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Expression of genes related to energy balance in adipose tissue of dairy cattle: effects of SCFA on mRNA abundance as quantified by qPCR and relevance of appropriate selection of reference genes

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Dedicated to my family, especially
to my father who passed away during my PhD study

Expression von Energie-Bilanz-assoziierten Genen im Fettgewebe von Milchkühen: Einfluss von SCFA auf die mRNA-Expression, quantifiziert mittels qPCR sowie Relevanz der Auswahl geeigneter Referenzgene

Ziel dieser Arbeit war es, den Einfluss von Propionat (C3) und ß-Hydroxybutyrat (BHB) auf die mRNA-Menge von verschiedenen Referenzgenen (RGs) und von Zielgenen (GOI), die mit der Energiehomöostase in Zusammenhang stehen, in Kurzzeitstimulationsversuchen (4 h) an Explantaten aus zwei unterschiedlichen Fettdepots (subkutan (SC) und retroperitoneal (RP)) von Milchkühen *in vitro* zu untersuchen. Hierbei haben wir uns auf drei Aspekte konzentriert: (1) die differenzielle Wirkung von C3 und BHB auf RGs sowie deren Verwendung in der Normalisierung zur Verbesserung der Zuverlässigkeit von Real-Time-quantitative-PCR- (qPCR-) daten. Zu diesem Zweck wurden das geNorm™- und das Normfinder©- Programm verwendet, um die stabilsten RGs zu identifizieren. Die geometrischen Mittelwerte der stabilsten RGs, gekennzeichnet durch geNorm™ als die stabilsten RGs bei C3- und BHB-Behandlung, wurden für die weitere Normalisierung verwendet. (2) Die Wirkung von C3 und BHB auf die mRNA-Abundanz der Gene, die mit der Energiehomöostase in Zusammenhang stehen, in SC und in RP Fettgewebsexplantaten zu untersuchen. Wir haben dabei gezeigt, dass die *in vitro* Stimulation von bovinem SC und RP Fettgewebe mit verschiedenen Konzentrationen von C3 oder BHB nach kurzer Zeit (4 h) differenzierte Auswirkungen auf die mRNA-Abundanz von Adiponectin, Adiponectin Rezeptor 1 (AdipoR1), AdipoR2, Peroxisomen-Proliferator-aktiviertem Rezeptor gamma 2 (PPARγ2), Insulinrezeptor-Substrat 1 (IRS-1), CCAAT/enhancer-binding-Proteine α (C/EBPα), Glucosetransporter 4 (GLUT4), Interleukin-6 (IL-6) und Sterol regulatory element-binding protein 1 (SREBP1) hat. Die mRNA von AdipoR1/R2 wurde im bovinen Explantat-Modell durch BHB weniger beeinflusst als durch C3; dies deutet darauf hin, dass das Adiponectin-System gegenüber C3 sensitiver sein könnte als gegenüber BHB. Die mRNA von PPARγ2, einem der Hauptregulatoren der Adipogenese, wurde von C3 in SC Fettgewebe erhöht, während C3 die mRNA von IRS-1 im RP Fettgewebe reduzierte. Die Insulin-induzierten Veränderungen waren auf die mRNAs des Freie Fettsäuren Rezeptors 3 (FFAR3) und von IL-6 in SC und RP Fettgewebe beschränkt, und zeigten bei beiden Genen einen Trend ($P \leq 0{,}15$) zur Erhöhung der mRNA-Menge. (3) Etablierung eines Zellkultursystems für primäre, bovine Präadipozyten zur Charakterisierung der mRNA-Expression der Gene von C/EBPα, FFAR2, FFAR3, Fettsäure-bindendem Protein 4 (FABP4) und PPARγ2 während der Differenzierung. Neben dem zeitlichen Anstieg der einzelnen mRNAs haben wir als Erste den Nachweis von FFAR2 und FFAR3 mRNA in beiden Depots des Fettgewebes erbracht.

