Retinaldehyde in Energy Balance

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submitted by
Patrick O'Brien
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Brigham And Women’s Hospital, Harvard Medical School, Boston,
Massachusetts, USA durchgeführt.

External Supervisor: Associate Prof. Jorge Plutzky, MD
Internal Supervisor: Ao. Univ.-Prof. Dr.med.vet. Alois Strasser
Reviewer: Univ.-Prof. Dr.med. Elena Pohl
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1 Introduction

1.1 Obesity

Adiposity is a main risk factor for many illnesses. Obesity has been associated with respiratory defects, cancer, reproductive abnormalities, arthritis, psychological syndromes, gout, and especially metabolic and cardiovascular diseases like diabetes, hypertension, non-alcoholic fatty liver disease, coronary artery disease and stroke (HASLAM & JAMES, 2005). These diseases are among the leading causes for worldwide morbidity and ultimately mortality. Currently it is estimated that 1.1 billion adults are overweight worldwide, of which more than 300 million are obese (JAMES et al., 2004). Overweight is characterised by a body mass index (kg/m²) of 25.0 or higher and obesity has a lower cut-off BMI of 30.0. Nature has provided mammals with the possibility to store excess energy in the form of lipids in adipose tissue to provide calories or energy in times when less food is available. Two forms of adipose tissue exist. White adipose tissue is located subcutaneously, visceral, intramuscular and around organs. The main functions of fat tissue are insulation, cushioning and energy storage. Another form exists, brown adipose tissue. It has a high mitochondrial content and is responsible for non-shivering thermogenesis by uncoupling the mitochondria’s membrane potential (CANNON & NEDERGAARD, 2004).

When energy needs to be released, triglycerides stored in adipocytes are hydrolysed to free fatty acids (FFAs) by action of lipases and released into the blood to reach bound to albumin - tissues in demand (KRAEMER & SHEN, 2002). Skeletal muscle is the primary site of fatty acid oxidation. Two main types of muscle fibres exist. White fast twitch fibres rely on glycolysis, generate force quickly, but can’t sustain this energy production for a long duration. In contrast, red slow twitch fibres are rich in myoglobin and mitochondria equipped for endurance tasks by relying on fatty acid oxidation. Skeletal muscles in mammals are made up of the two types of muscle fibres and can be divided into groups whether they are more fast, slow or mixed type. Hence some muscles rely more on glycolysis and others more on fatty acid oxidation.

Uptake of fatty acids into cells including myocytes is facilitated by numerous enzymes including fatty acid translocase (FAT/CD36), plasma-membrane associated fatty acid binding protein (FABP) and several fatty acid transporter proteins (FATPs) (LUIKEN et al., 1999). For energy production FFA are being oxidised. Therefore they need to be activated and transported through the mitochondrial membrane. Acyl-CoA
synthetase adds one molecule of Coenzyme-A to a fatty acid by using up two molecules of ATP. The resulting Acyl-CoA can then be transported through the outer mitochondrial plasma membrane by the action of carnitine palmitoyltransferase I (CPT1). This enzyme exchanges Coenzyme-A with a molecule of carnitine. The resulting acylcarnitine is then transported into the mitochondrion by Carnitine-acylcarnitine translocase and the carnitine is exchanged again for Coenzyme-A by carnitine palmitoyltransferase II (CPT2). Inside the mitochondrion fatty acyl-CoA is then broken down by several enzymes into acetyl-CoA molecules, a process called β-oxidation. The resulting acetyl-CoA molecules can then fuel the Krebs cycle and finally the oxidative phosphorylation which results in ATP generation. Very long fatty acids (>22 C-atoms) can be oxidised in peroxisomes to form shorter carbon chains which can then be shuttled into mitochondrial β-oxidation. Acyl-CoA oxidase (ACOX) initiates this process by oxidising acyl-CoA which generates a shorter acyl-CoA and acetyl-CoA. ACOX is regarded as the controlling step into peroxisomal oxidation (WANDERS & WATERHAM, 2006).

Adipose tissue not only consists of fat-storing adipocytes, but also connective tissue, nerve tissue, stromal-vascular cells and immune cells. These together can secrete and respond to hormones, cytokines and chemokines and therefore adipose tissue has now been accepted as an endocrine organ (KERSHAW & FLIER, 2004). Excess adipose tissue especially in the visceral compartment can act upon other organs like the liver, pancreas, intestine and the vascular system. The association of obesity as the main risk factor with other morbidities like insulin resistance, hyperglycaemia, dyslipidaemia and hypertension becomes clearer in this context. Obesity and its co-morbidities are now called the ‘metabolic syndrome’ characterised by abdominal obesity, dyslipidaemia, hypertension, prothrombotic states, insulin resistance and inflammation (GRUNDY et al., 2004).

Understanding the pathophysiological processes underlying obesity and discovering novel therapeutic approaches – either dietary or pharmaceutical – could help fight the rising obesity epidemic we are facing. Recent findings (ZIOUZENKOVA et al., 2007) have suggested a role for Vitamin A and its derivatives, the retinoids, in the regulation of obesity, insulin resistance and glucose homeostasis, which will be discussed in the following chapters.
1.2 Physiology of Vitamin A

Vitamin A is an essential micronutrient and its intestinal uptake is mandatory. This was first described by McCollum & Davis in 1913. Vitamin A is abundant in animal products like liver, butter, eggs and milk, but also in coloured vegetables and fruit like carrots, sweet potatoes, spinach and mango. Vitamin A can be taken up either as β-carotene (fruit & vegetable) or as retinyl esters from animal products. These are hydrolysed to retinol in the intestine and enterocytes, esterified with fatty acids and packed into chylomicrons. The retinyl esters then reach the liver and are again hydrolysed and re-esterified in the hepatocytes. The final storage are hepatic stellate cells, also called Ito cells, lipid droplet filled cells representing 5-8% of the total liver cells and storing 80% of all the Vitamin A in the body (GEERTS, 2001). The liver secretes retinol into the blood bound to retinol binding proteins (RBP) in combination with transthyretin in order to reach target organs (Fig. 1).

