UNIVERSITY OF VETERINARY MEDICINE VIENNA

Retinoid Metabolism in Brown Fat Function

Master Thesis

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List of abbreviations

Abbreviation	Full name
~	Approximately
&	And
°C	Degrees Celsius
36b4	Ribosomal protein, large, P0
Ab/ mAb	Antibody/ monoclonal antibody
ADH1/ 4/ 7	Alcohol dehydrogenase 1/ 4/ 7
ADHFe1	Iron containing alcohol dehydrogenase 1
ALDH1a1	Aldehyde dehydrogenase 1, subfamily a1
ALDH1a2	Aldehyde dehydrogenase 1, subfamily a2
ALDH1a3	Aldehyde dehydrogenase 1, subfamily a3
AGN	RAR antagonist
ATP	Adenosine triphosphate
ATRA/ RA	All-trans retinoic acid
BAT	Brown adipose tissue
Cidea	Cell death-inducing DNA fragmentation factor alpha subunit-like effector A
Co-IP	Protein complex immunoprecipitation
cAMP	Cyclic adenosine monophosphate
Crabp2	Cellular retinoic acid binding protein 2
CRE	cAMP response element
CREB	cAMP resonse element-binding
Ct	Cycle threshold
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
Fabp4	Fatty acid binding protein 4
FBS	Fetal bovine serum
g	Gram
Gs	Galphas

Abbreviation	Full name
IBAT	Interscapular brown adipose tissue
i.e.	Id est
L	liter
PBS	Phosphor buffered saline
PET	Positron emission tomography
PKA	Protein kinase A
Ppargc-1a	Peroxisome proliferative activated receptor gamma coactivator 1 alpha
Prdm16	PR domain containing 16
PVDF membrane	Polyvinylidenfluorid membrane
RAR	Retinoic acid receptor
RARalpha	Retinoic acid receptor alpha
RARbeta	Retinoic acid receptor beta
RARgamma	Retinoic acid receptor gamma
Rdh1, 10	Retinol dehydrogenase 1, 10
RNA/ mRNA	Ribonucleic acid
Rpm	Rounds per minute
RT-PCR	Reverse transcriptase- real-time PCR
SEM	Standard error of the mean
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
TBST	Tris-Buffered Saline and Tween 20
Tfam	Mitochondrial transcription factor A
TTNPB	4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid
UCP1	Uncoupling protein 1
Vol	Volume
WAT	White adipose tissue
WHO	World Health Organization

1. Introduction

1.1. Obesity – a public health issue

According to the World Health Organization (WHO), adiposity, as obesity is also termed, is described as health compromising excessive fat accumulation. Out of over 7 billion people worldwide approximately 1.4 billion adults are considered overweight or obese. In addition, 40 million obese children are younger than 5 years (WHO fact sheet No. 311, 2012). Main reasons for obesity are lack in physical exercise, exaggerated calorie intake and mass urbanization (NGUYEN & LAU, 2012). Body mass index (BMI) presents a tool to measure obesity and studies show that a BMI above 25 is associated with increased general mortality risk of about 30% and a 40% higher death risk caused by cardiovascular diseases (Prospective Studies Collaboration, 2009). Since obesity is highly prevalent worldwide and increases risks for numerous diseases, especially cardiovascular diseases like hypertension (NGUYEN & LAU, 2012) and type 2 diabetes (HILL et al., 2003), it is a serious public health concern. Nevertheless, obesity is preventable and studying adipose tissue biology contributes to understand and find therapeutic strategies against obesity.

1.2. Adipose tissue

Two different adipose tissues can be distinguished in mammals, white adipose tissue (WAT) and brown adipose tissue (BAT). While WAT is evenly distributed in the whole body, BAT is dispersed throughout the body in the form of smaller depots (Figure 1) (CANNON & NEDERGAARD, 2004). WAT cells have low number of mitochondria and store triglycerides in a single lipid drop. In contrast to WAT, BAT cells contain multiple smaller lipid droplets and are rich in mitochondria, which give BAT its brown color (ENERBÄCK, 2009). The most determining difference between WAT and BAT is the expression of uncoupling protein 1 (UCP1) in brown adipocytes, enabling the unique ability of non-shivering thermogenesis in BAT, which will be referred to later (CINTI, 2005). In obese mammals, WAT amount is increased. Positive energy balance leads to hypertrophic and hyperplastic changes of white adipocytes (CINTI,

2005). After reaching a certain size (DI GIROLAMO et al., 1998), it is hypothesized that these white adipocytes increase cell number instead of growing further (FAUST et al, 1978). However, adipose tissue is more than a mere fat storing tissue. Its contribution to immune response, bone mass and thyroid and reproductive function shows the diverse tasks of adipose tissue (ROSEN & SPIEGELMAN, 2006). Especially BAT with its special function to oxidize fatty acids and dissipate energy in form of heat, raises the question if not adipose tissue itself has a mechanism which can be used to decrease obesity (KOZAK & ANUNCIADO-KOZA, 2008).

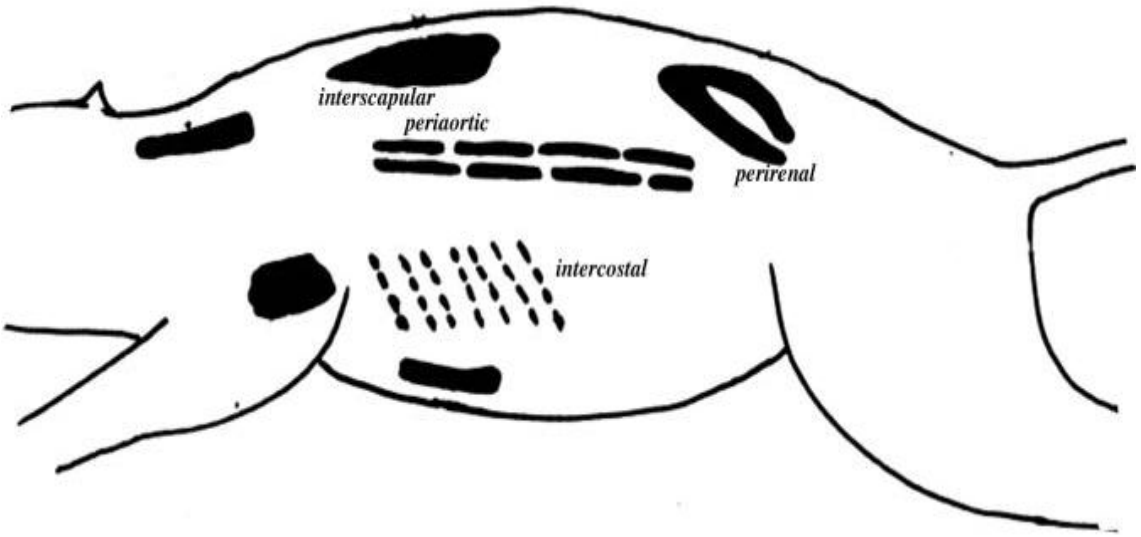

Figure 1: Distribution of brown adipose tissue depots in the body (might vary between different animals or strains) (CANNON & NEDERGAARD, 2004).

1.3. Brown adipose tissue

Brown adipose tissue is a special kind of fat depot exclusively found in mammals, especially in hibernating ones and newborns (CANNON & NEDERGAARD, 2004). It has the unique ability of non-shivering thermogenesis, a process in which respiration is uncoupled from adenosine triphosphate (ATP) synthesis which leads to energy dissipation in the form of heat (NICHOLLS et al., 1978) (Figure 4). One protein is considered to be most crucial in this process – UCP1. It is a mitochondrial membrane protein, responsible for the uncoupling of oxidative phosphorylation, by functioning as a proton transporter (Figure 5) (AQUILA et al., 1985). BAT and non-shivering thermogenesis are activated in response to cold, mainly by beta adrenergic signaling

(NICHOLLS & LOCKE, 1984), and allows mammals to survive in cold (CANNON & NEDERGAARD, 2004).

BAT has first been described in 1551 by Conrad Gesner (CANNON & NEDERGAARD, 2004). Nevertheless, it is a rather young organ. Its ability to produce heat was first discovered in the 1960s (SMITH, 1964), and uncoupling protein (UCP) was first detected in the late 1970s (NICHOLLS et al., 1978). Thus, many mechanisms of BAT are not fully understood until now. Due to BAT's potential role in decrease of obesity, understanding underlying mechanisms of thermogenesis and BAT itself is of importance.

1.4. Brown adipose tissue in humans

The relevance of BAT and thermogenesis in regard to obesity became even more important since BAT was found in humans (SAITO et al., 2009). Whereas BAT physiology is well studied in rodents, where BAT is robustly present, its relevance for humans had been a controversial issue. However, positron emission tomography (PET), originally used to detect malignant tumors, revealed symmetric uptake of fluorodeoxyglucose (Figure 2), a radiopharmaceutical, in human shoulders where no tumor could be found. Later, these areas were identified as brown adipose tissue (COHADE et al., 2003).

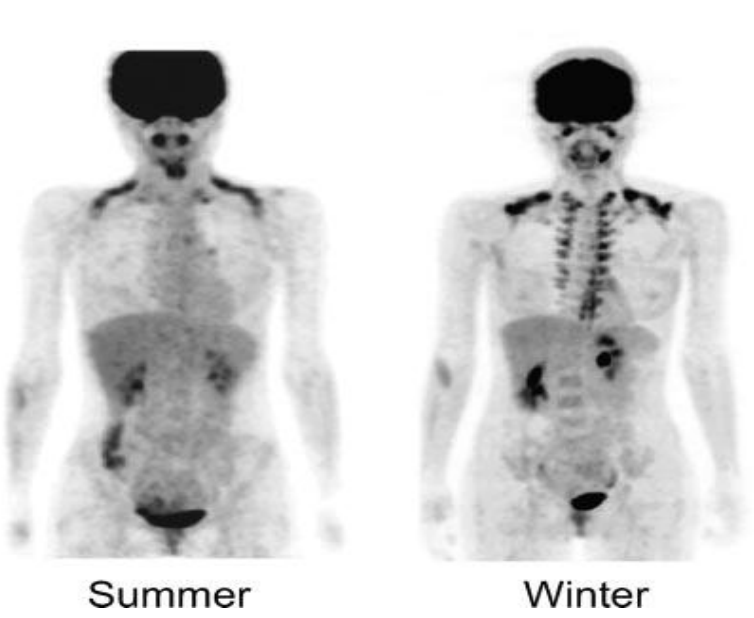

Figure 2: PET scan of distribution of BAT in humans, differences in summer and winter. In winter, due to exposure to cold, the presence of BAT in humans seems to be stronger (SAITO et al., 2009).

1.5. Thermogenesis

Non-shivering thermogenesis – the ability to produce heat without shivering – is the special feature, making BAT attractive for therapeutic strategies against obesity (KOZAK & ANUNCIADO-KOZA, 2008; KOZAK et al., 2010). Up to now, the main pathway activating thermogenesis in BAT is considered to be the beta adrenergic pathway (Figure 3). Norepinephrine, a neurotransmitter released from the sympathetic nervous system, triggers beta adrenergic induced *UCP1* gene transcription. Among others, norepinephrine is a ligand for beta receptors. In regard to non-shivering thermogenesis in mice, beta 3 receptors are the most important ones of all three beta receptor isoforms (others being beta 1 and 2 receptors) found in BAT of mice (CANNON & NEDERGAARD, 2004). Of note, beta 3 receptors do not seem to be present in all mammals, even if thermogenesis in these animals can be observed, suggesting that beta 3 receptors are not essential for thermogenesis (ATGIE et al., 1996). Beta receptors are G-coupled proteins. For thermogenesis, only G_{alphas} (Gs) type of G-coupled proteins are relevant (GRANNEMAN, 1988). Binding of norepinephrine to the Gs coupled beta 3 receptor leads to activation of cyclic adenosine monophosphate (cAMP), which then phosphorylates protein kinase A (PKA). Phosphorylated PKA leads to the activation of transcription factor cAMP response element-binding (CREB) protein which is needed for the transcription of *UCP1*, the protein responsible for non-shivering thermogenesis (Figure 3) (NICHOLLS & LOCKE, 1984).