Expression of genes related to energy balance in adipose tissue of dairy cattle: effects of SCFA on mRNA abundance as quantified by qPCR and relevance of appropriate selection of reference genes

The aim of this thesis was to study the effect of propionate (C3) and β-hydroxybutyrate (BHB) on the mRNA abundance of reference genes (RGs) and of the genes of interest (GOI) related to energy balance, by short term incubation (4 h) in bovine AT explants *in vitro*. Herein, we focused on three aspects: First, the differential effect of C3 and BHB on RGs and their use in gene expression normalization to improve the reliability of quantitative real-time PCR (qPCR) data. For this purpose, geNorm™ and Normfinder© programs were used to identify the RGs with highest stability. The geometric mean of the RGs identified by geNorm™ as being most stable in C3 and BHB treatment was used for accurate normalization. Second, to explore the effects of C3 and BHB on the mRNA abundance of energy balance related genes in subcutaneous (SC) and retroperitoneal (RP) adipose tissue (AT) explants. We demonstrated that *in vitro* stimulation of bovine SC and RP AT explants with different concentrations of C3 or BHB in short-term exerts differentiated effects on the mRNA abundance of the analyzed energy balance related genes adiponectin, adiponectin Receptor 1 (AdipoR1), AdipoR2, peroxisome proliferation-activated receptor gamma 2 (PPARγ2), insulin receptor substrate 1 (IRS-1), CCAAT/enhancer binding protein α (C/EBPα), facilitated glucose transporter 4 (GLUT4), interleukin 6 (IL-6) and sterol regulatory element-binding protein 1 (SREBP1). Adiponectin receptor 1 and AdipoR2 mRNA were less affected by BHB than by C3 in the bovine explant model, indicating that the bovine adiponectin system might be more sensitive to C3 than to BHB. The mRNA abundance of PPARγ2, a key regulator of adipogenesis, was increased by C3 in SC AT explants, while C3 suppressed mRNA abundance of IRS-1 in RP AT. The insulin-induced alterations were limited to the mRNAs of free fatty acid receptor 3 (FFAR3) and IL-6 in SC and RP AT, respectively, for which a trend ($P \leq 0.15$) for increased abundances was recorded. Third, we established a bovine primary preadipocyte culture for characterizing the mRNA expression of the genes encoding C/EBPα, FFAR2, FFAR3, fatty acid binding protein (FABP4) and PPARγ2 during differentiation. In addition to increased mRNA abundance of the GOI during differentiation, we demonstrated the presence of FFAR2 and FFAR3 mRNA not only in both AT depots but also in differentiating preadipocytes isolated from bovine SC AT.

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List of abbreviations

18S rRNA	18S ribosomal RNA
a.a.	Amino acids
AC	Adenylyl cyclase
AcAc	Acetoacetate
Acc. No.	Accession number
ACTB	Beta2 actin
Acetyl-CoA	Acetyl coenzyme A
AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
AMPK	5' adenosine monophosphate-activated protein kinase
APPL1	A pleckstrin homology domain-containing adaptor protein
AT	Adipose tissue
BBQ	Blackberry quencher
BHB	ß-hydroxybutyric acid
Bp	Base pair
bZip	Basic leucine zipper
C/EBPα	CAAT-enhancer binding protein α
C3	Propionate
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
Con.	Control
CPT-1	Carnitine Palmitoyltransferase-1
Ct.	Median cycle threshold
D	Day
DMEM/Ham's F-12	Dulbecco''s modified eagle''s medium/Ham''s nutrient mixture F-12
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
ERK	Extracellular-signal-regulated kinases
FABP4	Fatty acid binding protein 4
FAM	6-carboxyfluorescein
FAS	Fatty acid synthetase
FFAR2	Free fatty acid receptor 2
FFAR3	Free fatty acid receptor 3
Fig.	Figure
$G_{q/11}$	Subfamily of $_{q/11}$ G-protein