Retinol uptake at the target organ is either receptor-dependent (ROSS, 1993) or the retinol breaks free from the RBP and diffuses into the cell through the plasma membrane. Intracellular retinol is either bound to cellular retinoid binding proteins I or
Introduction

CRBP I & II or metabolised to its biologically active forms the so-called retinoids which can also be stored bound to cytoplasmic proteins (e.g. retinoic acid binds to cellular retinoic acid binding protein (CRABP)). If retinol is not stored it can be oxidised by alcohol dehydrogenases (Adh1, 3, 4) or retinol dehydrogenases (Rdh1 & 10) to form retinaldehyde (Rald). This step is reversible by the action of short-chain dehydrogenase/reductase enzymes (SDR) (DUESTER, 2000). Rald can be irreversibly oxidised by the family of aldehyde dehydrogenases (ALDH1a1, a2 and a3), also known as retinaldehyde dehydrogenases (Raldh1, 2 and 3). The resulting retinoic acid (RA) exists in several isomers, namely all-trans-retinoic acid (ATRA), 13-cis-retinoic acid (13cisRA) and 9-cis-retinoic acid (9cisRA). The conversion of Rald to RA is considered as the rate-limiting step of retinoid metabolism. RA can regulate gene transcription by entering the nucleus and binding to nuclear receptors, namely the retinoic acid receptors (RARα, β, γ) and retinoid X receptors (RXRα, β, γ). ATRA and 13cisRA can bind to RAR and RXR, whereas 9cisRA solely binds RXR in vitro (KUMAR et al., 2011). RAR and RXR can either form heterodimers or RXR can form homodimers to facilitate transcriptional events. RXR can also heterodimerise with several other nuclear factors including the thyroid hormone receptor (TR), Vitamin D receptor (VDR), Liver X receptor (LXR) and peroxisome-proliferator activated receptor (PPAR) (CHAWLA et al., 2001) (see Fig. 2E). The homo- and heterodimers are bound to the respective consensus sequence in the promoter region of a gene and kept quiescent by a co-repressor complex at their C-terminal AF2 domain (activation function 2). The structure of nuclear receptors is highly conserved and consists of an N-terminal activation function domain (AF1), the DNA binding domain, the ligand binding domain and the aforementioned AF2 domain (see Fig. 2A). Nuclear factors bind to conserved response elements e.g. “AGGTCA”. Response elements can occur as direct, inverted or everted repeats with ‘n’ nucleotides in-between (see Fig. 2B). The response elements are to a degree variable and hence selective for different nuclear factors. For example PPARs bind to peroxisome proliferator responsive elements (PPRE) or RAR binds to RA responsive elements (RARE). The nuclear factor dimers sit on the DNA and the binding of a ligand at the ligand binding domain triggers a conformational change and the co-repressor complex is released and a co-activator complex is able to bind. This leads to a local change of the chromatin structure and the transcriptional machinery is able to start gene expression (Fig. 2C+D) (SHULMAN & MANGELSDORF, 2005).
The numerous heterodimerisation partners of RXR illustrate how diverse the actions of RA are. Retinoids functions in many different tissues and plays a vital role in in adult physiology including vision, reproduction, immune function, skin health, haematopoiesis, bone formation, cellular differentiation and metabolism (BLOMHOFF & BLOMHOFF, 2006; ZIOUZENKOVA & PLUTZKY, 2008). The physiological importance of retinoids highlights how dysregulation of this pathway can be implicated in pathological conditions like infectious disease (STEPHENSEN, 2001), skin diseases (ORFANOS et al., 1997), cancer (NILES, 2004), metabolic diseases (YANG et al., 2005) and embryonic development.

Null mutants of Adh and Raldh isoforms (NIEDERREITHER et al., 2002) showed that embryonic development strongly relies on RA and its generation through the distinct enzymes. Different mouse models have demonstrated that lack of RA during the embryonic phase either leads to severe developmental defects or abortion of the embryo. While genetic deletion of Raldh2 and 3 is embryonically lethal, Raldh1
homozygous knockout mice are viable without obvious developmental defects. Therefore these animals provide a unique genetic tool to study retinoid metabolism \textit{in vivo}, in particular the function of the RA precursor Rald.

Rald plays a very important role in vision, namely the rod cells of the eye. The 11-\textit{cis}-configuration of Rald poses as a co-factor for opsin, a G-protein coupled receptor. Opsin together with its co-factor Rald is called Rhodopsin. Photons bleach 11-\textit{cis}-Retinal to the all-\textit{trans} isomer which induces a conformational change in opsin ultimately triggering a second messenger cascade leading to a nerve signal and enables vertebrates to detect light (RIPPS, 2010). Only recently a novel biological function of Rald outside the eye has been discovered by the Plutzky lab (ZIOUZENKOVA et al., 2007).

1.3 Retinaldehyde in energy metabolism

Besides the aforementioned role of Rald in vision, no other biologic function had been defined for this specific retinoid metabolite. In 2007, ZIOUZENKOVA et al. established a new role for Raldh1 and Rald in adipogenesis and obesity, linking retinoids to metabolism. The Plutzky lab showed that Rald is present in rodent adipose tissue and that mice lacking Raldh1 had higher endogenous tissue concentrations of Rald. In vitro, Rald blocked adipogenesis through inhibition of PPAR\textgamma/RXR transactivation, the main adipogenic transcription factors. Notably, Raldh1 deficient mice were completely protected against diet-induced obesity (Fig. 3A+B) and consequently had improved insulin sensitivity and glucose homeostasis (Fig. 3D). Furthermore these mice had a significantly lower respiratory quotient, a measure of increased fatty acid oxidation (Fig. 3C). In conclusion the Plutzky lab showed that Rald is not just an intermediate for RA, but a truly active metabolite \textit{in vivo} and \textit{in vitro} with distinct functions and discrete effects on metabolism.
1.4 Project overview & hypothesis