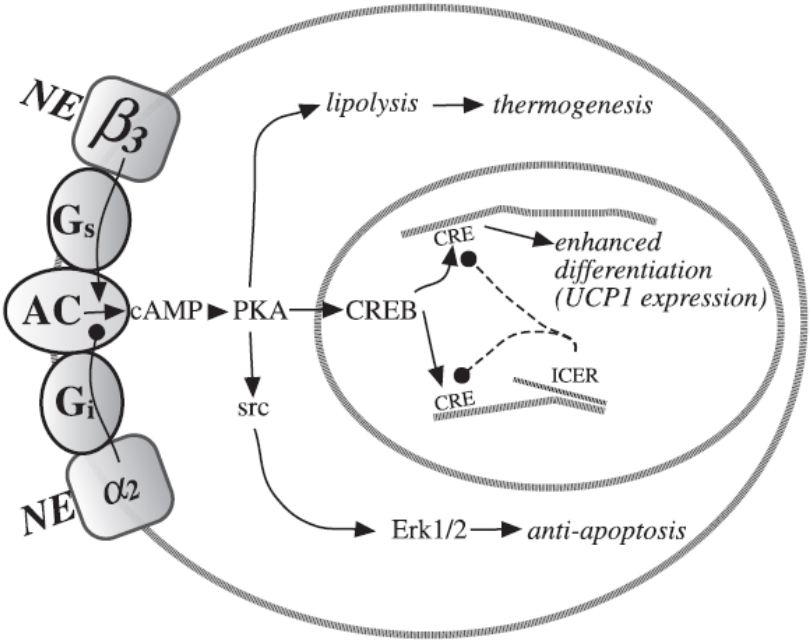

Figure 3: Beta adrenergic pathway for induction of UCP1; norepinephrine (NE), cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), cAMP response element-binding protein (CREB), cAMP response element (CRE), inducible cAMP early repressor (ICER) (CANNON & NEDERGAARD, 2004).

Norepinephrine activated cAMP and PKA do not only lead to transcription of *UCP1*, but also signal triglyceride breakdown, an important substrate for thermogenesis. Combustion of the fatty acids in the respiratory chain leads to extrusion of protons out of the mitochondrion. UCP1 protein, when activated, allows protons to re-enter the mitochondrion (AQUILA et al., 1985) uncoupled from oxidative phosphorylation, thereby interrupting adenosine triphosphate (ATP) synthesis. Instead of ATP, heat is produced (Figure 4) (NICHOLLS & LOCKE, 1984).

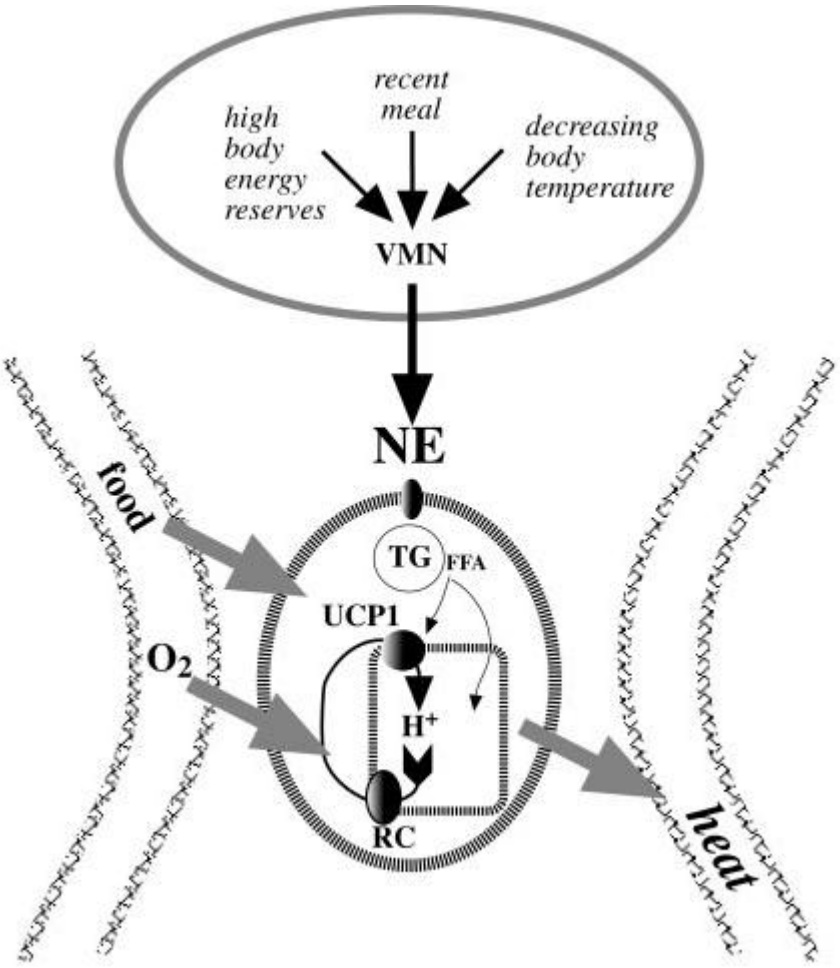

Figure 4: Mechanism of thermogenesis; ventromedial hypothalamic nucleus (VMN), norepinephrine (NE), triglyceride (TG), fatty acid (FFA), uncoupling protein 1 (UCP1), proton (H+), respiratory chain (RC) (CANNON & NEDERGAARD, 2004).

The above described mechanism was the general known way of non-shivering thermogenesis induction. However, nowadays more pathways are known to contribute to UCP1 expression and thereby thermogenesis (Figure 5). One of these pathways is the retinoic acid pathway.

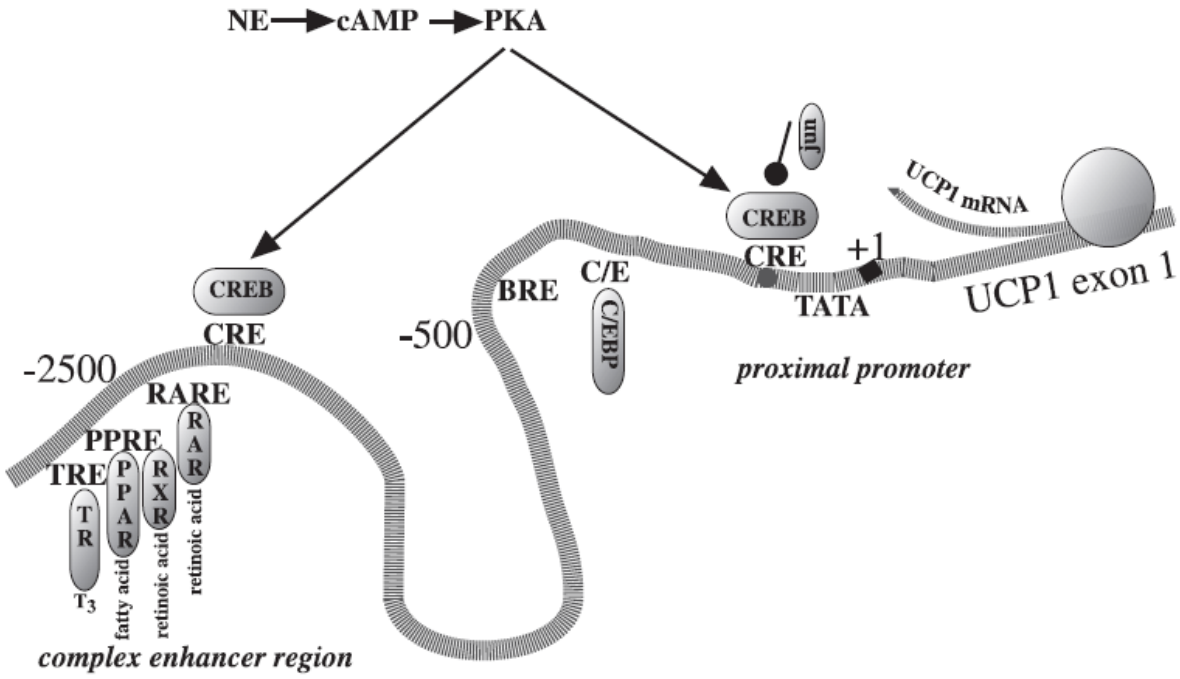

Figure 5: Alternative pathways for transcription of UCP1; retinoic acid response element (RARE), retinoic acid receptor (RAR), cAMP response element (CRE), cAMP response element-binding (CREB), uncoupling protein 1 (UCP1) (CANNON & NEDERGAARD, 2004).

1.6. Retinoid metabolism

Vitamin A metabolites, so called retinoids, are involved in many biological processes like cell differentiation and development (DE LUCA, 1991). Recently, retinoids were linked to regulation of adiposity (ZIOUZENKOVA et al., 2007), metabolic disease and even modulation of cancer (ALTUCCI et al., 2007). Most of these interactions are performed by the activation of retinoic acid receptors (RARs) and retinoid X receptor (RXR), two nuclear receptors which are involved in regulation of gene transcription (ZIOUZENKOVA & PLUTZKY, 2008). Retinoid metabolism is strictly regulated (NAPOLI, 1996). A network of enzymes is necessary to convert vitamin A (retinol), which is obtained through diet, to retinaldehyde and retinoic acid (RA). Alcohol dehydrogenases, especially the so called retinol dehydrogenases, oxidize retinol to retinaldehyde, followed by the irreversible conversion of retinaldehyde to retinoic acid by retinaldehyde dehydrogenases (Figure 6).

The role of retinoids in BAT metabolism and activation of thermogenesis is not well understood yet. Early studies show, that *UCP1* can be induced by pharmacologic concentrations of RA (ALVAREZ et al., 1995), suggesting a role of RA in BAT.

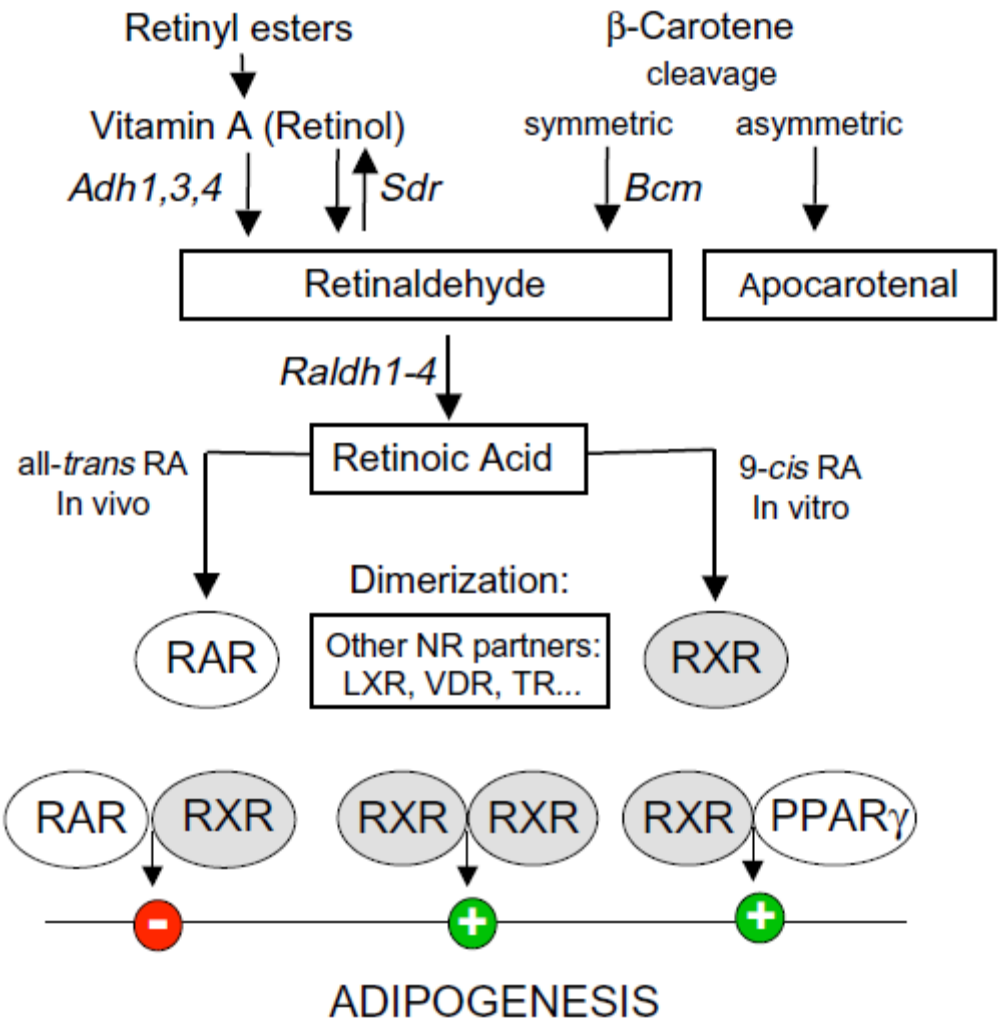

Figure 6: Retinol conversion and possible trancriptional interactions in regard to adipogenesis (ZIOUZENKOVA & PLUTZKY, 2008).

1.7. Aim of this study

The first aim of this study is to investigate the expression of critical components of the retinoid pathway, including converting enzymes and binding proteins, in mouse BAT. This will inform us whether the BAT possesses the transcriptional machinery essential for storing and metabolizing retinoids. Next, we seek to establish an *in vitro* model of brown adipocytes in order to study possible interactions between the retinoid and beta adrenergic signaling pathway on thermogenesis in aim two. Finally we will try to gain new mechanistic insight in how thermogenesis can be maintained through retinoid and beta adrenergic signaling. These studies will hopefully help to shed light on the role of retinoids in brown fat physiology and thermogenesis.