G12/13	Subfamily of $_{12/13}$ G-protein
G-6-P	Glucose 6-phosphate
GAPDH	Glyceraldehyde-phosphate-dehydrogenase
$G_{i/o}$	Subfamily of $_{i/o}$ G-protein
GLUT4	Facilitated glucose transporter 4
GOI	Genes of interest
GPR109A	G-protein coupled receptor 109 A
G_s	Subfamily of s G-protein
HPCAL1	Hippocalcin-like 1
IL-6	Interleukin 6
I-R	Insulin-receptor
IRS-1	Insulin receptor substrate 1
JAK	Janus family of kinase
Len.	Fragment length
LRP10	Low density lipoprotein receptor-related protein 10
M	Gene stability measure
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
N	Sample size
n-3 PUFA	n-3 polyunsaturated fatty acids
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NEFA	Nonesterified fatty acids
NF	Normalization factor
NO	Nitric oxide
No-RT	No reverse transcriptase control
P	Probability
p38 MAPK	p38 mitogen-activated protein kinase
PCR	Polymerase chain reaction
PDE-3B	Phosphodiesterase 3B
PI3K	Phosphatidylinositol 3-kinase
PKA	cAMP-dependent protein kinase
PkB	Protein kinase B
PKC	cAMP-dependent protein kinase C
PLC-β2	Phospholipase C β2
POL II	RNA polymerase II
PPARγ2	Peroxisome-Proliferator-actived receptor γ 2

qPCR	Quantitative real-time PCR
R	Correlation coefficients
RG	Reference gene
RNase-free	Ribonuclease-free
ROS	Reactive-oxygen species
RP	Retroperitoneal
RPS9	Ribosomal protein S9
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
SC	Subcutaneous
SCFA	Short chain fatty acid
SEM	Standard error of the mean
SREBP1	Sterol regulatory element-binding proteins 1
STAT	Signal transducer and activator of transcription
SVC	Stromal-vascular cell fraction
Ta.	Annealing temperature
Treat.	Treatment
TZD	Thiazolidiendiones
V	Pairwise variation
vs.	Versus
β-AR	β-adrenergic receptors

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1. Introduction

Today, ruminants account for almost all dairy lifestock and also contribute to about one-third of the meat production worldwide. The use of high-yielding breeds such as Holstein-Friesians has resulted in dramatic increases in milk production during the past decades. Compared to a suckler cow which would naturally produce around 4 litres of milk per day, a high yielding dairy cow will produce around 27 litres per day for a period of 10 months (305 d lactation period). After parturition, milk production peaks between wk 5 to 7 post-partum, whereas maximum feed intake is reached not until 8 to 22 wk after calving. The lag in feed intake during this period leads to a negative energy balance (Ingvartsen et al., 2000). Energy balance is defined as the difference between the energy consumed and the energy required for maintenance, growth, pregnancy, and lactation (Grummer, 2007) and is of particular importance during the transition from pregnancy to lactation (Drackley, 1999), particularly in high-yielding dairy cows. The mobilization of body stores, mainly in the form of lipids accumulated in various AT depots, to compensate the energy loss via milk can be excessive and is knowingly coupled to health disturbances, e.g. metabolic diseases and impaired immune function. Accretion of body stores occurs during the preceding pregnancy and might also reach an extent predisposing for metabolic disorders like obesity, reduced insulin sensitivity and fatty liver, which affect the health and production of dairy cattle (Ametaj et al., 2005). Positive and negative energy blance as well as their impact in relation with different factors will be discussed in further chapters. However, understanding the effect and interrelationships of both situations, i.e. energy surplus and deficit, will helps to develop concepts for improvement of dairy cattle health and performance. At the moment, there are several methods (e.g. *in vivo* and *in vitro*) to study the effects of energy surplus or deficit in dairy cattle with their advanteges and disadvanteges. The present thesis will foucus only on an *in vitro* model based on the reasons explained in the further sections.