Based on the observation by ZIOUZENKOVA et al. (2007) that Raldh1 deficient mice show decreased respiratory quotient and are protected against diet-induced obesity, we aimed at finding a molecular mechanism that could underlie this phenotype. Muscle tissue accounts for ~35% of body mass and for a fifth of the daily resting energy expenditure (JANSSEN et al., 2000). Adipose tissue, liver and skeletal muscle are the main organs controlling the fatty acid homeostasis. While fatty acids are mainly stored in adipose tissue in form of triglycerides, liver and even more important skeletal muscle are the sites of fatty acid oxidation. Based on the findings that Raldh1 deficient mice on high-fat diet have an increased metabolic rate and lower respiratory quotient, we hypothesised that enhanced fatty acid oxidation in skeletal muscle contributes to protection against diet induced obesity. It is therefore the aim of this study to investigate the role of Rald and Raldh1 in the regulation of skeletal muscle fatty oxidation.
2 Materials and Methods

2.1 Tissues and cell culture

2.1.1 Raldh1-/- mouse model & genotyping

Wildtype (WT) and Raldh1-/- knockout (FAN et al., 2003) C57BL/6 mice (B6.129-Aldh1a1tm1Gdu/J) were kindly provided by Gregg Duester, Sanford-Burnham Medical Research Institute, La Jolla, USA and bred according to IACUC standards. Experimental animals were age and weight matched and only female mice were used.

In the Raldh1-/- knockout mice exon 11 of the gene is exchanged by a neomycin resistance cassette (NeoR). DNA was extracted from tail tips by incubating for ~18h at 55°C in 100 µl DirectPCR Lysis reagent (Viagen, Los Angeles, USA) + 1 µL Proteinase K (Qiagen, Valencia, USA). The next day the lysed tail tip was centrifuged at 3000 RPM for 3 min and genomic DNA was isolated and used for PCR. The knockout was confirmed by PCR for Raldh1 and neomycin by performing PCR with the primers and protocol shown in Table 1. PCR products were then visualised on a 1.5% agarose gel including 0.1 µl/ml Ethidium bromide with a UVP BioDoc-It™ System (Upland, California, USA).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer: 5’-3’</th>
<th>Reverse primer: 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raldh1</td>
<td>TGGCATTTGCTCCACAGATA</td>
<td>CACAGTGATGGCCTTATCCA</td>
</tr>
<tr>
<td>NeoR</td>
<td>CTCCTGCCGAGAAAGTATCC</td>
<td>AATATCACGGGTTAGCCAACG</td>
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</table>

<table>
<thead>
<tr>
<th>Cycle 1: 1x</th>
<th>Cycle 2: 30x</th>
<th>Cycle 3: 1x</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C; 02:00</td>
<td>95°C; 00:30</td>
<td>72°C; 05:00</td>
</tr>
<tr>
<td>57°C; 00:40</td>
<td>72°C; 01:00</td>
<td></td>
</tr>
</tbody>
</table>

2.1.2 Murine gastrocnemius and soleus muscle

Mice were anesthetised by intraperitoneal injection of 100 µl Tribromoethanol (12.5 mg/ml) and then killed by cervical dislocation. The skin of the hind leg was removed and the soleus and gastrocnemius muscle were dissected and immediately
snap-frozen in liquid nitrogen to avoid any RNA or protein degradation. The soleus muscle was removed by cutting the Achilles tendon, pulling the calf forward to see and cut the insertion of the soleus on the tibia. The gastrocnemius muscle was removed subsequently. Tissues were then homogenised as described in 2.3.

### 2.1.3 C2C12 myoblast cell line

Murine C2C12 myoblasts were obtained from the American Type Culture Collection. This subclone (BLAU et al., 1985) obtained from dystrophic C3H mice established by Yaffe & Saxel in 1977 feature the main characteristics of muscle fibres once the cells are differentiated (i.e. multinucleated, fused and elongated cells; high expression of myogenic markers).

Cells were grown in high glucose Dulbecco’s modified Eagle’s medium (Catalogue No. 30-2002), containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate, as well as 10% fetal bovine serum and 1% Penicillin and Streptomycin (Pen/Strep). Cells were split at ~80% confluence. To differentiate the myoblasts into multinucleated and fused myotubes 100% confluent cells were serum starved (High glucose DMEM + 2% horse serum + 1% Pen/Strep) and differentiated for 4 days.

### 2.2 Giemsa staining

For the staining of cells with Giemsa a fresh 5% solution of Giemsa Stain (EMD chemicals, Gibbstown, US) in ddH$_2$O was prepared. Cells were then washed with cold PBS, fixed for 20s in Methanol and then stained for 20-30min. Finally cells were rinsed with tap water and images were taken using a DIGITAL CAMERA DXM1200F attached to an OPTIPHOT-2 light microscope (both Nikon Inc., Melville, NY, USA).

### 2.3 Tissue harvest and homogenisation

To isolate RNA from cultured cells 500 µl Trizol (Invitrogen, Carlsbad, USA) were added to each well (6- or 12 well plate), scraped with the end of a P1000 tip and RNA was extracted as explained in 2.4.

Tissues from in vivo experiments were snap-frozen in liquid nitrogen and stored at -80°C. For the homogenisation a steal bead was added to a 2ml tube and precoolied on
dry ice. Tissues were transferred to tubes, 200 µl of Trizol added. These were placed in precooled (-20°C) adapter sets for the QIAGEN TissueLyser II and samples were homogenised for 3 x 30s bouts at 30Hz for full tissue breakup. Before subsequent RNA extraction 300 µl Trizol were added (500 µl total) and samples were centrifuged at 10000RPM for 10min to remove tissue and cell debris.

2.4 RNA extraction

100 µl chloroform and 120 µl RNAse-free water were added to 500 µl Trizol, vortexed thoroughly for 15s and incubated at room-temperature for 10min. This was transferred to Phase Lock Gel Heavy 2 ml tubes (5Prime, Hamburg, Germany) and phases were separated for 10min at 13000RPM. 400 µl of the aqueous phase were transferred to a new tube and 1.5 µl GlycoBlue (Ambion Inc., Austin, USA) and 280 µl cold Isopropanol were added to precipitate RNA for 30min at 4°C. After 30min centrifugation at 13000RPM and 4°C the supernatant was removed and the RNA pellet washed twice with 1ml 75% ethanol (5min centrifugation at 10000RPM and 4°C). Finally Ethanol was removed and the pellet dried for approximately 10min until residual Ethanol had evaporated. The remaining RNA was resuspended in 12 µl RNAse-free water and RNA yield was measured on a NanoDrop2000 (Thermo Fisher Scientific, Wilmington, USA).