2. Materials and Methods

2.1. Materials

2.1.1. List of products

Product name	Provider
AGN 193109	Santa Cruz Biotechnology (Santa Cruz, California)
ATRA	Sigma (St. Louis, Missouri)
Autoradiography Film	Denville Scientific (Metuchen, New Jersey)
Extra thick blotting paper	Bio-Rad (Hercules, California)
Cell lysis buffer 10x	Cell Signaling Technology® (Danvers, Massachusetts)
Chloroform	American Bioanalytical (Natick, Massachusetts)
CREB Inhibitor	Sigma (St. Louis, Missouri)
DeliverX™	Affymetrix (Santa Clara, California)
Dexamethasone	Sigma (St. Louis, Missouri)
DMSO	Sigma (St. Louis, Missouri)
DNase	Invitrogen (Grand Island, New York)
Dynabeads®	Invitrogen (Grand Island, New York)
Ethanol	Decon Labs (King of Prussia, Pennsylvania)
FBS	Sigma (St. Louis, Missouri)
FSK	Invitrogen (Grand Island, New York)
GlycoBlue™	Invitrogen (Grand Island, New York)
H-89	Sigma (St. Louis, Missouri)
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems (Carlsbad, California)
Trypsin	Invitrogen (Grand Island, New York)
High Glucose DMEM	Invitrogen (Grand Island, New York)
Indomethacin	Sigma (St. Louis, Missouri)
Insulin	Sigma (St. Louis, Missouri)
iQ™ 96-well PCR plate	Bio-Rad (Hercules, California)

Product name	Provider
iQ™ SYBR®Green Supermix	Bio-Rad (Hercules, California)
Isobutylmethylxanthine	Sigma (St. Louis, Missouri)
Isopropanolol	Sigma (St. Louis, Missouri)
Methanol	Fisher Scientific (Fair Lawn, New Jersey)
MicroBCA™	Thermo Scientific (Rockford, Illinois)
Microseal® 'B' Adhesive Seals	Bio-Rad (Hercules, California)
NE-PER® Nuclear and Cytoplasmic Extraction Reagents	Thermo Scientific (Rockford, Illinois)
TTNPB	Sigma (St. Louis, Missouri)
Trypsin	Sigma (St. Louis, Missouri)
NuPAGE 4-12% Tris-Bis Gels	Invitrogen (Grand Island, New York)
NuPAGE LDS Sample Buffer 4x	Invitrogen (Grand Island, New York)
Nu PAGE MOPS SDS Running Buffer 20x	Invitrogen (Grand Island, New York)
NuPAGE Transfer Buffer 20x	Invitrogen (Grand Island, New York)
Norepinephrin	Sigma (St. Louis, Missouri)
PBS	Invitrogen (Grand Island, New York)
Penicillin-streptomycin	Sigma (St. Louis, Missouri)
Protease inhibitor	Sigma (St. Louis, Missouri)
Phase lock tubes	5 Prime (Gaithersburg, Maryland)
PVDF membrane	Bio-Rad (Hercules, California)
Sodium azide	Sigma (St. Louis, Missouri)
T3	Sigma (St. Louis, Missouri)
TBST	Boston Bioproducts (Ashland, Massachusetts)
TRIzol®	Invitrogen (Grand Island, New York)
Western Lightning®Plus ECL	PerkinElmer (Waltham, Massachsetts)

2.1.2. List of devices

Name of device	Provider
Denville 260D centrifuge	Denville Scientific (South Plainfield)
GPCR centrifuge	Beckman (Brea, California)

Name of device	Provider
Kodak X-omat 2000A	Kodak (Rochester, New York)
MyIQ iCycler	Bio-Rad (Hercules, California)
NanoDrop2000	Thermo Scientific (Rockford, Illinois)
Select Heatblocks	VWR Scientific Products (Radnor, Pennsylvania)
SpectraMax Plus	Molecular Devices (Silicon Valley, California)
Trans-Blot® SD Semi-Dry Transfer Cell	Bio-Rad (Hercules, California)
Vortex Genie 2	Scientific industries (Bohemia, New York)
xCellSureLock	Invitrogen (Grand Island, New York)

2.1.3. List of siRNA pools

Name of siRNA pool	Provider
FITC labeled scrambled RNA	Invitrogen (Grand Island, New York)
siRARalpha	Ambion (Austin, Texas)
siRARgamma	Ambion (Austin, Texas)

2.1.4. List of Abs

Name of Ab	Provider
mGAPDH	Santa Cruz Biotechnology (Santa Cruz, California)
mCREB	Abcam (Cambridge, Massachusetts)
mRARalpha	Santa Cruz Biotechnology (Santa Cruz, California)
mRARgamma	Abcam (Cambridge, Massachusetts)
HRP goat anti mouse	Invitrogen (Grand Island, New York)
HRP goat anti rabbit	Invitrogen (Grand Island, New York)

2.1.5. List of mouse primers

All primers were obtained from Integrated DNA Technologies IDT (Coralville, Iowa)

Primer Name	Forward Primer	Reverse Primer
36b4	5' – CAA CCC AGC TCT GGA GAA AC – 3'	5' – GAG GTC CTC CTT GGT GAA CA – 3'

Primer Name	Forward Primer	Reverse Primer
Adh1	5’ – ACA AAC CCT TCA CCA TCG AG – 3’	5’ – GTG GCC ACC ATC TTA ATT CG – 3’
Adh4	5’ – CCC AGG GAG CTA GAC AAA CCT – 3’	5’ – CCC CAG CCT AAT ACA GTG CAG – 3’
Adh7	5’ – AGG ACT CTA CCA AGC CCA TCA G – 3’	5’ – ACC ACC ACA CTG GTC CCA TAG – 3’
Adhfe1	5’ – CGA GTC ACA CAC TTG CTG AGG – 3’	5’ – CTT CCT TCG TAA CCC CTG CTC – 3’
Aldh1a1	5’ – AAG AAG GGG ACA AGG CTG AT – 3’	5’ – TGA AGA GCC GTC AGA GGA GT – 3’
Aldh1a2	5’ – ACA TCG ATT TGC AGG GAG TC – 3’	5’ – GTC CAA GTC AGC ATC TGC AA – 3’
Aldh1a3	5’ – AGC CAC AGG AGA GCA AGT GT – 3’	5’ – AAG TTC CAC GGG ATG ATC TG – 3’
Cidea	5’ – TTC CTC GGC TGT CTC AAT GTC – 3’	5’ – GGG ATG GCT GCT CTT CTG TAT C – 3’
Crabp2	5’ – CAA GCC AGC AGT CGA GAT CA – 3’	5’ – TCT TAC AGG GTC TCC CAT CCA C – 3’
Ppargc-1a	5’ – CCC TGC CAT TGT TAA GAC C – 3’	5’ – TGC TGC TGT TCC TGT TTT C – 3’
Prdm16	5’ – TGG CCT TCA TCA CCT CTC TGA – 3’	5’ – TTT CTG ATC CAC GGC TCC TGT GA – 3’
Rdh1	5’ – GGG CTG TGA CTC TGG CTT T – 3’	5’ – GTT CCC AAC ACG CTC CTT CA – 3’
Rdh10	5’ – ACA CGC AGA GCA ATG AGG AG – 3’	5’ – GTC CAG AAA CCA CAC CAG CA – 3’
Tfam	5’ – CCG AAG TGT TTT TCC AGC ATG – 3’	5’ – GGG CTG CAA TTT TCC TAA CCA – 3’
UCP1	5’ – CTT TGC CTC ACT CAG GAT TGG C – 3’	5’ – ACT GCC ACA CCT CCA GTC ATT – 3’

2.1.6. List of buffers and media

Growth medium	High glucose DMEM (4.5 g/L) 10% FBS 1% Streptomycin-penicillin

Standard adipogenic induction cocktail for BAT cells	High glucose DMEM (4.5 g/L) 20 nM insulin 1 nM T3 1 µM dexamethasone 0.5 mM isobutylmethylxanthine 0.125 mM indomethacin

2.2. Methods

2.2.1. Animals

All tissue samples used in this study originated from C57BL/6J wild type mice of the Jackson Laboratory (Bar Harbor, Maine). For tissue analysis, brown adipose tissue from 15 week old females was harvested prior to this study. All procedures were fully approved by the Harvard Medical School Institutional Animal Care and Use Committee.

2.2.2. BAT cells

For *in vitro* studies, SV40 immortalized stromal-vascular cells isolated from C57BL/6J mice were used. These immortalized BAT cells were provided by C. R. Kahn. The cells were cultivated in 12-well, 24-well, or 10 cm culture plates and differentiated to mature brown adipocytes as described below.

2.2.3. *In vitro* adipose differentiation

BAT cells were grown in high glucose (4.5 g/L) DMEM containing 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin. When fully confluent, the cells were induced using a standard adipogenic cocktail with 20 nM insulin, 1 nM T3, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine, 0.125 mM indomethacin. Forty-eight hours past induction, the growth medium was supplemented with insulin (20 nM) and T3 (1nM). At day four, the cells were stimulated as indicated for 4 hours, followed by cell harvest for either RNA or protein extraction as explained below.

2.2.4. Cell transfection and siRNA knockdown

BAT cells were transfected 48 hours after induction with FITC labeled scrambled RNA (siControl), siRARalpha, or siRARgamma using DeliverX™ according to manufacturer's instruction. Forty-eight hours after transfection, the cells were stimulated with retinoids or beta-adrenergic agonists as indicated (Figures 18–21) and harvested for RNA extraction 4 hours after stimulation or stated otherwise.

2.2.5. RNA extraction, reverse transcription and gene expression

For extraction of RNA, TRIzol® was used. Cells were washed with PBS, scraped with 500 µL TRIzol® per well and transferred into 1.5 mL tubes. After incubation (room temperature, 5 minutes) samples were kept at -80°C for at least 1 hour. Then, 120 µL DEPC treated water and 100 µL chloroform were added to each tube. Samples were incubated (room temperature, 10 minutes), transferred into phase lock tubes, and centrifuged (4°C, 13000 rpm, 10 minutes). The aqueous phase (exactly 380 µL) was transferred into new 1.5 mL tubes, supplemented with 280 µL isopropanol and 1.5 µL GlycoBlue™, incubated (-20°C, 30 minutes), and centrifuged (4°C, 14000 rpm, 30 minutes). All samples were washed twice with 75% ethanol and the RNA pellet was resuspended in 12 µL DEPC treated water. RNA concentration was measured using NanoDrop 2000. Prior to reverse transcription with the High Capacity cDNA Reverse Transcripton Kit, RNA was treated with DNase. Gene expression was analyzed via real-time RT-PCR with MyiQ cycler (Bio-Rad, Hercules, California) using iQ™ SYBR®Green supermix and 96-well PCR plates. One PCR reaction (25 µL reaction volume) contained 12.5 µL iQ™ SYBR®Green supermix, 0.8 µL forward primer, 0.8 µL reverse primer, 0.8 µL cDNA and 10.1 µL DEPC treated water. The PCR was set up as following:

-	Initial denaturation	95°C (10 minutes)
-	40 cycles of	95°C (15 seconds)
		60°C (1 minute)
-	Melt curve analysis	95°C (15 seconds)
		60°C (1 minute)
		95°C (15 seconds)

For normalization, gene expression was compared to the expression of housekeeping gene 36b4 unless stated otherwise. Outliers were sorted out by visual inspection.

2.2.6. Protein complex immunoprecipitation

Cells were harvested using NE-PER® Nuclear and Cytoplasmic Extraction Reagents according to manufacturer's instruction. Harvested cells were stored at -80°C for at least 1 hour before protein concentration measurement using MicroBCA™. In short, samples were diluted 1:200 and 100 µL of BSA standard or diluted sample was pipetted into a 96-well plate. Dyes were mixed according to manufacturer's instruction and added to the standards and samples. For protein concentration measurement, SpectraMax Plus was used. Co-IP was performed using Dynabeads® according to manufacturer's instructions. Protein lysates were supplemented with NuPAGE® LDS sample buffer, heated at 75°C for 10 minutes and stored at -80°C until further use.

2.2.7. Immunoblotting

After thawing, protein lysates were heated at 75°C for 1 minute and loaded onto a NuPAGE® 4-12% Bis-Tris gel. SDS-PAGE was performed at 180V for 60 minutes. Afterwards, the gel was transferred onto a PVDF membrane. Western blotting was conducted at 20 V for 50 minutes. Incubation with the primary antibody (1:500 in TBST) was conducted overnight at 4°C, followed by 1 hour incubation with the respective secondary antibody (1:20000 in 5% milk in TBST) at room temperature. For visualization, membranes were developed onto autoradiography films.

2.2.8. Statistical analysis

Data is stated as means ± SEM. For comparison of two groups, unpaired two-tailed Student's *t*-test was performed. P-values <0.05 were considered as significant.