1.1. Adipose tissue

Adipose tissue represents a special loose connective tissue containing lipid-loaden adipocytes and other cell types. The main focus of this study is white AT, whereas brown AT, which is almost exclusively found in neonates, will not be addressed. White AT stores lipids as trigly-

cerides and mobilizes them for systemic utilization when other tissues require energy. Besides its role as energy store, it is an active endocrine organ producing different types of hormones, cytokines and chemokines, collectively termed as adipocytokines to regulate homeostasis. Adipose tissue produces also transcriptional factors and in this way influences many aspects of energy metabolism through a network of local and systemic signals. The present study focuses on selected energy balance related genes like adiponectin, AdipoR1/2, C/EBPα, FFAR2/3, GLUT4, G-protein-coupled receptor 109A (GPR109A), IL-6, IRS-1, PPARγ2, and SREBP1 (Caimari et al., 2010; Herwig et al., 2009; Liu et al., 2003; Nilaweera et al., 2003) for which the backgrounds will be provided in the following chapters. Adipose tissue modulates energy expenditure, appetite, insulin sensitivity, endocrine and reproductive functions, bone metabolism, inflammation, and immunity (Shoelson et al., 2007).

It is localized in different places of the body. The different localizations (depots) display various functions. Subcutanous depots are located directly underneath the dermis in the subcutis (e.g. at the tail-head, withers, sternum); whereas the visceral depots reside in the abdominal and thoracic cavity as fat pads (e.g. heart, intestine, liver and kidney fat). Visceral and SC AT differ according to their structure and metabolic function. Fat accumulation in SC and visceral depots is prone to metabolic disorders in man, particularly when visceral fat deposition is abundant (Lafontan et al., 2003). However, the amount of SC AT generally exceeds the visceral fat mass by 3 to 4 times (Chowdhury et al., 1994); both depots can interact in a coordinate and compensatory manner. The intra-abdominal fat is subdivided into preperitoneal and RT depots (Shen et al., 2003). The preperitoneal depots surrounding the intestine, i.e. that the omental-mesenteric blood vessels drains into the liver via the portal circulation, whereas RP blood reaches the systemic circulation via the inferior vena cava; the release of mediators affecting insulin sensitivity from the RP AT depot will thus exert systemic, rather than specifically hepatic effects (He et al., 2008).

The cell types forming AT comprise adipocytes, which might contribute to about 35% to 70% of adipose mass in human adults, but form only 25% of the total cell population (Frühbeck, 2008). In addition to adipocytes, AT also contains preadipocytes, endothelial cells, fibroblasts, leukocytes and, most importantly, macrophages (Tilg et al., 2006; Trayhurn et al., 2004) (Fig. 1). These diverse cell types account for the remaining 75% of the total cell population; the multicellularity implies a wide range of targets for an extensive autocrine/paracrine crosstalk (Frühbeck, 2008) in AT as a local effect and/or elicits endocrine effects in different organs and thus affects energy homeostasis in organism via the aforementioned energy balance

related genes. The AT of obese individuals contains a large number of macrophages, which also are an additional source of soluble mediators in AT (Fig. 1). However, macrophages in AT of monogastrics seem to be the main source of proinflammatory cytokines like IL-6 (Galic et al., 2010), which will be in detail discussed in the next chapters. Adipose tissue contributes 30% of the IL-6 concentration in the circulation of obese individuals (Mohamed-Ali et al., 1997). The various mediators produced by adipocytes and resident macrophages might contribute to local and systemic inflammation (Tilg et al., 2006). Less is known about the infiltration and resident population of the macrophages in lactating dairy cattle during different physiological situation.

