase dissipates energy of H^+ gradient as heat. UCP proteins also regulate respiratory chain also. UCP1 is located in the inner membrane of the mitochondria of brown adiposities, and is likely to function as a homodimer. UCPs are regulated by purin nucleotides.

It was a milestone when sequence homologues of UCP1 were discovered. It is very important that UCP2 and UCP3 occur in the human tissues. Nevertheless, we should keep in mind that UCP2 and UCP3 are expressed relatively very poor amount compared to UCP1 and can be regarded as mild uncoupling proteins. The UCP2 is located ubiquitous in mammals. UCP3 has a skeletal muscle- and BAT-specific expression. Although the mRNA levels of UCP2 and UCP3 are rather high in brown adipose tissue, the protein level may not be well expressed.

As cold acclimation-recruited norepinephrine-induced nonshivering thermogenesis is intact in both UCP2- and UCP3-ablated mice, these proteins are apparently not essential for the

thermogenic process of brown adipose tissue. Since no detailed studies of brown adipose tissue or brown fat mitochondria have been reported in UCP2- or UCP3-ablated mice, more subtle effects of the absence of these proteins on BAT function may have gone unnoticed.

UCP1 and UCP3 have an opposite protein expression in cold environment in BAT. Protein level of UCP1 is increased while the protein level of UCP3 decreased in cold environment. Both mRNA level of UCP1 and UCP3 have been shown an increased transcription.

The most important hormonal regulation of BAT depends on insulin and glucagons. The increased level of insulin leads to phosphorilation of insulin-receptor signal (IRS-1) protein. Activation of phosphadidyl-inozitol-3-kinase (PI3K) activates the protein dependent kinase (PDK). Protein kinase B (AKT) is a substrae of PDK, and phospoho-AKT is the active form of AKT. GST3ß will be phosphorilated by AKT, phospho-GST3ß is the inactive form of GSK3ß. The net result of insulin signalling leads to the activation of glycogen synthase and phosphoprotein phosphatase, and glycogen content of BAT is increased dramatically under reacclimatization.

In mature brown adipocytes, norepinephrine interacts with three types of adrenergic receptors: β, α2, and α1. The most significant and the most studied pathway is the pathway for β3-adrenergic stimulation of thermogenesis. NE generates cAMP, it activates protein kinase A, then it phosphoryalates perilipin and hormone sensitive lipase (HSL), resulting fatty acids releasing in WAT. Another effect of NE is the up-regulation of UCP1 transcription in BAT.

There is cooperation between BAT and WAT under cold environment. WAT provides the BAT with free fatty acid to allow the enough energy for thermogenesis and ATP synthesis. The flux of the fatty acid is increased in BAT parallel with a much more intensive ß-oxidation.

Acetl-CoA carboxylase (ACC) and citrate lyase (CitLy) is the most significant enzyme of lipogenesis. Acetl-CoA carboxylase is the committed step of fatty acid synthesis. CitLy converts the cytosolic citrate to acetyl-CoA and oxaloacetate. Cytosolic acetyl-CoA is the carbon source of lipid synthesis. We investigated the ACC, CitLy activity and the enzymes of glycogen metabolism in cold adapted and reacclimatized rats of BAT and WAT. Tricium (^{3}H) incorporation shows the rate

of fatty acid synthesis. Tritium incorporation was measured into the newly synthesised fatty acid, and the total fat of BAT.

This part of the works has been based on the observation that there is a dramatic accumulation of glycogen after cold-exposure in BAT. The glycogen accumulation was detected as early as 3h after the replacement to neutral temperature from 1-week cold exposure, and was peaked by the 24th hour exceeding the control value 16-fold and reaching. Glycogen repletion during the recovery from cold exposure was also observed in muscle of rats; however, in these tissues glycogen levels did not significantly exceed the original control values.

AIMS OF THE STUDY

1.) Large protein bands were discovered under the gel electrophoresis in cold acclimated BAT. Mass-spectroscopy analysis shows ACC and CitLy enzymes were increased in cold exposure. We wanted clarify the flux of the fatty acid synthesis at the uncoupled mitochondrion in BAT. We wanted analyse the contribution of the WAT in the thermogenesis focusing on the two-lipogenic enzyme feature.

2.) Farkas et al. (1999) discovered a dramatic glycogen accumulation after re-acclimatization in the BAT of rats that has an intensive atrophy under reacclimatization. We planed the investigation of signal transudation of BAT in the background of glycogen synthesis. We were curious that muscle tissue has or has not similar feature.