3. Results

3.1. Expression of retinoid metabolism machinery in BAT

Retinoid metabolism in tissue is tightly regulated by a complex network of enzymes and binding proteins. We set out to study if the key components of retinoid metabolism are present in brown adipose tissue (BAT). We therefore analyzed gene expression of retinoid converting enzymes and retinoid binding proteins (Figures 7–10) in BAT of 15 week old female C57BL/6J mice which were kept at room temperature and were fed a standard chow.

We first determined the expression pattern of retinol (vitamin A) converting enzymes, which catalyze the initial step of retinoid metabolism. Thus, mRNA expression of alcohol dehydrogenase 1, 4 and 7 (*Adh1*, *Adh4*, *and Adh7*), and iron containing alcohol dehydrogenase 1 (*AdhFe1) was* studied in BAT. In addition, retinol dehydrogenase 1 and 10 (*Rdh1*, *Rdh10*) mRNA expression was analyzed (Figures 7 – 8). Among the alcohol dehydrogenases, *AdhFe1* expression was highest with a six fold difference in mRNA expression compared to *Adh1* (mean Ct_{Adh1} = 22.56) (Figure 7). *Adh4* and *Adh7* expression levels were the lowest of all tested alcohol dehydrogenases with mean cycle threshold (Ct) values of 30.50 and 30.80, respectively.

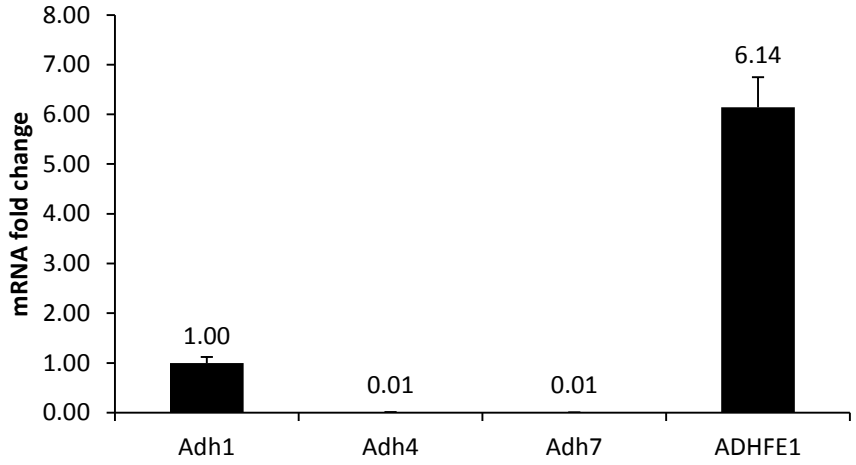

Figure 7: Relative mRNA expression of alcohol dehydrogenases in BAT: BAT from C57BL/6J mice (n=5-6); mRNA analysis of alcohol dehydrogenases in relation to *Adh1* (mean Ct = 22.56); no p-value given, since unrelevant in this context; data is given as means ± SEM.

Among the retinol dehydrogenases, *Rdh10* was almost 10 fold higher expressed than *Rdh1* (mean Ct_{Rdh1} = 30.86) (Figure 8).

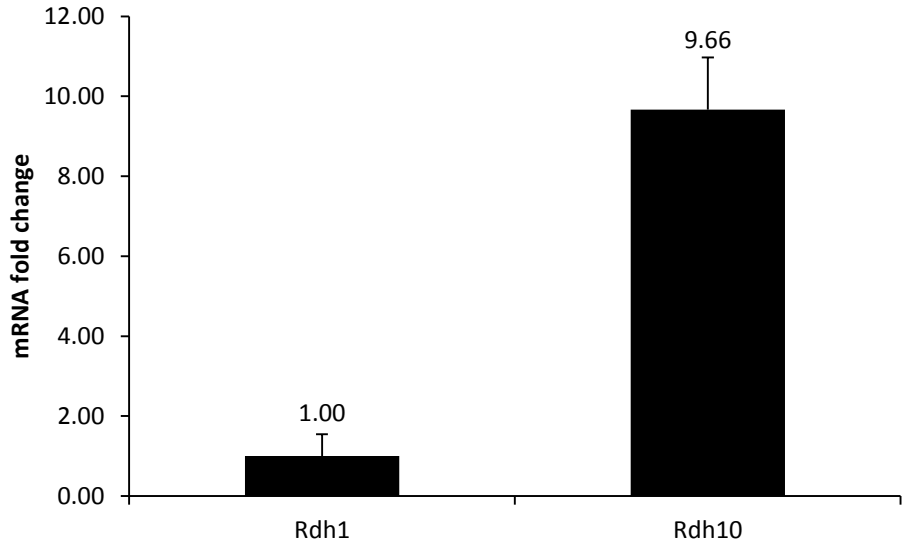

Figure 8: Relative mRNA expression of retinol dehydrogenases in BAT: in BAT of C57BL/6J mice (n=6), mRNA analysis of retinol dehydrogenases in relation to *Rdh1* (mean Ct = 30.86); no p-value given, since unrelevant in this context; data is given as means ± SEM.

Next we analyzed BAT gene expression of retinaldehyde dehydrogenases, the enzymes responsible for oxidizing retinaldehyde to retinoic acid (RA), which is considered the rate-limiting step of retinoid metabolism (Figure 9). Gene expression of retinaldehyde dehydrogenase 1, 2, and 3 (*Aldh1a1*, *Aldh1a2*, and *Aldh1a3)* was measured. All three enzymes were present in BAT. *Aldh1a1* was the predominant isoform in BAT (mean $Ct_{Aldh1a1}$ = 28.24), followed by *Aldh1a3* and *Aldh1a2*.

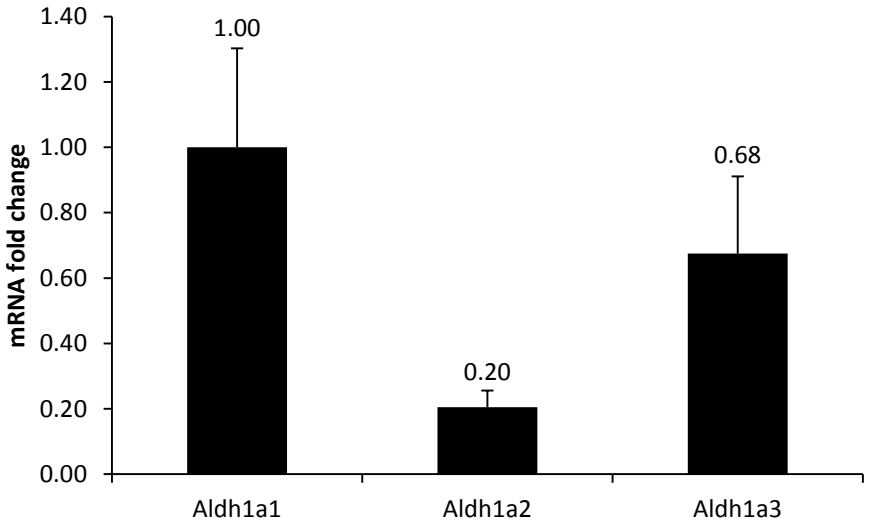

Figure 9: Relative mRNA expression of retinaldehyde dehydrogenases in BAT: in BAT of C57BL/6J mice (n=5), mRNA analysis of retinaldehyde dehydrogenases in relation to *Aldh1a1* (mean Ct = 28.24); no p-value given, since unrelevant in this context; data is given as means ± SEM.

We then went on to characterize the expression of intracellular retinoid binding proteins. We measured mRNA expression of cellular retinoic acid binding protein II (*Crabp2*) and fatty acid binding protein 4 (*Fabp4*). Even though *Crabp2* expression was low (mean Ct_{Crabp2} = 29.84), it was still present in BAT. In contrast, *Fabp4* was robustly expressed with >26000 times higher gene expression compared to *Crabp2*. Given the crucial role of *Fabp4* in binding fatty acids in adipose tissue (HERTZEL & BERNLOHR, 2000), the high expression in BAT was not unexpected (Figure 10).

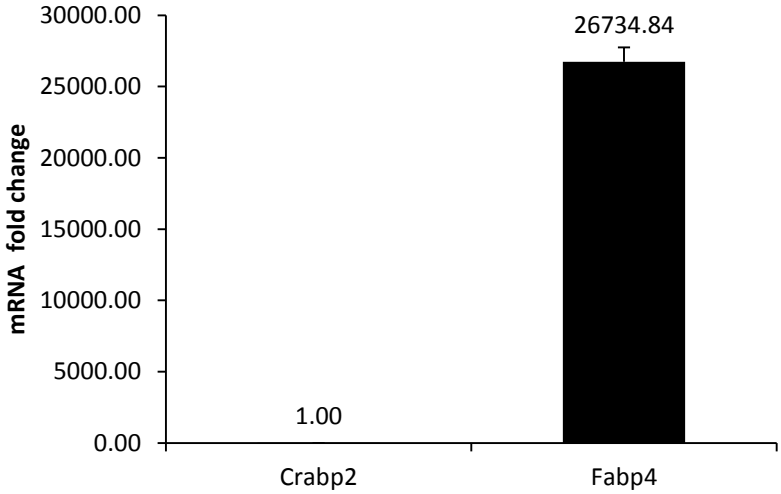

Figure 10: Relative mRNA expression of *Crabp2* and *Fabp4*: in BAT of C57BL/6J mice (n=5), mRNA analysis of *Fabp4* in relation to *Crabp2* (mean Ct = 29.84); no p-value given, since unrelevant in this context; data is given as means ± SEM.

The robust expression of all relevant retinoid converting enzymes and binding proteins indicates that the transcriptional machinery for active retinoid metabolism is present in murine BAT and therefore implies a functional role of retinoids within BAT.

3.2. Establishment of an *in vitro* model

Based on the transcriptional evidence for BAT as retinoid metabolizing organ we sought to establish a cell culture model to study effects of retinoids on brown adipocytes *in vitro* (Figures 11–12). Therefore, SV40 immortalized stromal vascular cells isolated from BAT from C57Bl/6 mice (provided from C. R. Kahn) were differentiated to brown adipocytes. In short, BAT cells were cultured until totally confluent, followed by 48 hours induction using an adipogenic induction cocktail as described in “Materials and Methods”. Then, medium was changed to growth medium containing insulin and T3 (see “Materials and Methods”). At day four, the differentiated cells were stimulated as indicated. During the process of differentiation, the shape of the cells changed from fibroplastic shape to mature adipocytes with significant lipid accumulation (data not shown). To verify successful differentiation of BAT cells to brown adipocytes, mRNA expression of classical brown fat markers such as cell death-inducing DNA fragmentation factor alpha subunit-like effector A (*Cidea*), peroxisome proliferative activated receptor gamma coactivator 1 alpha (*Ppargc1a*), PR domain containing 16 (*Prdm16*), mitochondrial transcription factor A (*Tfam*), and uncoupling protein 1 (*UCP1*) was analyzed at day 0, day 2 and day 4 of adipocyte differentiation (Figure 11).

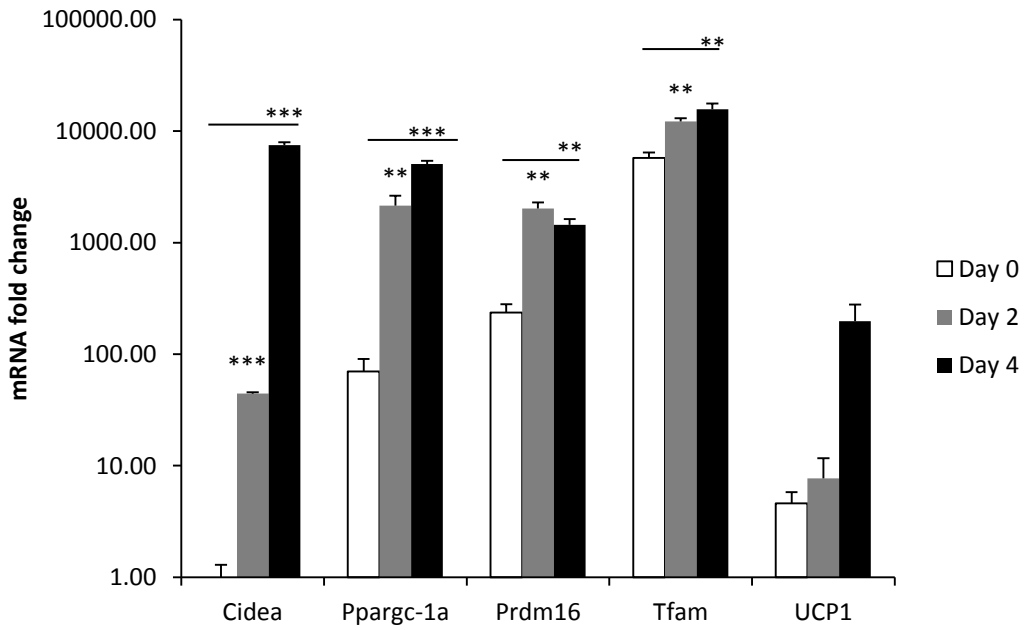

Figure 11: Relative mRNA expression of brown fat markers in differentiated BAT cells: BAT cells were differentiated as described in "Methods" and mRNA was analyzed at day 0, day 2, and day 4 past induction (n=3 per point of time); note logarithmic scale on y-axis; data is given as means ± SEM (**p<0.01, ***p<0.001).