1.2. Energy balance and related genes

As mentioned above, negative energy balance during early lactation in dairy cows leads to an altered metabolic state by drawing on body fat reserves (Bertics et al., 1992). The depletion of AT in energy-deficit conditions is accompanied by comprehensive metabolic and endocrine changes in dairy cows during early lactation, and may result in an increased risk for many diseases and metabolic disorders like ketosis. In a situation of negative energy balance during early lactation, nonesterified fatty acids (NEFA) are released from AT by lipolysis. The oxidation of NEFA to acetyl-CoA represents the first step of energy generation for most organs. In the following steps, the activated acetyl enters the tricarboxylic acid cycle by reacting with oxaloacetate to form citrate; in a succession of reactions the acetyl is converted into carbon dioxide and energy (ATP). The availability of oxaloacetate, however, depends on an adequate supply of its precursors (e.g. pyruvate, which is the product of glucose degradation in glycolysis). Acetyl-CoA cannot enter the citric acid cycle if the concentration of oxaloacetate is lowered (e.g. when carbohydrate supply is limited and gluconeogenetic conditions develop). In fasting or diabetes, oxaloacetate is consumed to form glucose by the gluconeogenic pathway and is unavailable for condensation with Acetyl-CoA. The Acetyl-CoA is then directed to the formation of ketone bodies, i.e. acetone, BHB and acetoacetate (Fig. 3) (Berg et al., 2006). Ketone bodies may also originate from butyrate from ruminal fermentation and subsequent metabolization in the ruminal epithelium (Kristensen et al., 2000), but its concentrations in blood, milk, and urine are closer linked to lipolysis than to ruminal absorption. Therefore, BHB is used as an indicator for lipolysis in dairy cows and increased concentrations occur during energy deficit and are indicative for metabolic stress which might finally result in clin-

ical symptoms like ketosis. When the hepatic uptake of lipids exceeds the oxidation and secretion of lipids by the liver, which usually is preceded by high concentrations of plasma NEFA mobilized from AT, excess lipids are stored as triacylglycerol in the liver and are associated with decreased metabolic functions of the liver and thus open out into the fatty liver syndrom. In dairy cattle, the increased NEFA and BHB concentrations in blood are related to decreased protein and mRNA abundance of adipokines like leptin in SC AT from 10 d before parturition to 10 d thereafter (Duske et al., 2009). It is also known that BHB and butyrate inhibit adipocyte lipolysis in bovine AT *in vitro* (Metz et al., 1974). The effect of BHB and its effect on energy balance related genes will be discussed in coming chapters.

As mentioned before, surplus energy load can cause metabolic disorders like obesity, reduced insulin sensitivity and fatty liver, which in turn affect health and production of dairy cattle. Short chain fatty acids (SCFA), mainly acetate, C3 and butyrate, are known as the main energy source in ruminants. In ruminantes, most of the carbohydrates from feed stuffs are fermented to the SCFA, carbon dioxide, and methane in the rumen. The SCFA blood concentration is different between monogastrics and ruminants; besides SCFA concentrations differ in blood depending on the distance to the place of absorption, i.e. when comparing portal, hepatic and peripheral values (Bjorkman et al., 1986; Cummings et al., 1987). The peripheral concentration of acetate is between 1.2 and 2.1 mM. Acetate is the major substrate for lipogenesis and oxidation (Brockman, 2005). Propionate is efficiently extracted (80–85%) by the liver in first pass and provides the main substrate for hepatic gluconeogenesis (Baird et al., 1980). About 20% of the absorbed C3 thus reach the circulation; the periperheral C3 serum concentrations reportedly range between 0.06–0.08 mM (Bjorkman et al., 1986). Previous studies demonstrated that in contrast to acetate, C3 stimulates insulin secretion and increases glucagon concentration immediately after the infusion in dairy cattle and sheep (Bradford et al., 2006; Lee et al., 2002; Sano et al., 1995). Insulin increases leptin mRNA in bovine AT explants (Houseknecht et al., 2000). Therefore, the present thesis focuses on insulin dependent or independent effect of C3 on AT.