3.) A question was arising about the role of UCPs connecting with atrophy in BAT. We investigated the changes in the level of mRNA of UCP1 and UCP3 and their protein expression under the cold and reacclimatized condition, to clarify the role of the UCPs in cold induced thermogenesis.

MATERIALS AND METHODS

Rat treatment

Male Wistar rats weighing 200-250 g were used. A group of cold exposed animals was replaced for 6, 24 and 48 hours to neutral temperature. Throughout the text, cold means +5^{o}C, while neutral temperature means +29^{o}C.

Immunoblotting analyses (Western blotting)

Protein components were separated on 12% acryl amide gels and blotted onto nitro-cellulose membrane, which was followed by immunoblotting with appropriate antibodies. Rabbit antibody for UCP1 was a kind gift from Barbara Cannon (Stockholm University, Sweden), which was tested not to cross-react with UCP3. Guinea pig antibody for UCP3 was purchased from Linco Research, Inc. (St. Charles, USA). This antibody was specific for UCP3; however, it reacted only with the long form of UCP3 (UCP3L) Rabbit antibodies for Akt, P-Akt; GSK-3, P- GSK-3; ATP-citrate lyase, P-ATP citrate lyase, acetyl-CoA carboxylase and P-Acetyl-CoA carboxylase were purchased form Cell Signaling (Kvalitex Kft., Budapest, Hungary) Anti-rabbit peroxidase conjugated secondary antibody was purchased from Sigma-Aldrich, (Budapest, Hungary).. Anti-guinea pig peroxidase conjugated secondary antibody was bought from Rockland, (Gilbertsvill, USA). Blots were developed with ECL plus (Amersham Pharmacia, UK.) on X-ray films.

Production of antibodies against human frataxin

A cDNA fragment encoding human frataxin was amplified from a human brain cDNA library using AAAAGATCTATGATAGCAGCGGCAGGAGGA as forward and AAACTCGAAGAGAGTCGATGGATAAGTG as reverse primers. Product of the reaction was cloned into pGEX 4T-1 plasmid resulting in a fusion between glutathione-S-transferase (GST) and frataxin. The fusion protein was expressed in BL21 *E.coli* cells and isolated using glutathione

sepharose as suggested by the supplier (Pharmacia). Isolated GST-frataxin fusion protein was used as antigen to generate antibodies in rabbits.

Northern blot analyses

Total RNA from rat interscapular BAT was purified and separated in a 1.2% agarose gel containing formaldehyde and transferred to Hybond-N membranes. The DNA probes both for UCP1 and UCP3 were kind gifts from Barbara Cannon (Stockholm University, Sweden). PhosphorImager detected the blots and the data were analysed by OptiQuant software.

Identification of proteins by mass spectrometry following SDS–PAGE separation

Digests were purified with ZipTipC18 pipette tips (Millipore, Bedford, MA, USA) using a procedure recommended by the manufacturer. One μL elute from the ZipTips was mixed at a 1:1 ratio with saturated DHB matrix (2,5-dihydroxybenzoic acid) solution and applied to an Achor chip MALDI plate (Bruker-Daltonics, Bremen, Germany). A Bruker Reflex III MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) was used in positive ion reflector mode with delayed extraction. Autolysis products of trypsin served as internal standards for mass calibration. The monoisotopic masses for all peptide ion signals in the acquired spectra were determined and the information was entered in a database searching against a non-redundant database (NCBI), using the Mascot program (Matrix Science; http://www.matrixscience.com/).

Materials for enzyme activities, glucose uptake and in vivo fatty acid synthesis

2-Deoxy-D-[U-^{14}C]glucose (Amersham), $^{3}H_2O$ and [γ-^{32}P]ATP were purchased from the Institute of Isotopes Co. (Budapest, Hungary). GSK3β was from Calbiochem (La Jolla, CA, USA). Insulin determination kit was from Crystal Chemicals (Downers Growe, IL, USA). Antibody against actin, amyloglycosidase and other chemicals were from Sigma-Aldrich (Budapest,

Hungary). SB 415286 specific GSK-3 inhibitor was from Tocris, (Avonmouth, UK). GSK-3 substrate peptide derived from Upstate (USA).