In course of the differentiation, the mRNA expression of all BAT markers was significantly increased with peak levels at day 4, indicating that we were indeed able to differentiate stromal vascular cells to mature brown adipocytes.

To test the responsiveness of the differentiated BAT cells to beta adrenergic stimulation and, hence, their potential for the induction of thermogenesis, chronic stimulation over 4 days was performed using forskolin (FSK, Figure 12), a cAMP agonist which can increase intracellular cAMP levels and thereby mimics beta adrenergic signaling downstream of cAMP. Gene expression analysis of the key thermogenic factor *UCP1* revealed a dramatic increase following FSK stimulation at day 2 compared to unstimulated cells (Figure 12). Afterwards, *UCP1* induction strongly decreased to almost similar *UCP1* expression level of unstimulated cells. This can be explained by cellular desensitization in response to chronic adrenergic stimulation a phenomenon that has previously been described (CANNON & NEDERGAARD, 2004; VASUDEVAN et al., 2011).

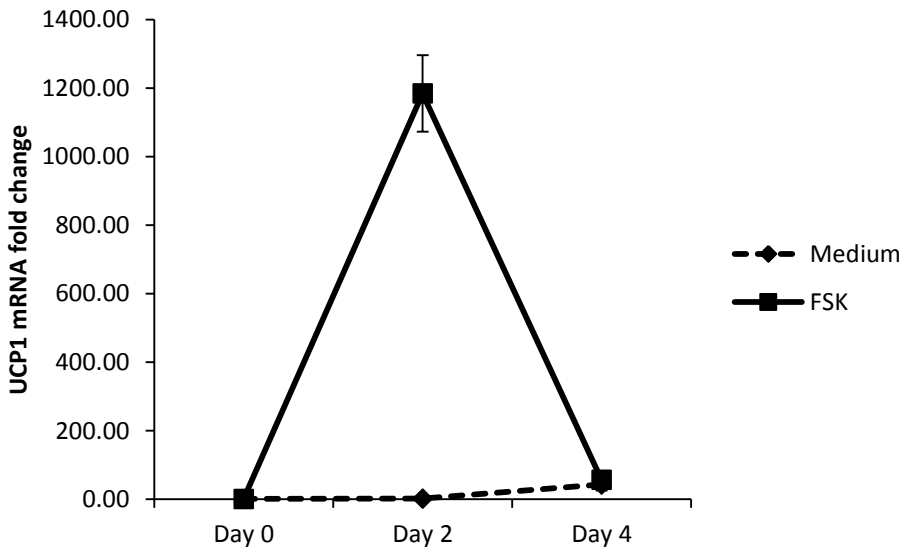

Figure 12: *UCP1* mRNA expression in the course of BAT cell differentiation and FSK responsiveness during differentiation: Differentiated BAT cells were chronically stimulated with FSK (10 µM) every 12 hours for 4 days (n=3 per point of time and condition); UCP1 mRNA analysis of day 0, day 2 and day 4; data is given as means ± SEM.

Here, we showed successful differentiation of SV40 immortalized BAT cells to mature brown adipocytes. In addition, the findings indicate responsiveness of these adipocytes to FSK stimulation.

3.3. Effect of retinoic acid and beta adrenergic stimulation on *UCP1* induction in BAT cells

To consider the role of RA in thermogenesis, differentiated BAT cells were stimulated with all-trans RA (ATRA) and adrenergic stimuli as described (Figure 13). Norepinephrine and FSK, a stimulator of cAMP synthesis, significantly increased *UCP1* expression (23 fold and 55 fold respectively) compared to cells stimulated with vehicle. Also ATRA significantly raised *UCP1* expression by 39 fold. Of note, UCP1 induction by ATRA was significantly stronger than induction by norepinephrine. More interestingly, combined stimulation with ATRA and norepinephrine or FSK led to dramatic and significant increase of *UCP1* mRNA levels (270 fold and 295 mRNA fold change respectively).

These data indicate synergistic effects on *UCP1* induction through combined stimulation with RA and beta adrenergic agonists and implicate a possible enhancing interaction of retinoic acid and the beta adrenergic pathway.

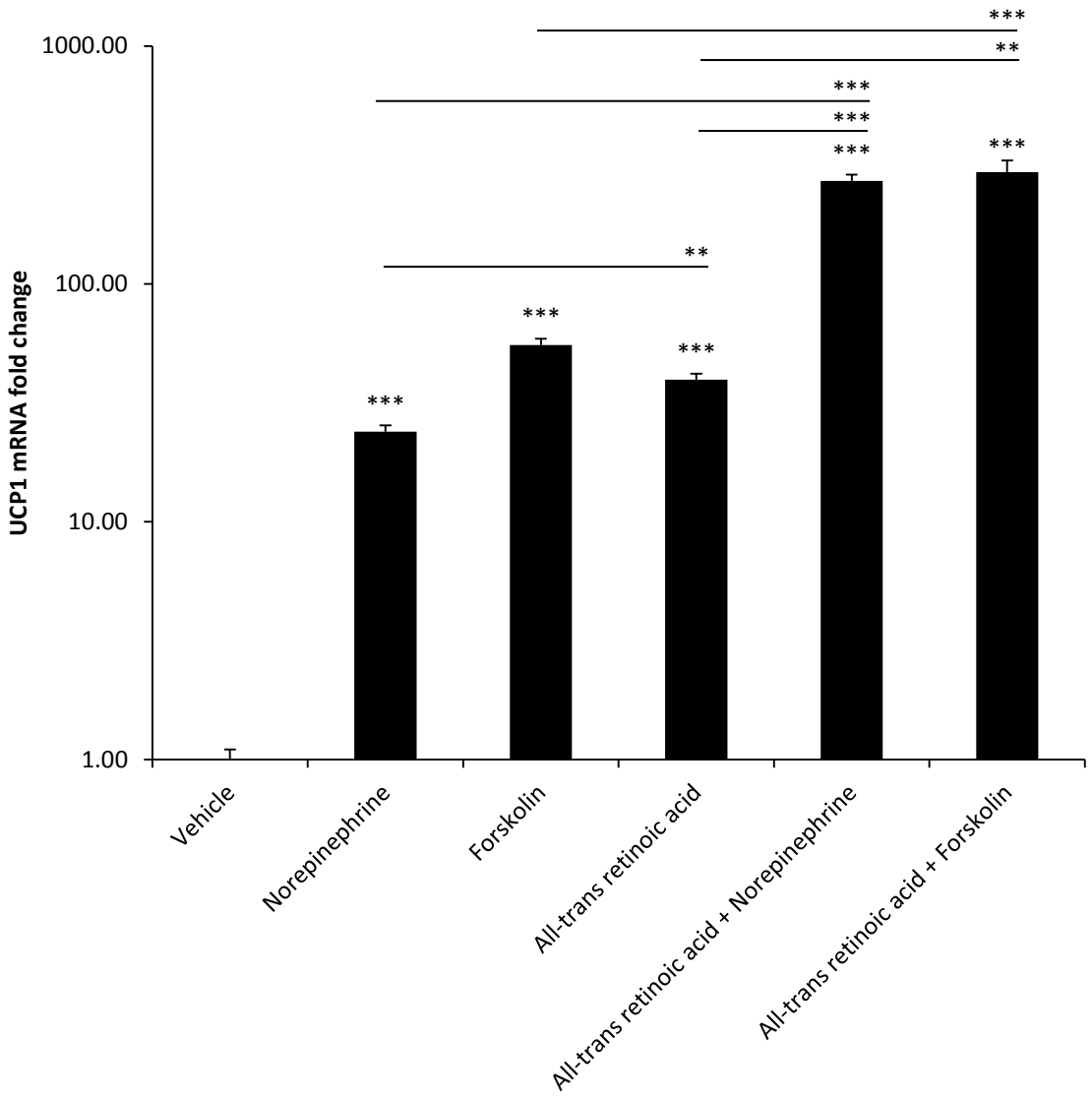

Figure 13: Effect of stimulation with beta adrenergic/ or cAMP agonists and all-trans retinoic acid on UCP1 induction in BAT cells: at day 4 after induction of differentiation, BAT cells were stimulated with vehicle (DMSO), norepinephrine (1 µM), forskolin (10 µM), ATRA (1 µM), and combinations as indicated for 4 hours (n=3 per condition); note logarithmic scale on y-axis; data is given as means ± SEM (**p<0.01, ***p<0.001).

3.4. Role of retinoic acid receptors in synergistic *UCP1* induction

Next we investigated whether the synergistic effects between ATRA and beta agonists on UCP1 expression were retinoic acid receptor (RAR) dependent or due to non-genomic effects (Figures 14–17). Therefore, differentiated BAT cells were stimulated with TTNPB, a selective RAR agonist, and FSK as indicated (Figure 14) Both, TTNPB and FSK were able to induce *UCP1* on their own with comparative efficiency (9 fold or 11 fold change respectively). Combined stimulation of BAT cells

with TTNPB and FSK showed strongly increased *UCP1* mRNA levels compared to single stimulation (50 fold mRNA increase) suggesting involvement of RARs in synergistically enhanced *UCP1* induction (Figure 14).

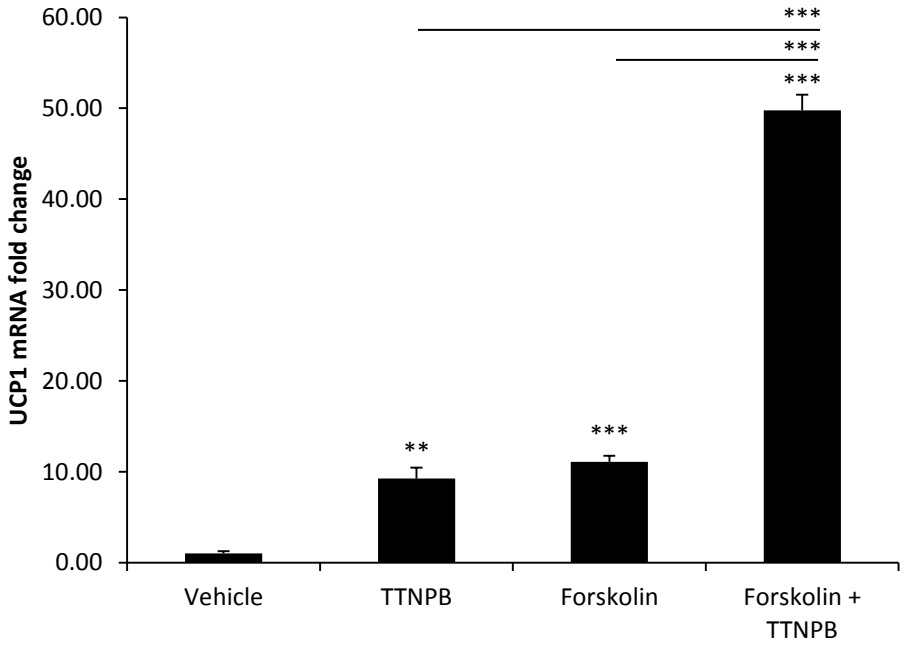

Figure 14: Effect of selective RAR agonist on *UCP1* induction: BAT cells, stimulated at day 4 after induction with vehicle (DMSO), TTNPB (1 µM), or forskolin (10 µM) 4 hours stimulation (n=3 per condition); data is given as means ± SEM (**p<0.01, ***p<0.001).

In order to further study RAR's implication in *UCP1* induction we used the selective RAR antagonist AGN 193109 .Differentiated BAT cells were stimulated with AGN, ATRA or FSK as indicated (Figure 15). AGN alone did not induce *UCP1* gene expression. Co-stimulation of ATRA with AGN significantly decreased ATRA-mediated *UCP1* transcription. Surprisingly also FSK-mediated UCP1 expression was markedly downregulated. These results suggest that cAMP-mediated effects on UCP1 are in part RAR dependent but this would need further investigation.