After the discovery of leptin as the first adipokine (Taniguchi et al., 2002; Zhang et al., 1994) our perspective about the functions of AT and adipocytes was changed. The role of adipokines like adiponectin, and resistin, but also cytokines such as IL-6, which is also secreted at high levels by the AT, became more obvious. In mouse colon, C3 elicits anti-inflammatory effects on IL-6 mRNA and protein expression (Tedelind et al., 2007), which in turn inhibit adiponectin gene expression and secretion (Fasshauer et al., 2003). In monogastrics, an exten-

sive association was shown between body mass and expression and secretion of some of energy balance related genes like leptin and adiponectin. In different physiological situations like obesity, leptin increases and thus shows an inverse correlation with adiponectin. Adiponectin and its receptors, which will be discussed in the coming chapters, influence energy homoeostasis and increase insulin sensitivity (Guerre-Millo, 2008). Decreased insulin sensitivity is defined as a pathologic state of decreased responsiveness of target tissues to normal circulating levels of insulin. Adiponectin acts as an autocrine/paracrine factor, it is involved in adipocyte lipid accumulation and differentiation, and affects other energy metabolism related genes like C/EBPα and SREBP1 expression (Fu et al., 2005), and regulates the expression of its receptors in AT (Liu et al., 2008b). Several studies demonstrated the effect of C3 on mRNA abundance of nutrient sensing receptors and adipokines in ruminant AT *in vivo* and *in vitro* (Mielenz et al., 2008; Soliman et al., 2007). The concentrations dependent SCFA silencing by demethylation (Benjamin et al., 2001) might also lead to repression of mRNA abundance of energy balance related genes as it was shown for mouse leptin promoter activity (Yokomori et al., 2002).

As mentioned before, the transcriptional pattern and the protein expression in AT under dynamic physiological regulation may change. However, it is important to note that adipokines are not all exclusively derived from AT. The cross talk between energy balance related genes in different organs such as AT, liver, skeletal muscle, and central nervous system is assumed to provide an important link between obesity, insulin sensitivity, immunity, appetite and energy balance, lipid metabolism, inflammatory disorders and acute phase response (Trayhurn et al., 2004). Under the different physiological situations, energy homeostasis depends on many factors, among which some of these factors act as nutrient sensor like FFAR2/3 (Ichimura et al., 2009) and GPR109A. The regulation of nutrient sensing receptors trough different signal transduction pathways leads to the expression of adipokines or other transcriptional factors like C/EBPα, PPARγ2, GLUT4, IRS-1, SREBP1, FABP4 (Caimari et al., 2010; Fernyhough et al., 2007), adiponectin, AdipoR1/R2, and IL-6. Most of the studies describe the regulation of protein expression but not the regulation of mRNAs like the ones for adiponectin or IL-6 (Trayhurn et al., 2004). The aforementioned energy balance related genes will be discussed in the following chapters in details.