2.5 Reverse transcription

In order to digest residual genomic DNA before transcribing RNA to cDNA 1 µl of DNase reaction Buffer was added to 1ug of RNA in 9 µl ddH₂O. 1 µl of DNase was added and incubated at room temperature for 15min to digest any DNA contamination in the samples. 1 µl of EDTA was added and tubes were placed on 65°C to inactivate further DNase activity.

Per sample 2.0 µl 10xRT Buffer, 0.8 µl 25x dNTP Mix s(100 mM), 1 µl of MultiScribe™ Reverse Transcriptase, 1 µl RNase Inhibitor, 2.2 µl Nuclease-free H₂O were added and RNA was reverse transcribed using the following protocol on a PTC-200 thermocycler (MJ RESEARCH, Waltham, USA):
2 Materials and Methods

Table 2 Reverse transcription protocol.

<table>
<thead>
<tr>
<th></th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>25 °C</td>
<td>37 °C</td>
<td>85 °C</td>
<td>4 °C</td>
</tr>
<tr>
<td>Time</td>
<td>10 min</td>
<td>120 min</td>
<td>5 min</td>
<td>∞</td>
</tr>
</tbody>
</table>

2.6 Quantitative Real-time PCR

Samples were prepared in duplicates (30 µl BIO-RAD iQ™ SYBR® Green Supermix (Hercules, CA, USA), 24 µl ddH2O, 2 µl each of forward + reverse primers (10 µM concentration), 2 µl cDNA sample) and 2x25 µl were analysed on a BIO-RAD MyiQ™ or Applied Biosystems OneStepPlus™ Real-time PCR system (Carlsbad, CA, USA) with the following protocol:

Table 3 Real-time PCR protocol.

<table>
<thead>
<tr>
<th>Cycle 1: 1x</th>
<th>Cycle 2: 40x</th>
<th>Cycle 3: 1x</th>
<th>Cycle 4: Melting curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C; 03:00</td>
<td>95°C; 00:15</td>
<td>95°C; 01:00</td>
<td>55°C – 95°C</td>
</tr>
<tr>
<td>60°C; 01:00</td>
<td></td>
<td></td>
<td>0.5°C increments; 00:10</td>
</tr>
</tbody>
</table>

2.7 Primer sequences

Primers were obtained from IDT (Coralville, USA) and reconstituted with DEPC treated water. For qPCR assays primers were diluted to a stock concentration of 10 µM and used at a final concentration of 170nM.

Table 4 Primer sequences used for gene expression analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer: 5’-3’</th>
<th>Reverse primer: 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>36B4 (reference gene)</td>
<td>CAACCCAGCTCTGGAGAAAC</td>
<td>GAGGTCCTCTCTTGTTGGAACA</td>
</tr>
<tr>
<td>ACOX</td>
<td>GACCGTCTCGACTGACATCAT</td>
<td>CCAGTCAGTCGGATGAGTCTTTA</td>
</tr>
<tr>
<td>CPT1β</td>
<td>CGAGGATTCTCTGGAACTGC</td>
<td>GGTCGCTTCTTTCAAGGTCTGT</td>
</tr>
<tr>
<td>MCK</td>
<td>GCAAGCCACCCCAAGTTTTGA</td>
<td>ACCTGTGGCCGGCTTCTT</td>
</tr>
<tr>
<td>MyoD</td>
<td>AGCATAGTGAGCAGCATCTC</td>
<td>CTTGGGTCTCTGTTTCTGTGT</td>
</tr>
<tr>
<td>myogenin</td>
<td>GGAAGTCTCTGTGGGTTGAC</td>
<td>GCGCAGATCTCCACTTTAG</td>
</tr>
<tr>
<td>Raldh1</td>
<td>CCATGGATGCTTCAGAGAG</td>
<td>ACTTCCCACCATTGACTGC</td>
</tr>
<tr>
<td>RARα</td>
<td>CCAGCTTCAGTCAGTGTTTA</td>
<td>TCCTTGTTGCTGATGTT</td>
</tr>
<tr>
<td>RARβ</td>
<td>GCATTGATCCAGGATTCTCCA</td>
<td>ACAGATCTCCGAGCATCAG</td>
</tr>
</tbody>
</table>
### Materials and Methods

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RARγ</td>
<td>CCATGCTTTTGATGCAATGACA</td>
<td>TTCTGAATGCTGGGTCTGAAG</td>
</tr>
<tr>
<td>RXRα</td>
<td>CGGAACACGGCTCACAGT</td>
<td>AGCTCGGTCTGTCATCTG</td>
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<tr>
<td>RXRβ</td>
<td>CAAAGGGCTCTGATGCAAATCT</td>
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<tr>
<td>RXRγ</td>
<td>GCCACCCTGGAGGCCTATA</td>
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<tr>
<td>UCP2</td>
<td>TGTAGCCAGCCCTACAGATG</td>
<td>GCTCTGGTATCTCCGACCAC</td>
</tr>
<tr>
<td>UCP3</td>
<td>CCACGGCCTCTACAAAGGA</td>
<td>AGATTCCGCAGTACCTGGA</td>
</tr>
</tbody>
</table>

### 2.8 Western blotting

For the polyacrylamide gel electrophoresis on 4-12% gradient gels (ready-made) we used 14µg of protein per sample in NuPAGE® LDS Sample Preparation Buffer and Reducing agent and separated proteins in a XCell SureLock™ chamber filled with NuPAGE® MOPS buffer. 200V were applied for a total of 50 minutes (all by Invitrogen, Carlsbad, USA).