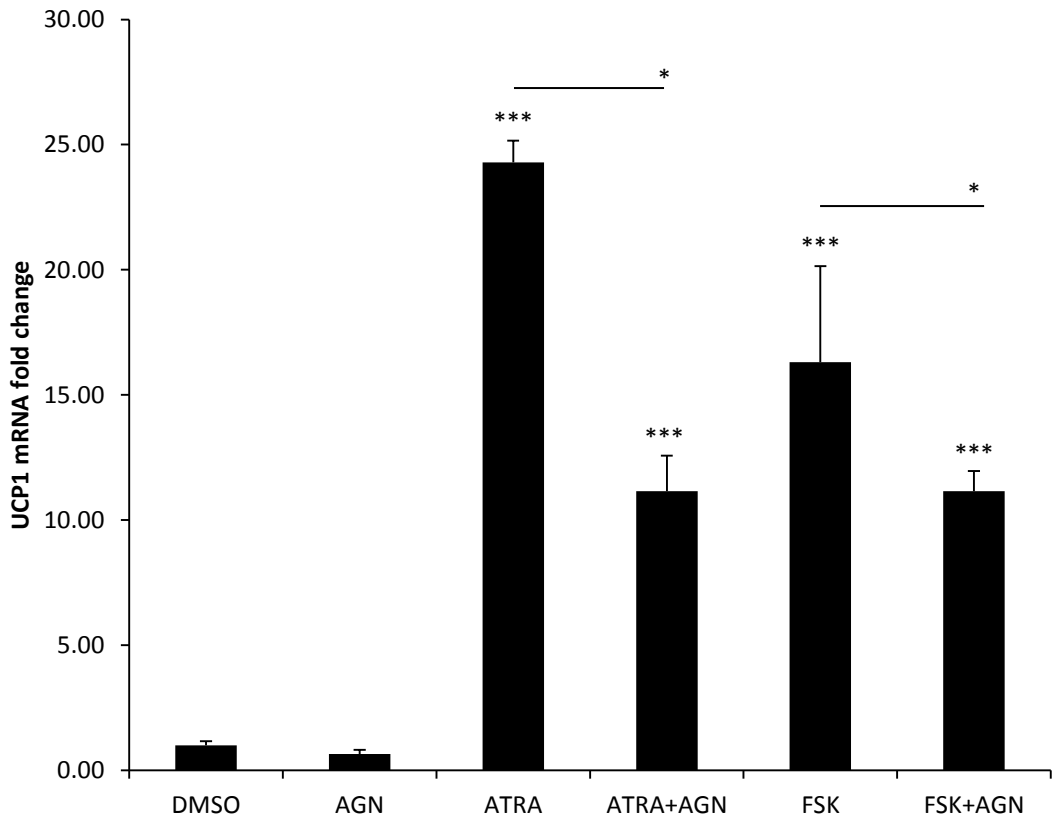

Figure 15: Effect of RAR antagonist AGN on *UCP1* transcription: BAT cells, stimulated at day 4 after induction with vehicle (DMSO), AGN (1 µM), ATRA (1µM), FSK (10 µM), or combinations as indicated for 4 hours following mRNA analysis (n=3 per condition); data is given as means ± SEM (**p<0.01, ***p<0.001).

In an opposite approach we inhibited adrenergic signaling by a cAMP activated protein kinase A (PKA) inhibitor or by a cAMP response element binding protein (CREB) inhibitor. Both PKA and CREB are key components of the adrenergic signaling pathway (CANNON & NEDERGAARD, 2004). The PKA inhibitor H-89 not only blunted FSK-induced but also ATRA-induced UCP1 expression (Figure 16). The CREB inhibitor N-(4-Chlorophenyl)-3-hydroxy-2-naphtamide significantly decreased FSK- but not ATRA-mediated *UCP1* expression (Figure 17). Together these results suggest a complex interaction between retinoid and adrenergic signaling to regulate and enhance transcription of *UCP1*, a key mediator of thermogenesis.

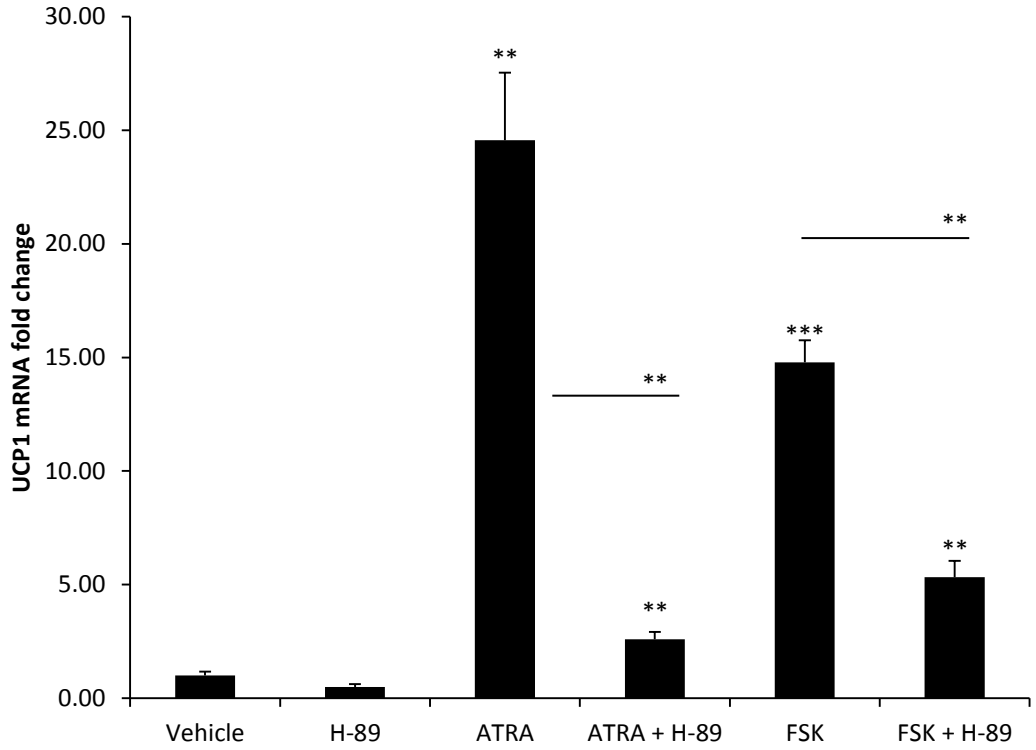

Figure 16: Effect of PKA inhibitor H-89 on *UCP1* mRNA levels in BAT cells: BAT cells, stimulated at day 4 after induction with vehicle (DMSO), H-89 (20 µM), ATRA (1µM), FSK (10 µM), or combinations as indicated for 4 hours following mRNA analysis (n=3 per condition); data is given as means ± SEM (**p<0.01, ***p<0.001).

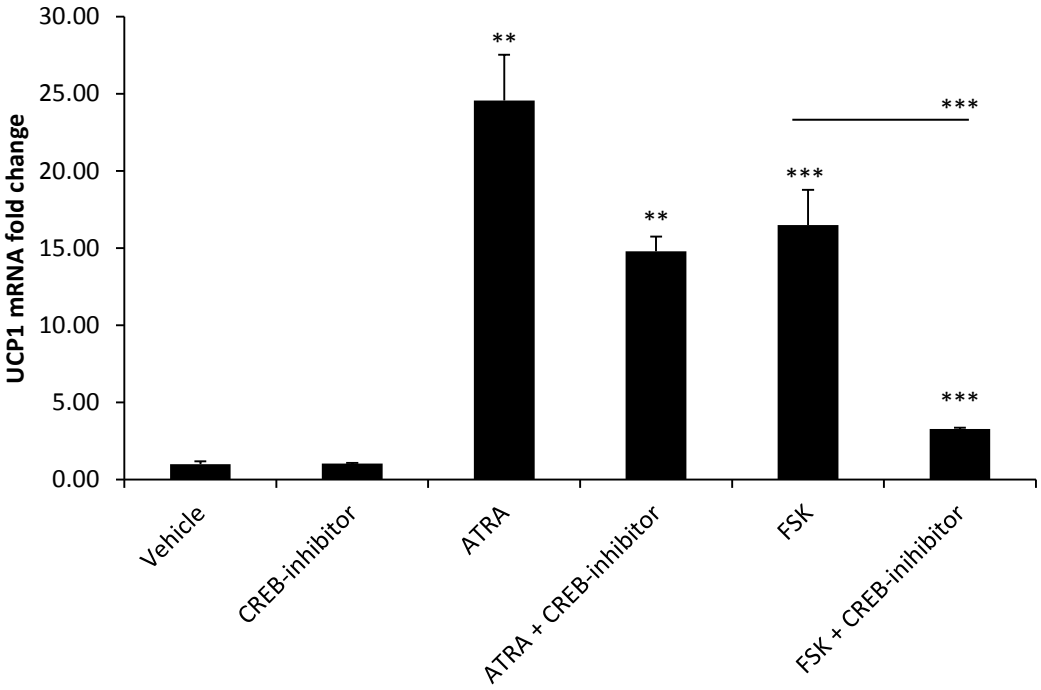

Figure 17: Effect of CREB inhibitor on *UCP1* transcription: BAT cells, stimulated at day 4 after induction with vehicle (DMSO), CREB inhibitor (20 µM), ATRA (1µM), FSK (10 µM), or combinations as indicated for 4 hours following mRNA analysis (n=3 per condition; data is given as means ± SEM (**p<0.01, ***p<0.001).

3.5. RAR knockdown experiments

To further strengthen our observation that RAR might be involved in the synergism between retinoid and beta adrenergic signaling we performed RAR knockdown experiments using RARalpha and RARgamma siRNA. Forty-eight hours after transfection we assessed mRNA knockdown by quantitative real time RT-PCR.

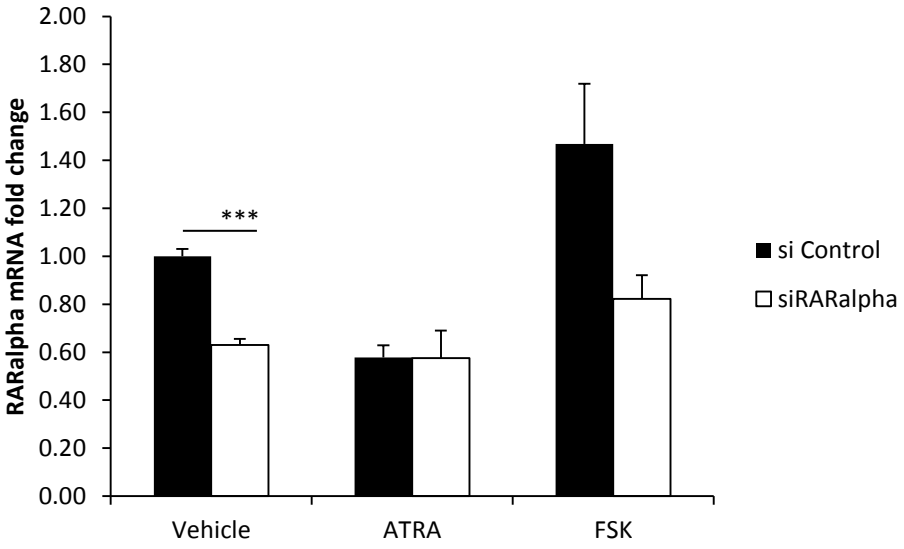

Figure 18: Gene expression of *Rara* after siRARalpha knockdown: 48 hours after adipogenic induction of BAT cells, transfection with siControl (FITC labeled scrambled RNA) and siRARalpha was performed, 48 hours past transfection, cells were stimulated with vehicle, ATRA (1 µM), or FSK (10 µM) as indicated and *Rara* mRNA expression was analyzed 4 hours after stimulation (n=3 per condition); data is given as means ± SEM (**p<0.01, ***p<0.001).

Knockdown against RARalpha (siRARalpha) resulted in a 40% repression of RARalpha gene expression (Figure 18). Of note, ATRA stimulation itself decreased the level of *Rara* mRNA in siControl transfected cells. However, siRARalpha did not inhibit ATRA- or FSK-induced *UCP1* expression (Figure 20).

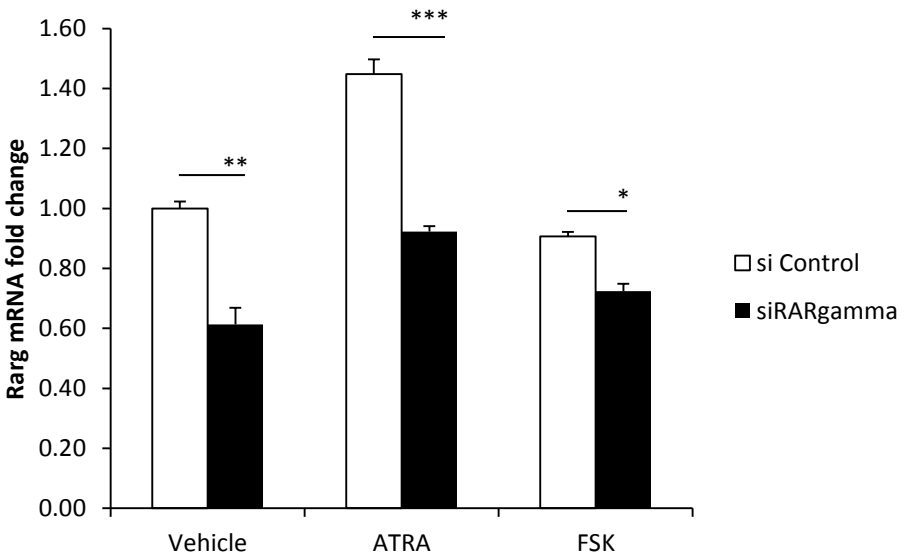

Figure 19: Gene expression of *Rarg* after siRARgamma knockdown: 48 hours after adipogenic induction of BAT cells, transfection with siControl (FITC labeled scrambled RNA) and siRARgamma was performed, 48 hours past transfection, cells were stimulated with vehicle, ATRA (1 µM), or FSK (10 µM) as indicated and *Rarg* mRNA expression was analyzed 4 hours after stimulation (n=3 per condition); data is given as means ± SEM (**p<0.01, ***p<0.001).