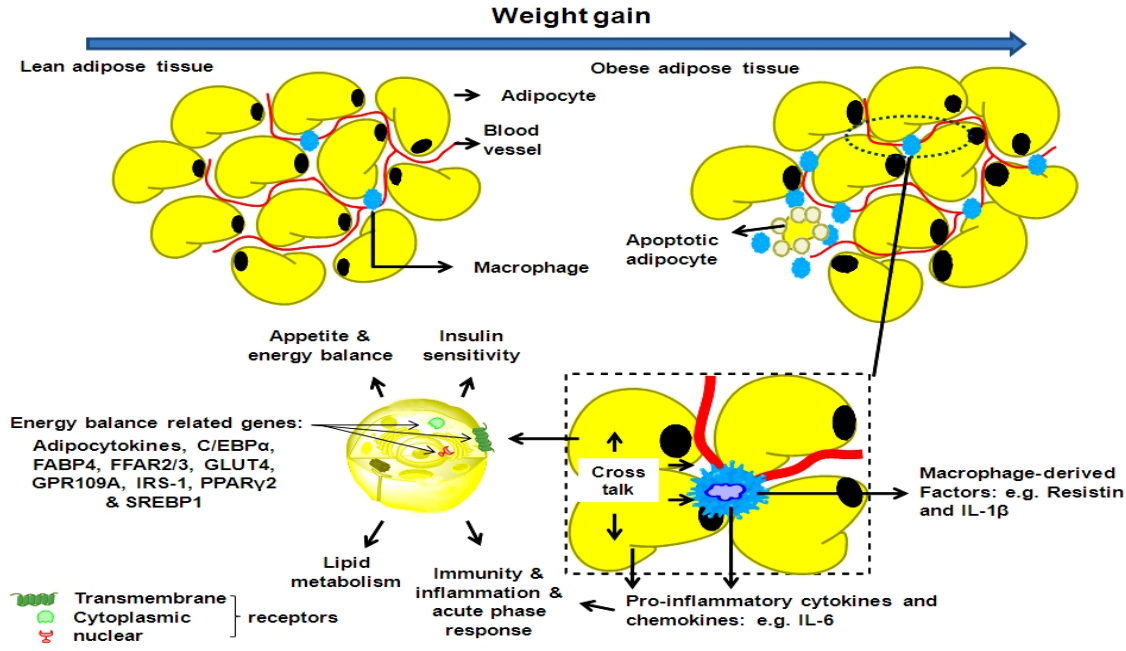

Fig. 1. Adipose tissue: cellular components, molecules synthesized and their classified functional rules. Expansion of the AT during weight gain leads to the recruitment of macrophages shown in monogastrics through various signals, which might include energy balance related genes produced by adipocytes. These macrophages are found mainly around apoptotic adipocytes. Energy balance related genes include C/EBPα, PPARγ2, GLUT4, IRS-1, SREBP1, FABP4, ,adipocytokines such as adiponectin, AdipoR1/2 and IL-6 and nutrient sensing receptors like FFAR2/3 and GPR109A shown as different receptors types (Modified after Tilg et al., 2006; Trayhurn et al., 2004).

1.2.1. Free fatty acid receptors regulate energy homeostasis

In association with the aforementioned proteins and mRNAs, the family of G-Protein coupled receptors (GPRs) is involved in fatty acid metabolism. The members of this family are involved in different physiological functions like nutritional regulation and they are of importance as pharmaceutical targets. They are localized in different organs including intestine (e.g. GPR120), adipocytes, taste buds, and lung (Ichimura et al., 2009) or in liver, heart, and skeletal muscle (e.g. GPR40). The family of GPR40 is activated by medium and long-chain fatty acids and is involved in different metabolic functions like potentiating insulin secretion, adipogenesis and lipolysis (Covington et al., 2006). In the present study, we focused on those members of this family (e.g. GPR41 and GPR43) that are activated by SCFA (Brown et al., 2005; Le Poul et al., 2003) and on one receptor that is activated by BHB and nicotinic acid, i.e. GPR109A (Gille et al., 2008).

GPR41/43 are alternatively called free fatty acid binding membrane receptors 3/2, respectively (FFAR2/3 labeled in the body of the text). The expression of FFAR2 and FFAR3 mRNA was addressed in AT, isolated leukocytes (Lemor et al., 2009), mammary epithelial cells and lactating mammary gland of bovine species (Yonezawa et al., 2009) and some other tissues like liver, heart, spleen, and skeletal muscle (Wang et al., 2009). The highest expression was observed for FFAR2 in spleen and immune cells such as neutrophils and monocytes (Stoddart et al., 2008), whereas for FFAR3 the highest expression was observed in human AT (Brown et al., 2005). However, there is one report indicating that both receptors are not expressed in bovine AT (Wang et al., 2009).

Short chain fatty acids are identified as ligands for human FFAR2 and FFAR3, they show differences in SCFA specificity, have key differences in the signalling pathways they activate, and in their tissue expression patterns (Ge et al., 2008). The rank order of potency starts with acetate (C2), C3 being equipotent followed by butyrate (C4), then valerate (C5), and formate (C1) (Stoddart et al., 2008). Several studies indicated that the different SCFA have different affinities to bind the receptor (Lee et al., 2008; Tedelind et al., 2007), but it should be realized that the SCFA concentration differ largely between human and ruminants (Bjorkman et al., 1986; Cummings et al., 1987). FFAR2/3 have been reported to stimulate leptin release and adipogenesis, and are known as energy storage monitoring receptors (Covington et al., 2006). Both receptors are regulated in dairy cows during the transition from pregnancy to lactation (Lemor et al., 2009). The bovine FFAR2/3 were cloned and characterized by Wang et al. (2009). The bovine FFAR2 and FFAR3 comprise 329 and 326 amino acids (a.a.), respectively; the multiple alignment (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_multalin.html; (Corpet, 1988)) shows 36.4% homology between the a.a. of these two proteins (Fig. 2a). The phylogram using the treeview of ClustalW shows (http://www.ebi.ac.uk/Tools/clustalw2/index.html; (Larkin et al., 2007)) that the bovine, murine and human FFAR2 is closer related than the FFAR3 in these three species; the a.a. of men and mice are more homolog compared to the bovine FFAR2. In addition, the murine FFAR3 is much more similar to FFAR2 of cattle, mice and humans (Fig. 2b). The bovine putative GPR109A is not yet characterized; therefore no sequence comparisons across species were possible.