Proteins were then blotted in a semi-dry method onto a 0.45micron Polyscreen PVDF Membrane by applying 10V for 5min followed by 45min at 20V. PVDF membranes were then blocked in 5% dry milk in TBST (0.1% Tween 20) for 1 hour, rinsed shortly three times in TBST and then washed for three times for 5 minutes. The primary antibody was prepared in TBST (Raldh1 1:2000, GAPDH 1:1000) containing 0.1% Sodium azide for reuse. The next morning the antibody was removed and membranes rinsed and washed as before, blocked for 10min and then incubated with the secondary antibody in 5% dry milk in TBST (anti-rabbit 1:2500) for 45min. After 3 short rinses and three 10min washes in TBST membranes were incubated in Western Lightning® Plus-ECL (PerkinElmer, Waltham, USA) for 2min to visualise the horse-radish peroxidase activity of secondary antibody on HyBlot CL™ Autoradiography films.

### 2.9 Statistical analysis

Data analysis and statistics were performed using Excel included in Microsoft Office Pro 2010, Version 14.0.4763.1000. Data are given as arithmetic mean ± standard error of means (SEM). Significancies were tested using the inbuilt two-sided T-Test of Excel. A P-value of 0.05 or less was considered statistically significant.
2.10 Ethical approval

The animal protocol was reviewed and approved by the HMA Standing Committee on Animals and The Institutional Animal Care and Use Committee (IACUC).
3 Results

3.1 Enzymatic machinery of retinol metabolism is present in muscle

First we tested whether the enzymatic machinery regulating Rald and RA is present in skeletal muscle. We therefore isolated soleus and gastrocnemius muscle from C57BL/6 WT mice and analysed gene and protein expression of Adh1 and Raldh the main enzymes controlling retinoid metabolism. Fig. 4 shows that Raldh1 is the predominant isoform expressed in both gastrocnemius and soleus muscle.

Fig. 4 Expression of alcohol dehydrogenase and Raldh isoforms in skeletal muscle. a) mRNA expression of Adh1 and Raldh1, 2, 3 was analysed in gastrocnemius and soleus muscle of C57Bl/6 WT mice. n = 4 b) Western blot analysis for Raldh1 in gastrocnemius (Gas) and soleus (Sol) muscle. n = 2.

3.2 Genotyping of experimental animals

Raldh1-/- mice, an established model of high endogenous Rald concentrations, have no obvious developmental defects. Raldh1-/- mice were generated by deletion of exon 11 of the Aldh1a1 gene and replacement by a Neomycin resistance cassette yielding an unfunctional protein (FAN et al., 2003). Genomic PCR using DNA obtained from mice tails resulted in a 354bp PCR product of exon 11 of the Aldh1a1 gene only in WT and not in Raldh1-/- mice. On the other side genomic PCR for the Neomycin cassette resulted in a 362bp PCR product in Raldh1-/- but not in WT DNA. The genotyping of the 20 animals used for the following experiments can be seen in Fig. 5. Animals are separated whether they were fed or fasted for 13h before harvesting the tissues for experiment 3.3.
Fig. 5 Genotyping of WT and Raldh1⁻/⁻ mice. Shown are PCR products of exon 11 of the Raldh1 gene (*Aldh1a1*) and the Neomycin resistance cassette on a 1.5% agarose gel and visualised with Ethidium Bromide on an UVP BioDoc-It™ System.

### 3.3 Raldh1 deficiency induces β-oxidation in skeletal muscle

In order to examine whether Raldh1 deficiency affects β-oxidation in muscle *in vivo* we fed WT (*n* = 8) and Raldh1⁻/⁻ mice (*n* = 12) a normal chow diet for 13 weeks. Half of each group were then fasted overnight for 13h. Soleus and gastrocnemius muscle were dissected the next morning. After tissue homogenisation and RNA extraction we performed reverse transcription. We then analysed the gene expression of ACOX (initial enzyme of peroxisomal β-oxidation), CPT1β or muscle type (shuttle for fatty acids) and Uncoupling proteins 2 and 3 (UCP-2 & 3) and normalised them to the reference gene 36B4.

As shown in Fig. 6 β-oxidation markers are significantly higher expressed (filled bars) in soleus muscle which predominantly consists of red fibres and therefore relies mainly on oxidative metabolism compared to gastrocnemius with a mix of white and red fibres. Especially CPT1β (see Fig. 6b) demonstrates this difference with a twofold increase of CPT1β in oxidative soleus vs. gastrocnemius muscle. Comparing the gene expressions between WT and Raldh1⁻/⁻ in the fed state (filled bars) none of the genes appeared to show a significant difference. However, when mice were overnight fasted (empty bars) expression of oxidative genes generally increased in both genotypes with a much more pronounced upregulation in the Raldh1 deficient muscle. In fasted mice Raldh1 deficiency increased ACOX by ~40% in both gastrocnemius and soleus compared
to WT animals. UCP-2 expression was about twofold upregulated in fasted knockout gastrocnemius and soleus, respectively. The most striking difference was seen for UCP-3 mRNA expression which was ~2.5 fold (gastrocnemius) and about twofold (soleus) higher in Raldh1 deficient compared to WT muscle. Taken together these data indicate the Raldh1 deficiency influences expression of critical mediators of fatty acid oxidation in skeletal muscle.

Fig. 6 Gene expression of key β-oxidation markers in skeletal muscle tissue before and after fasting for 13h (full bar = fed, empty bar = fasted). WT = Wildtype, KO = Raldh1-/-. Gastroc = M. gastrocnemius, Soleus = M. Soleus. *P < 0.05, ** P<0.01. Sample size: n=4 for random fed and n=6 for fasted mice for both genotypes.
3.4 Characterisation of the C2C12 myoblasts cell line

To test whether Rald could account for increased expression of fatty acid oxidation markers observed in Raldh1 deficient mice we went on to study Rald effects on fatty acid oxidation *in vitro* using the C2C12 myoblast cell line.

First we established myotube formation of C2C12 myoblasts. Therefore cells were grown to confluence and differentiation was initiated by changing the medium to differentiation medium (see 2.1.3). Cells were fixed and Giemsa stained to visualise myoblast formation. Alignment of cells was first seen on day 1. Fusion to elongated and multinucleated myotubes followed in the subsequent days and reached a maximum on days 4 – 6 (Fig. 7).