Knockdown of siRARgamma resulted in a significant 40% knockdown of RARgamma mRNA expression in unstimulated cells and gene repression was even less in cells stimulated with ATRA or FSK (Figure 19). RARgamma transfected ATRA treated cells showed a significant reduction of *UCP1* mRNA compared to non-transfected ATRA treated cells, indicating a possible RARgamma involvement in ATRA induced *UCP1* expression. Since RARalpha was not repressed in ATRA treated cells, the relative contribution of RARalpha versus gamma cannot be addressed. Here, siRARgamma did not affect FSK-induced UCP1 expression (Figure 21), however, the RARgamma knockdown in these cells was modest with 21%.

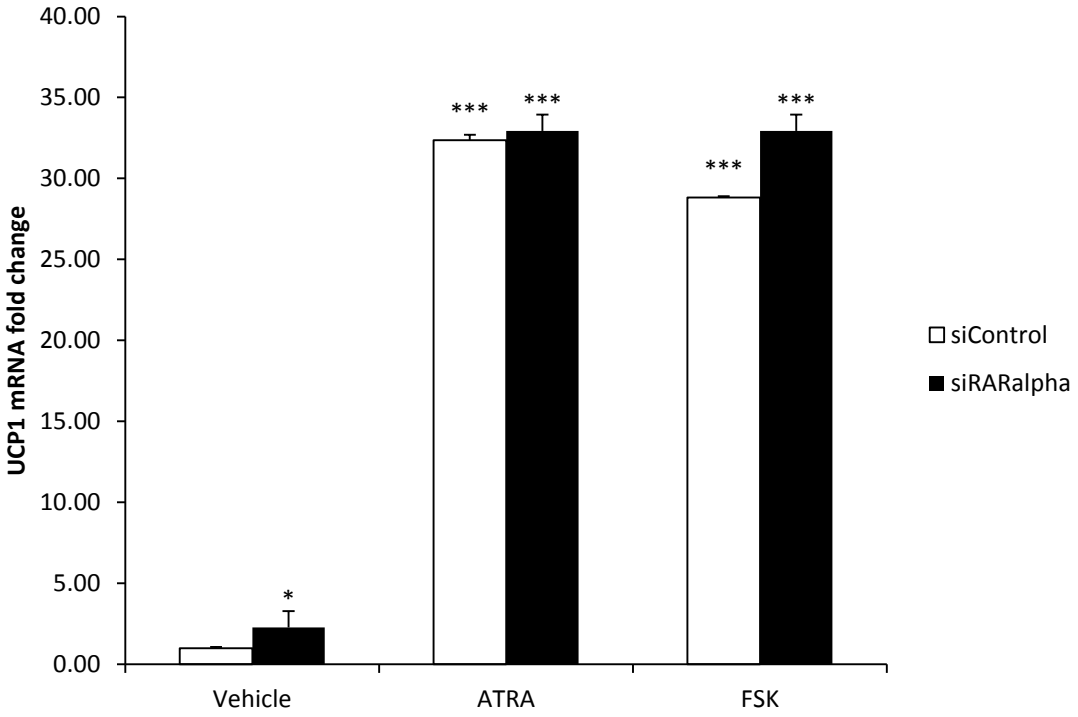

Figure 20: *UCP1* mRNA fold change after siRARalpha knockdown: 48 hours after adipogenic induction of BAT cells, transfection with siControl (FITC labeled scrambled RNA), and siRARalpha was performed, 48 hours past transfection, cells were stimulated with vehicle, ATRA (1 µM), or FSK (10 µM) as indicated and *UCP1* mRNA expression was analyzed 4 hours after stimulation (n=3 per condition); data is given as means ± SEM (**p<0.01, ***p<0.001).

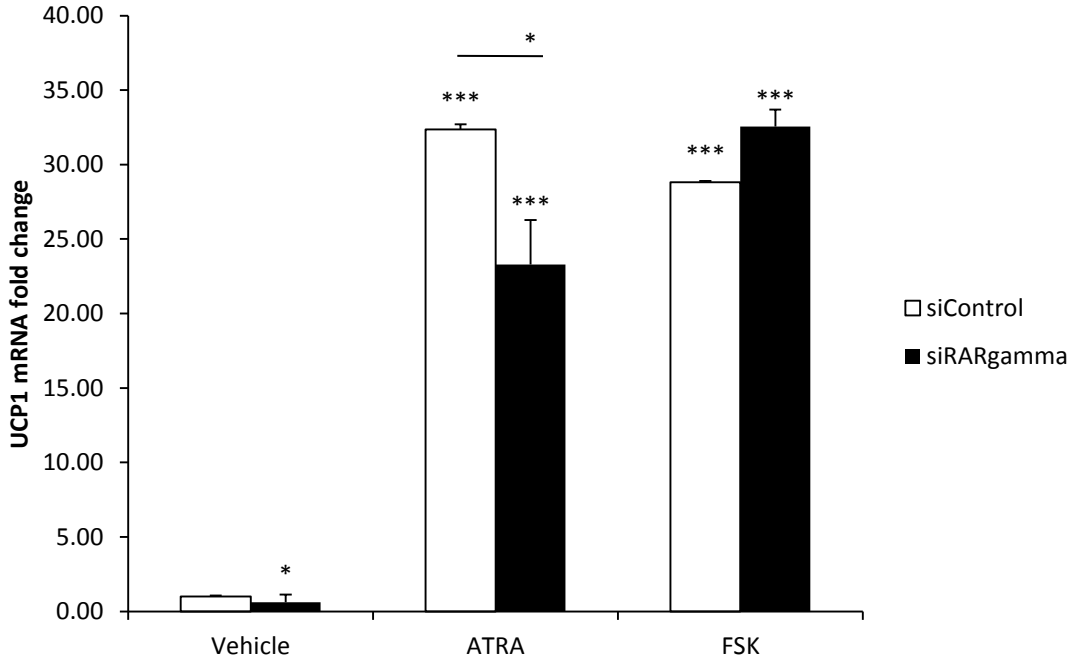

Figure 21: UCP1 mRNA fold change after siRARgamma knockdown: 48 hours after adipogenic induction of BAT cells, transfection with siControl (FITC labeled scrambled RNA), and siRARgamma was performed, 48 hours past transfection, cells were stimulated with vehicle, ATRA (1 µM), or FSK (10 µM) as indicated and *UCP1* mRNA expression was analyzed 4 hours after stimulation (n=3 per condition); data is given as means ± SEM (**p<0.01, ***p<0.001).

3.6. Protein complex immunoprecipitation

To study a direct interaction between RAR and the adrenergic pathway, here with the transcription factor CREB, we performed co-immunoprecipitation.
First, BAT cells were differentiated to mature brown adipocytes and protein lysates were isolated. We separated the cytoplasmic and nuclear fraction since the transcription factors RAR and CREB are mainly located in the nucleus. Immunoblot analysis for KU70, a protein involved in DNA repair and therefore only found in the nucleus, and Tubulin alpha, a cytosolic protein, showed successful separation of both fractions (Figure 22).

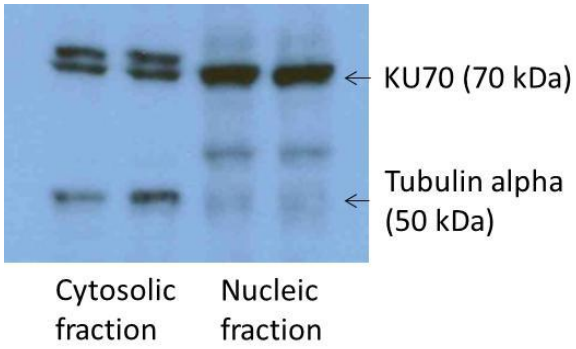

Figure 22: Immunoblot analysis of cytosolic and nucleic fraction of differentiated BAT cells; anti mouse KU70 Ab (~70 kDa) and anti mouse Tubulin alpha Ab (~50 kDa).

Immunoblot analysis against CREB also showed that we were able to detect CREB in the nuclear fraction (Figure 23).

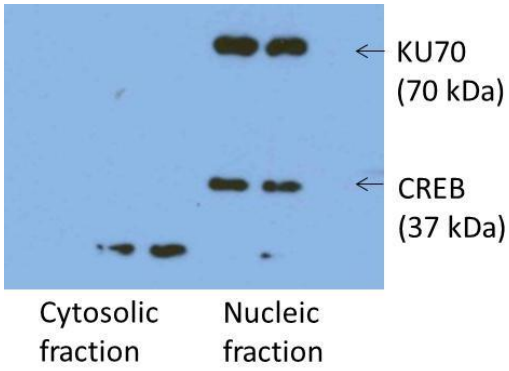

Figure 23: Immunoblot analysis of cytosolic and nucleic fraction of differentiated BAT cells; anti mouse KU70 Ab (~70 kDa) and anti mouse CREB Ab (~37 kDa).

In addition, immunoblot analysis against RAR gamma showed that we were able to detect RAR gamma in the nucleic fractions (Figure 24).

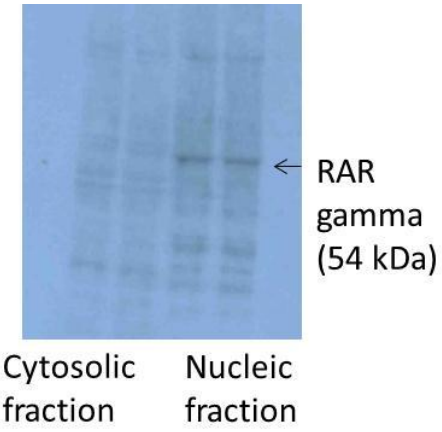

Figure 24: Immunoblot analysis of cytosolic and nucleic fraction of differentiated BAT cells; anti mouse RAR gamma Ab (~54 kDa).

We then went on to immunoprecipitate CREB using ChIP-grade antibodies and Dynabeads®. We were able to precipitate CREB in the nuclear lysate (not shown), however neither RAR alpha nor gamma co-precipitated with CREB. It cannot be excluded that the levels of RAR present in the purified nuclear lysates are too low (Figure 24) to detect significant amounts by Co-IP.

4. Discussion

In this study, we set out to examine retinoid metabolism and its effect on BAT function and thermogenesis. We showed the presence of all components necessary for the retinoid pathway in mouse BAT. Furthermore, an *in vitro* model could be established which demonstrated the ability of RA to induce the thermogenic factor *UCP1* and synergistic effects when acting together with beta adrenergic agonists (norepinephrine or FSK). Our data suggest that RAR (alpha/gamma) is involved in the interaction between retinoid and adrenergic signaling but further studies are needed.

4.1. Retinoid machinery in BAT

In the 1980s, BAT was already described as an organ involved in energy balance with potentially high impact regarding control of obesity (HIMMS-HAGEN, 1989), due to its unique ability of energy dissipation. Despite that, mechanistic insight in regulation of thermogenesis in BAT under physiologic and pathologic conditions is still incomplete. Understanding the mechanisms within BAT in regard to non-shivering thermogenesis might help finding therapeutic strategies to decrease obesity (KOZAK & ANUNCIADO-KOZA, 2008). Whereas cold-induced thermogenesis via beta adrenergic signaling is well characterized and considered to be the main contributor to non-shivering thermogenesis (CANNON & NEDERGAARD, 2004), other pathways which might contribute to non-shivering thermogenesis are not fully understood yet. One of the discussed alternative contributors to non-shivering thermogenesis is vitamin A (retinol) and its metabolites, the retinoids (retinoic acid in particular) (BONET et al., 2012).

Studies of ALVAREZ and colleagues (1995) showed that retinoic acid raised *UCP1* mRNA levels in mouse BAT as efficient as norepinephrine, even in the absence of adrenergic signaling (ALVAREZ et al. 1995).