Enzyme activities

Pyruvate dehydrogenase enzyme (PDH) and phosphofructokinase-1 (PFK-1) acetyl-CaA carboxylase (ACC), citrate lyase (CitLy) and GSK3 activities were measured spectrophotometrically as reported in the original papers of the author. Glycogen content was measured as previously described.

Glucose uptake

Glucose uptake was measured by using 2-deoxy-[^{14}C]glucose, basically as described. Briefly, 1h after an intraperitoneal injection of 7.4 kBq radioactive 2-deoxy-glucose, glucose concentration was measured in the plasma, while radioactivity was measured in the plasma and BAT. The plasma specific radioactivity was then used to calculate glucose uptake.

In vivo fatty acid synthesis

Fatty acid (FA) synthesis was evaluated in vivo by measuring incorporation of $^{3}H_2O$ into FA. This method measured rate of de novo FA synthesis independent of the precursor carbon source. An intraperitoneal injection of 370 kBq tritiated water in 1 mL saline was administered to the animals and 1 h later they were killed. The tissues were processed and radioactivity in FA in BAT and WAT was counted in a liquid scintillation counter. Results were given as µg H atom incorporated into 1g tissues in 1h.

RESULTS AND DISSCUTION

The opposite regulation of lipid synthesis by ACC and CitLy in BAT and WAT plays an important role in the cooperation between the two organs in favour of thermogenesis. WAT acts primarily as a net FFA provider, whereas BAT acts as a net FFA user. The level of fatty acid synthesis is increased parallel with ß-oxidation. Flux of ß-oxidation is much higher than fatty acid synthesis. The situation is not unknown in BAT. ACC1 isoenzyme is phosphorylated under cold environment only in BAT. ACC2 is not phosphorylated in the same condition in BAT. ACC2 is responsible for regulation of carnitine acyl transferase. The question is: what is the explanation of increased fatty acids synthesis and ß-oxidation. Detailed mechanism of uncoupling is not clear yet. A theory is indicating that UCPs need fatty acid for uncoupling the mitochondria. Increased fatty acid synthesis may be a compensation mechanism of increased level of fatty acid oxidation.

The considerable increasing of active forms of ACC and CitLy indicates a massive FA synthesis in cold in BAT. On the contrary the activity of FFA synthesis down regulated in WAT by both lipogenic enzymes. The ^{3}H incorporation shows clearly a converse regulation of FFA synthesis between BAT and WAT. To understanding the deeper relation between BAT and WAT we have measured the FFA content in BAT in cold and reacclimatized environment. We have gotten indirect evidence that the FFA oxidation (ß-oxidation) is much intensive than FFA synthesis in BAT.

Glucose uptake is increased at cold environment and at reacclimatization phase also in BAT. Glucose transporter-4 (GLUT4) shows over expression under both environments in BAT. Insulin level of serum is decreased under cold acclimatization and started to return to normal level at neutral temperature. Activity of phosphofructokinase-1 did not change significantly in BAT at same temperature condition see above. Activity of pyruvate dehydrogenase (PDH) decreased in cold environment and this trend line was followed under reacclimatization. While PDH activity was repressed the glycogen amount of BAT was increased. This effect showed that glucose was taken

up and its degradation is inhibited by glycolysis, so there is a free way to glycogen conversation of glucose. We investigated the signal pathway of glycogen synthesis in BAT and muscle tissue. AKT and GSK3ß were studied and their phosphorylation. The results showed that glycogen synthase is inhibited in cold, which was well presented in the measurement of glycogen content in BAT. The glycogen amount of BAT is dramatically elevated under reacclimatization as it has been shown on the changes of p-AKT and (active) and p-GSK3ß (inactive). Muscle tissue has a remarkable content of glycogen. It is mentionable that the upstream signalling pathway of glycogen accumulation is activated only in the reacclimatization phase of rats in muscle.

We have showed that UCP1 protein expression increased markedly during the cold exposure and their level is maintained after 24h-reacclimatized condition to neutral temperature. On the contrary to the UCP3 protein expression has decreased greatly in the same condition but started to a gentle return to normal level on neutral temperature. mRNAs of UCP1 and UCP3 are markedly increased in cold adaptation, but the protein expression didn't followed it. It would be suppose a post-transcription and post-translation mechanism in the background. This results show that UCP1 has a key regulator function in the thermogenesis. UCP3 takes plays especially in regulation of respiratory chain.

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PUBLICATION

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