Fig. 7 Giemsa staining of C2C12 myoblast/myotubes at different time points. Cells were grown to confluence in High glucose DMEM + 10% FBS and then differentiated to myotubes by withdrawing serum (High glucose DMEM + 2% horse serum). a) day 0, b) day 2, c) day 4, d) day 6. Magnification = 40x, Bar indicates 200 µm.
To characterise the gene expression profile of differentiating C2C12 cells we isolated RNA on days 0, 2, 4 and 6 during differentiation and analysed the gene expression of various myogenic markers, as well as β-oxidation and retinoid metabolism related genes (Fig. 8, Fig. 9 and Fig. 10).

By differentiating the myoblasts to myotubes we observed a strong induction of key myogenic markers such as muscle creatine kinase (MCK), myogenin and MyoD. MCK is a protein necessary for the function of muscle tissue and was induced 113 fold on day four when myotubes were fully differentiated. myogenin and MyoD are transcription factors belonging to the group of “Myogenic regulatory factors” or MRFs (PERRY & RUDNICK, 2000). These basic helix-loop-helix transcription factors are key inducers of myogenic transformation by binding to E-Boxes of many muscle specific genes (PERRY & RUDNICK, 2000). Similar to MCK induction of myogenin was highest on day 4 with a fold change of ~91 compared to day 0. MyoD was only induced 2 fold during differentiation which could be due to the fact that MyoD is more important for myogenic determination (i.e. somitic cell → myoblast) rather than terminal differentiation (i.e. myoblast → myotubes); the latter is mainly driven by the transcription factor myogenin Fig. 8.

Fig. 8 Gene expression of myogenic markers in C2C12 myotubes day 0 – 6. MCK = muscle creatine kinase. Results reflect three independent experiments with 3 wells per condition each time.*P < 0.05, **P<0.01, *** P<0.001.
In order to characterise expression of genes relevant for retinoid metabolism in C2C12 cells we quantified mRNA expression of Raldh1 and the α, β and γ isoforms of RARs and RXRs (see Fig. 9). Raldh1 was highly induced during differentiation of C2C12 cells and was expressed ~12 fold higher on day 2 compared to undifferentiated myoblasts on day 0. This initial induction declines over time and stays about 5-7 fold higher in fully differentiated myotubes compared to undifferentiated myoblasts. Among RARs the γ isoform is the most abundant with 3 fold higher mRNA levels compared to the α isoform. The latter is stably expressed over the course of the six days, whereas RARγ expression decreases with differentiation. RARβ is the least abundant isoform (20-fold lower than RARα) and expression does not change during differentiation (see Fig. 9b).

Concerning the RXRs (Fig. 9c) the α isoform was most highly expressed in C2C12 cells while RXRβ expression was ~40% lower than RXRα. RXRβ and RXRα were induced until day 2 and 4, respectively but expression dropped back to the baseline levels by day 6. Expression of RXRγ was very low and therefore almost not detectable.

Fig. 9 Expression of Raldh1 and RAR and RXR isoforms in C2C12 myotubes over the course of 6 days during differentiation. Results reflect three independent experiments with 3 wells per condition each time.*P < 0.05, ** P<0.01.
Next we assessed expression of β-oxidation markers in C2C12 myoblasts (day 0) and during differentiation to myotubes (day 2 – 6). Acyl-CoA-oxidase (ACOX) expression showed modest (30%) but significant induction on day 2, but went back to baseline by day 6 (see Fig. 10). CPT1β was induced twofold with a maximum on day 6. Uncoupling protein 2 was induced twofold, reached its maximum on day 4 and decreased slightly towards day 6. Uncoupling protein 3 was highly induced with a 50x upregulation on day 6.

![Gene expression graphs](image)

Fig. 10 Gene expression of oxidation markers in C2C12 myotubes over the course of 6 days during differentiation. Results reflect three independent experiments with 3 wells per condition each time. **P < 0.05, *** P < 0.001.

### 3.5 Retinaldehyde stimulates β-oxidation *in vitro*

In order to test the hypothesis that Rald could have accounted for the increased in β-oxidation markers in Raldh1 deficient muscle we now stimulated C2C12 myotubes
with synthetic Rald. After differentiating the cells to myotubes for four days we treated the cells with 1 μM Rald and 0.5 mM oleic acid as a positive control.

Uptake of fatty acids into cells is mediated by albumin. Although there is albumin in fetal bovine serum (FBS) in many studies fatty acids are complexed to bovine serum albumin (BSA) before adding it to the medium. Hence we compared adding oleic acid alone or oleic acid complexed with BSA. We dissolved 2% fatty acid free BSA in DMEM and added the oleic acid directly into the medium. We also complexed Rald with BSA as β-carotene and its derivatives have a long carbon backbone like fatty acids and therefore hypothesised the complexing could facilitate Rald uptake into the cell. Oleic acid was used in a concentration of 0.5 mM which is approximately within the range of the physiological concentration of oleic acid in the blood of fasted rats (LOWELL & GOODMAN, 1987). The concentration of Rald was 1 μM which is within the physiologic range as well and has been used in vitro by other groups before (ZIOUZENKOVa et al., 2007).

![Gene expression of key β-oxidation markers in differentiated C2C12 myotubes after 24h stimulation with 0.5 mM Oleic acid complexed to BSA (black column), 0.5 mM oleic acid alone (dark grey column), 1 μM Rald with or without BSA (3rd and 4th column), control = differentiation medium. Results reflect three independent experiments with 3 wells per condition each time. *P < 0.05, **P<0.01.](image)
As shown in Fig. 11 both oleic acid alone and complexed to BSA potently induced expression of genes relevant for fatty acid oxidation with the exception of ACOX. CPT1β increased by 2 fold while UCP-2 was 4-6 fold and UCP-3 6-12 fold upregulated when compared to control.