In 2000, BONET and colleagues showed that diet induced vitamin A deficiency in mice had effect on BAT. They described increase in body weight, hypertrophied interscapular BAT (IBAT), and decrease in *UCP1* expression, all *in vivo*, indicating less thermogenic potential of BAT in vitamin A deficient mice *in vivo*. All these

observations could be rescued by the administration of ATRA, suggesting that retinoids have their own physiological role as regulators in BAT (BONET et al., 2000). In our study, we showed that all necessary components for retinoid production, storage and signaling are present in mouse BAT (Figures 7–10), complementing previous findings by other investigators. Further studies will address whether and how different components of retinoid pathway change during pathologic conditions such as obesity.

4.2. Retinoic acid and beta adrenergic pathway and the expression of *UCP1*

Previously, the beta adrenergic pathway has been considered the main effector of adaptive thermogenesis through transcriptional regulation of *UCP1*, even though alternative enhancer regions and response elements have been identified at the *UCP1* promoter (CANNON & NEDERGAARD, 2004). For example, ALVAREZ and colleagues (2000) reported retinoic acid response elements (RARE) with binding sites for RAR and retinoid X receptor (RXR), both transcription factors. Previous studies revealed that both RA and beta agonists can induce *UCP1* in brown adipocytes (ALVAREZ et al., 1995). However, the combined action of the retinoid and beta adrenergic pathway in thermogenesis is not well studied.

A potential link between retinoic acid and beta adrenergic signaling with effects on thermogenesis has been described in a mouse cell line (1B8) (GONZALEZ-BARROSO et al., 2000). GONZALEZ-BARROSO and colleagues (2000) showed that transcription of human *UCP1* gene transfected into the mouse cell line needed both norepinephrine and RA to induce human *UCP1* gene expression. Beta adrenergic stimulation was not sufficient enough to induce UCP1 expression, although they noted that the transfection of the human gene into the rodent cell line could have affected the normal activity of the human gene. HERNANDEZ and colleagues (2011) showed that *UCP1* gene transcription in rat primary brown adipocyte culture was not induced by RA alone but only in combination with norepinephrine, whereas in mice, RA stimulation alone was sufficient. However, the authors mentioned that they also observed insulin-dependent decrease of *UCP1* transcription in rats, which could not be observed in mice, suggesting that insulin would modulate the response of cultured rat adipocytes. Hence, they concluded that further investigation is needed to

understand *UCP1* expression in rats (HERNANDEZ et al., 2011). These somewhat controversial observations highlight the need for more in depth investigations of these two pathways in the context of thermogenesis.

Our observation of amplified UCP1 mRNA expression in BAT cells in presence of RA and beta agonists are consistent with the idea of enhancing interactions between the retinoid acid and beta adrenergic pathway. Furthermore, stimulation with RAR agonist TTNPB (Figure 14) or RAR antagonist AGN (Figure 15) showed that the underlying mechanism could involve RAR. Indeed, the *UCP1* promoter and enhancer regions contain RAREs and cAMP response elements (CREs) relevant for retinoid receptor and CREB binding (ALVAREZ et al., 1995; 2000), however, interaction of RARs with the beta adrenergic pathway, for example by formation of heterodimers with CREB has not been described yet. Our RAR agonist and antagonist data in combination with forskolin strongly suggested an interaction between the RAR and the cAMP-dependent *UCP1* induction. However, our attempts to identify a specific RAR isoform participating in this interaction failed because RAR isoform knockdown resulted in insufficient mRNA repression. Different experimental approaches were chosen including transfection before and after differentiation of BAT cells with no satisfactory results.

4.3. Potential ligands of the retinoid pathway for CREB

As previously shown, we were not able to see interaction between RAR and CREB.

Interestingly, AGGARWAL and colleagues demonstrated that RA was able to activate CREB in human bronchial epithelial cells (AGGARWAL et al., 2006) in RAR/RXR depleted cells, suggesting that the observed effect of an increase of *CREB* expression and the presence of activated CREB upon RA treatment would be RAR/RXR independent in human bronchial epithelial cells. This leaves the question whether a similar effect might also be observed in differentiated BAT cells or if the interaction of retinoic pathway with beta adrenergic pathway is regulated differently from that.

4.4. Future implications

Our results support the hypothesis that retinoids play a physiologically important role in BAT thermogenesis. We showed synergistic enhancement of *UCP1* transcription in the presence of retinoic acid and beta adrenergic agonist. Together, the data suggest interaction between the retinoid and beta adrenergic pathway. However, the interacting partners and the exact mechanism of action require further studies.

The main function of BAT is the acute production of heat in response to cold. It is also known that BAT thermogenesis can counteract obesity at least in animal models. Active BAT has also been identified in humans where it shows a negative correlation with the body mass index. BAT can be activated by cold exposure in humans. However pharmacologic attempts e.g. by ephedrine have failed to promote BAT thermogenesis in humans (CYPESSA et al., 2012) which highlights the current challenges in harnessing BAT activity as a therapeutic target for obesity. The role of retinoids in human BAT physiology is unclear but our *in vivo* and *in vitro* studies suggest that retinoids, especially retinoic acid, might have an impact on BAT function. Shading new light on the role of retinoids in brown fat activation and function in humans could have crucial impact on the discovery of novel tools to manage energy balance and metabolic disease.

5. Summary

Obesity is a risk factor for numerous diseases including cardiovascular disease, hypertension and type 2 diabetes. Obesity has therefore become a serious public health concern which emphasizes the need to study adipose tissue biology in more detail. Two kinds of different adipose tissues are present in mammals – the more prevalent white adipose tissue (WAT) and brown adipose tissue (BAT). While WAT mainly stores energy in the form of triglycerides, BAT can dissipate energy through uncoupled respiration and heat production. This process is mediated through uncoupling protein 1 (UCP1) and is called non-shivering thermogenesis. Recently, promoting BAT function has been suggested to counteract obesity. Non-shivering thermogenesis in BAT is typically activated via beta adrenergic stimulation. Nevertheless, alternative pathways have been discussed including the vitamin A metabolites: the retinoids. Retinoids have recently been linked to the regulation of energy balance and thermogenesis. However, the mechanisms still remain unclear. Hence, understanding the role of retinoids in BAT biology and their potential interaction with the beta adrenergic pathway might help to develop novel therapeutic strategies to fight the obesity epidemic. Therefore, it is the goal of this study to determine the effects of retinoid metabolism in BAT function and thermogenesis.

Retinoid formation in the tissue is tightly regulated by a network of enzymes and binding proteins. Thus, we set out to study if components of the retinoid pathway are expressed in BAT. Gene expression analysis of BAT isolated from C57BL/6J mice revealed significant expression levels of major regulatory enzymes, binding proteins and retinoid target genes. To test the effects of retinoids in brown adipocyte thermogenesis we applied immortalized tissue BAT precursor cells from C57BL/6J mice. These precursor cells were then differentiated to mature brown adipocytes followed by stimulation with retinoids and beta receptor agonists. Both, retinoic acid and beta receptor agonists dramatically induced expression of the major thermogenic factor UCP1 in brown adipocyte. Of note, the combination of retinoic acid and beta agonists amplified the effects on UCP1 induction, suggesting synergistic effects. This hypothesis was further strengthened by the observation that selective retinoid receptor antagonists decreased beta agonist mediated UCP1 induction, whereas inhibition of beta adrenergic signaling reduced retinoid mediated UCP1 induction. We

then sought to investigate potential interactions between distinct transcription factors of these two pathways. Hence we performed co-immunoprecipitation assays for the key transcription factors cAMP response element-binding (CREB) and retinoic acid receptor (RAR). However, so far we have not been able to detect any interactions yet, probably due to the very low abundance of RAR in brown adipocytes. In summary, this study has demonstrated that all major components of the retinoid pathway are present in BAT and therefore suggest BAT as a relevant tissue of retinoid action. Retinoic acid induces thermogenic gene expression in brown adipocytes and acts synergistically with beta receptor agonists. The present data suggest that these two pathways can enhance each other's effects on BAT activation which could have implications for the development of novel therapeutic approaches to augment BAT function.

6. Zusammenfassung

Adipositas ist ein Risikofaktor für zahlreiche Erkrankungen zu denen auch Herz-Kreislauferkrankungen und Typ 2 Diabetes gehören. Da Übergewicht aus diesem Grund ein ernsthaftes volksgesundheitliches Problem darstellt, ist es umso wichtiger das Fettgewebe zu studieren und die Vorgänge darin näher zu untersuchen. Bei Säugetieren unterscheidet man zwei unterschiedliche Arten von Fettgewebe – braunes (BAT) und weißes Fettgewebe (WAT). Während weißes Fett hauptsächlich Energie in Form von Triglyceriden speichert, verbrennt braunes Fett Energie durch Entkopplung der Atmungskette von der ATP Synthese und gibt diese in Form von Wärme ab. Dieses Phänomen wird Thermogenese genannt, vermittelt durch das Protein „Uncoupling Protein 1“ (UCP1), welches für den Entkopplungsvorgang verantwortlich ist. Aufgrund dieser Eigenschaft stellt sich die Frage, ob Thermogenese genutzt werden kann um Fettleibigkeit zu bekämpfen.

Thermogenese in braunen Adipozyten wird hauptsächlich durch adrenerge Stimulation aktiviert. Allerdings werden auch andere Signalwege diskutiert, die an der Aktivierung der Thermogenese beteiligt sein könnten, darunter auch die Rolle der Retinoide. Seit kurzem werden Retinoide mit der Regulierung von Energiebilanz und Thermogenese in Zusammenhang gebracht, die genauen Mechanismen sind jedoch weiterhin unklar. Für die Bekämpfung von Adipositas könnte es von großer Bedeutung sein die Rolle der Retinoide in braunem Fettgewebe und deren Zusammenspiel mit dem beta-adrenergen Signalweg zu verstehen, um neue Therapieansätze zur Bekämpfung der Adipositas zu finden. Ziel dieser Studie ist es also, die Wirkung des Retinoidstoffwechsels in braunem Fettgewebe zu untersuchen.

Die Bildung von Retinoiden im Gewebe ist streng geregelt. Daher haben wir untersucht, ob alle Komponenten für die Bildung und Regulierung von Retinoiden in braunem Fett vorhanden sind. Genexpressionsanalysen in braunem Fettgewebe von C57BL/6J Mäusen ergab, dass alle wichtigen Komponenten zur Bildung und Regulierung von Retinoiden im braunen Fett vorhanden sind. Um die Effekte von Retinoiden in braunem Fettgewebe beobachten zu können, haben wir ein *in-vitro* Modell aus immortalisierten Vorläuferzellen der braunen Adipozyten etabliert. Zunächst wurden diese Zellen zu reifen braunen Adipozyten differenziert und anschließend mit Retinoiden und Agonisten des beta-adrenergen Signalwegs

stimuliert. Die Stimulation mit Retinsäure oder beta-adrenergen Agonisten (Noradrenalin oder Forskolin) führte jeweils zu einer sehr hohen Expression von *UCP1*. Beachtenswert war, dass eine Co-Stimulation mit Retinsäure und einem beta adrenergen Agonisten zu einer derart verstärkten Expression von *UCP1* führte, dass eine synergetische Verstärkung beider Signalwege vermutet wurde. Diese Beobachtung konnte durch weitere Experimente untermauert werden, in denen nachgewiesen werden konnte, dass bei Stimulierung mit Retinsäure und gleichzeitiger Hemmung des beta adrenergen Signalwegs durch einen CREB Hemmer eine niedrigere Expression von *UCP1* beobachtet werden konnte, im Vergleich zur *UCP1* Expression bei ausschließlicher Stimulierung mit Retinsäure. Ähnliches konnte auch umgekehrt bei Hemmung des Retinoidsignalweges durch einen RAR Antagonisten gleichzeitiger Stimulation mit beta adrenergen Agonisten erreicht werden. Anschließend versuchten wir mögliche Interaktion der Komponenten beider Signalwege zu identifizieren. Dazu wurden „knockdown“ Experimente und eine Proteinkomplex Immunpräzipitation durchgeführt um einen möglichen Zusammenhang zwischen den Transkriptionsfaktoren RAR und CREB zu untersuchen. Bisher konnten jedoch keine Interaktionen festgestellt werden.

Zusammenfassend zeigten unsere Untersuchungen, dass alle wichtigen Komponenten zur Bildung und Regulierung von Retinoiden in braunem Fettgewebe vorhanden waren. Ebenso konnte eine synergistische Verstärkung der UCP1 Expression bei gleichzeitiger Stimulation mit Retinsäure und beta adrenergen Agonisten (Noradrenalin oder Forskolin) beobachtet werden. Dies weist darauf hin, dass beide Signalwege sich gegenseitig unterstützen könnten und die Expression von UCP1 verstärken, was für die Entwicklung neuer Therapieansätze zur Behandlung von Adipositas von Bedeutung sein könnte.

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8. Declaration

Herewith I declare that the thesis

“Retinoid Metabolism in White versus Brown Fat Function”

Has been conducted entirely on my own without any impermissible help. All used references and sources of information are quoted and listed.

This master thesis has not been previously submitted to any university.

_____

Tan An Dang

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