Interestingly, Rald also increased expression of fatty acid oxidation markers similar to our findings in Raldh1 deficient skeletal muscle. ACOX was induced by 30%, while CPT1β did not show any significant induction. UCP-2 was induced by ~50% and UCP-3 by 150%. Hence, these data suggest that Rald could be the mediator of increased fatty acid oxidation in skeletal muscle of Raldh1 deficient mice.
4 Discussion

Vitamin A (retinol) and its derivatives, the retinoids were already described in 1937 and how they can influence health and disease (MOORE, 1937). Since then this field has been researched extensively. Several enzymes in the cell can produce active metabolites from retinol by oxidation. Adh forms Rald and then a Raldh isoform oxidises Rald to RA. This last step is irreversible and rate limiting for the generation of RA in the cell. RA previously known as the biologically active form of Vitamin A has been investigated intensively and its role in development is now well established (NIEDERREITHER & DOLLÉ, 2008). The involvement of retinoids in metabolism and obesity so far has been vague and unclear. Here we provide new evidence that Raldh1 deficiency increases fatty acid oxidation by regulating a transcriptional programme in skeletal muscle that facilitates β-oxidation. We could demonstrate in vivo and in vitro that Raldh1 and Rald are involved in the regulation of key β-oxidation markers.

Retinoids play a crucial cellular role by action on nuclear receptors. RA can act as a ligand for RAR and RXR. These can heterodimerise and activate transcription as seen in Fig. 2. RXR can also form homodimers or heterodimers with several other nuclear receptors including the PPARs, Vitamin D receptor, Liver X receptor and other key regulators of metabolism and the immune system (CHAWLA et al., 2001; SHULMAN & MANGELSDORF, 2005). This function of gene activation and transcription makes the retinoids as ligands for these receptors an interesting field. Levels of RA are tightly regulated in the cell. Oxidation of Rald to RA is controlled by action of Raldh enzymes. Intracellular RA concentrations can be further regulated. CYP26 enzymes belonging to the Cytochrome P450 superfamily hydroxylate RA leading to its degradation and easier excretion (SAKAI & DRÄGER, 2010). The strict catabolism of Rald and RA and the many and diverse dimerization partners of RXR illustrate how sensitive the retinoids can act on development or adult tissue homeostasis.

Synthetic ligands for RXR and its dimerization partners (especially PPARs, LXR and TR) have been extensively studied with respect to its function in adiposity, diabetes, metabolic syndrome and immunity (SHULMAN & MANGELSDORF, 2005). Drugs in the class of Thiazolidinediones and Fibrates, PPARγ & PPARα agonists, are in clinical use for the treatment of type II Diabetes mellitus and dyslipidaemia (BROWN & PLUTZKY, 2007). Lipids are the known ligands for PPARs. Recently it was reported that RA is not
only a ligand for RAR and RXR, but also a high affinity ligand for PPARβ/δ (SHAW et al., 2003; BERRY & NOY, 2009). Similarly new evidence has emerged that Rald is not only an intermediary metabolite of RA, but itself can act upon nuclear receptors (ZIOUZENKOVA et al., 2007). Rald so far had only been described to play a major role in the eye for night vision. 9-cis-Rald is bound to opsin and can accept photons leading to a conformational change and ultimately to a nerve signal. Rald levels are tightly regulated by Adh and Sdr converting Retinol to Rald and vice versa and by Raldh converting Rald to RA. Apart from the eye, Rald was not described to be an active metabolite. The authors found that Rald had its own effects aside of RA with an important impact on metabolism. They showed that Rald is present in rodent fat and that its concentrations are decreased in obesity. Using a Raldh1 deficient mouse model lacking the ability to catabolise Rald they demonstrated that these mice were protected from diet induced obesity, had smaller adipocytes and enhanced glucose and insulin metabolism (Fig. 3). Direct administration of Rald could mimic these effects. Furthermore they could show that Rald had an antagonistic effect on RXR:PPARγ dimers in vitro. Raldh1-/- mice had a higher metabolic rate and a lower respiratory quotient compared to WT controls. However, distinct molecular mechanisms underlying the protection against obesity remained to be shown.

We therefore hypothesised that increased fatty acid oxidation in skeletal muscle oxidation could contribute to the metabolic phenotype of Raldh1 deficiency. A third of the human body is skeletal muscle and accounts for a fifth of daily resting energy expenditure (JANSSSEN et al., 2000). Skeletal muscle is the primary site of fatty acid oxidation. Two main types of muscle fibres exist of which muscles are made up of. White, fast-twitch fibres relying on glycolysis for fast force production and red fibres equipped with high levels of myoglobin and mitochondria to oxidise fatty acids for slow and steady force generation. At first we show that the enzymatic machinery for retinoid generation is present in skeletal muscle. Adh1 and Raldh1 are highly expressed in skeletal muscle while Raldh2 and Raldh3 are expressed 33-fold lower than Raldh1 (Fig. 4). We found that β-oxidation markers were highly induced in gastrocnemius and soleus muscle of fasted mice. More importantly we saw that fasting increased fatty acid oxidation genes significantly more in Raldh1-/- compared to WT mice (Fig. 6). Our data indicate that Raldh1 deficiency is associated with increased fatty acid mobilisation and subsequent oxidation in muscle. Hence, enhanced fatty acid turnover could contribute to the leanness seen in Raldh1-/- mice on high-fat diet (ZIOUZENKOVA et al., 2007).
As reported Raldh1 deficient mice are a model for high endogenous Rald concentrations (ZIOUZENKOVA et al., 2007). To test whether Rald could be a mediator of increased β-oxidation we performed *in vitro* experiments in C2C12 myoblast cell line. First we differentiated C2C12 myoblast to myotubes and characterised the gene expression profile relevant for myogenesis, retinoid metabolism and β-oxidation in order to find out whether this cell line is a suitable myocyte model to study effects of retinoids on β-oxidation. C2C12 myoblasts can be induced to form myotubes by serum withdrawal. We showed that myoblast formation is fully established at day 4 (Fig. 7). This is congruent with other studies (HWANG & LANE, 1999; LIU et al., 2010). Gene expression of myogenic markers confirmed the differentiation to myotubes Fig. 8. Furthermore we showed that Raldh1, RAR and RXR isoforms are expressed in C2C12 Fig. 9. Finally, we measured expression of β-oxidation markers of myoblasts and myotubes Fig. 10. All tested genes were expressed, some were induced during differentiation. We therefore proved that the genetic machinery for retinoid metabolism and β-oxidation is present in this cell model.

To elucidate Rald effects on fatty acid oxidation genes in myocytes we stimulated differentiated C2C12 with Rald and monitored gene expression. In short Rald significantly induced expression of genes relevant for fatty acid metabolism such as ACOX, UCP2 and UCP3 (Fig. 6). While Rald induced ACOX, oleic acid did not. A possible explanation is that ACOX as a peroxisomal β-oxidation enzyme is mainly necessary for very long fatty acids. Oleic acid has a C:18 backbone and can be fuelled directly into mitochondrial acetyl-CoA production and a chain shorting cycle in the peroxisomes is not necessary. A possible explanation for the Rald-mediated upregulation of ACOX could be that Rald enhances ACOX expression through a RAR or RXR heterodimer with a PPAR nuclear factor, since a PPRE exists in the promoter region of the ACOX gene (AMMERSCHLAEGER et al., 2004). The greatest Rald-mediated induction in C2C12 myotubes was seen for UCP-2 and in particular UCP-3. These two proteins have a high homology to UCP-1 (BOSS et al., 1997; FLEURY et al., 1997), a known mitochondrial membrane uncoupler important for non-shivering thermogenesis in brown adipose tissue (NICHOLLS et al., 1978). Evidence has accumulated that UCP-2 and 3 play a minor role in the uncoupling of the mitochondrial electron transport chain, but are rather involved in fatty acid metabolism (reviewed in SCHRAUWEN et al., 2006). UCP-3 is highly expressed in skeletal muscle and is induced upon rising levels of free fatty acids. Physiologic triggers for increased fatty acid concentrations are fasting, exercise or...
dietary intake (BRUN et al., 1999; CHOU et al., 2001; SCHRAUWEN et al., 2002). Previous reports showed that UCP-2 & 3 positively correlate with free fatty levels in vastus lateralis biopsies from obese patients (BOSS et al., 1998). Furthermore UCP-3 seems to be under the regulation of PPARs. Overexpression of PPARα leads to higher levels of UCP-3, as did ligand dependent PPARβ/δ activation (NAGASE et al., 1999; FINCK et al., 2005). The ability of PPARα and PPARβ/δ to induce UCP-3 is of big interest as these two nuclear hormone receptors induce a whole array of genes necessary for lipid utilisation.

Several theories have been postulated how UCP-3 functions in the cell (SCHRAUWEN et al., 2006). The accumulated evidence suggests that UCP3 does not directly participate in fatty acid breakdown and/or energy production but rather has a protective role during fatty acid oxidation. UCP-3 is located in the inner mitochondrial membrane and has a transport function, however it is not entirely clear yet which substrates are being transported by UCP3, although several have been suggested. When the mitochondrial oxidation capacity is at limit fatty acids accumulate in the mitochondrion and increase reactive oxygen species (ROS) production and lipid peroxidation (ST-PIERRE et al., 2002). To defend the mitochondria from possible ROS and peroxidation induced damage three UCP3 dependent protective mechanisms were hypothesised. On the one hand UCP-3 could export excess fatty acid anions to avoid lipid peroxidation. On the other hand UCP-3 could export the actual peroxides. Finally, a similar function as for UCP-1 has been hypothesised, namely that UCP-3 lowers the ROS production by uncoupling oxidative phosphorylation and the mitochondrial membrane. Hence one could speculate that Raldh1 may be protective against oxidative stress and ROS damage by increasing UCP3 tissue concentrations. However, further studies would be needed to elucidate any such effects.
5 Conclusion

Obesity has reached an epidemic character over the last decades and is growing rapidly. Currently ~300 Mio worldwide are considered to be obese and a doubling of this number till the year 2025 has been predicted (FORMIGUERA & CANTÓN, 2004). New dietary or pharmaceutical strategies in the clinician’s toolbox to fight against obesity are sought after and well needed.

Vitamin A’s role in development, but also in adult tissue physiology is well established. Over the past years more research has been conducted to describe the role of retinoids in various disease models (BLOMHOFF, 1994b; BLOMHOFF & BLOMHOFF, 2006). In the field of obesity and metabolism my sponsor’s lab could show that the RA precursor Rald also has biological function distinct from RA and that high levels of Rald protect mice from diet-induced obesity (ZIOUZENKOVA et al., 2007). Here we presented new mechanistic insight how the skeletal muscle could contribute to protection against obesity in Raldh1 deficiency.

By using the same Raldh1 deficient mouse model we demonstrated that the enzymatic machinery of retinol metabolism is present in skeletal muscle by showing protein and/or gene expression for Adh1 and Raldh1. Our studies revealed that β-oxidation markers are significantly higher in skeletal muscle of Raldh1−/− mice compared to WT mice. In vitro experiments established Rald as a transcriptional regulator of genes relevant for fatty acid metabolism in myocytes. Hence, Raldh1 deficiency and Rald appear to enhance muscle fatty acid oxidation in vivo and in vitro.

Taken together our data add to a growing body of literature that retinoids and its regulatory enzymes play an important role in the control of glucose and lipid metabolism. Our findings further highlight the importance of the Vitamin A pathway in pathophysiologic conditions such as obesity, diabetes and the metabolic syndrome. Targeting Raldh1 could therefore become a novel therapeutic strategy to fight obesity and related metabolic complications.
6 Zusammenfassung


Aktivatoren der PPAR nukleär Rezeptoren, werden heute intensiv in der Behandlung des metabolischen Syndroms benutzt.


Zusammenfassend tragen unsere Daten zum Feld der Retinoide und wie diese im Körper regulatorisch auf den Glukose- und Fetthaushalt wirken bei. Vor allem im Hinblick auf pathophysiologische Zustände wie Adipositas, Diabetes und dem Metabolischen Syndrom deuten unsere Funde auf die Bedeutung des Vitamin A Metabolismus hin und erlauben sogar zu erwägen, die Vitamin A Maschinerie
therapeutisch einzusetzen bzw. zu beeinflussen. Eine genaue Evaluierung möglicher Therapeutika, die hier eingreifen, ist jedoch unbedingt nötig, da Vitamin A eine so zentrale Rolle im Körper hat und sehr sensibel von der Enzymmaschinerie reguliert wird.
7 Bibliography