GPRs are highly important in cell function, and are membrane-spanning proteins with seven-transmembrane spanning-helices that respond to various stimuli such as light, tastes, or hormones (Hendriks-Balk et al., 2008). FFARs serve to transduce a extracellular signal to inside a cell through a variety of second messenger cascades, such as cAMP-dependent protein ki-

nase A (PKA) and cAMP-dependent protein kinase C (PKC) signalling pathways (Hong et al., 2005). The heterotrimeric G proteins are divided into four families: G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ based on similarity of α-subunits (Hendriks-Balk et al., 2008). FFAR2 can couple to both G_i and G_q; they may activate the $G_{i/o}$ pathway in adipocytes to inhibit lipolysis, and are involved in immune and inflammatory response and reduce plasma FFA levels *in vivo*. FFAR3 couples only to G_i (Ge et al., 2008), resulting in inhibition of adenylylcyclase and elevation of intracellular calcium through activation of PLC-β2 (Brown et al., 2005).

a

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                  10        20        30        40        50        60
                   |         |         |         |         |         |
FFAR2      -------MPDWDSSLILTAYIIILLTGLPANLLALRAFLGRVRQPHPAPVHILLLSLTLA
FFAR3      MTNPDHSFFLGNHWLFFSVYLFTFLVGLPLNLMALVIFVGKLRR-RPLAVDVLLLNLTLS
                  70        80        90       100       110       120
                   |         |         |         |         |         |
FFAR2      DVLLLLLLPFKIIEAASDFRWELSNLACALMGFGFYGSIYCSTLLLAGISVERYLGVAFP
FFAR3      DLVLLLFLPFRMVEAASAMHWSLPFVFCPFSRFLFFTTIYLTSLFLAAVSTERFLSVAYP
                 130       140       150       160       170       180
                   |         |         |         |         |         |
FFAR2      VQYKLSRRPVYGVIAALIAWVMSFGHGTVVIIVQYLNSTQRAPKENETTCYENFTQEQLR
FFAR3      LWYKTRPRPGQAGLVSGACWLLAAAHCSVVYVIEFSGNSSPSQGINGT-CYLEFREDQLA
                 190       200       210       220       230       240
                   |         |         |         |         |         |
FFAR2      LLLPIRLELCLLLFFFPMVVTTFCYSRFVWIMLTQPHMGAQKQRRAMGLAIVSLLNFLLC
FFAR3      LLLPVRLEMAVVLFGVPLFISSYCYSRLVCIL--GRGASHRRRKRVAGLAAATLLNFLVC

                 250       260       270       280       290       300
                   |         |         |         |         |         |
FFAR2      FGPYNISHLVGFYMKASPKWRREAVVFGSLNASLDPLLFYFSSSVVRRSFGKGLQALYHW
FFAR3      FGPYNMSHIVGYIQGKSPTWRSYVLLLSTLNSCVDPLVYYFSSS--------GFQADFHG
                 310       320       330
                   |         |         |
FFAR2      GSSLLGRRCKETAEAANEDPGVSQAEGAPSSDFTLD--
FFAR3      LLGRLTGSWGPWRQENGVTSKKSEGEGPPQELFNIEAS
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b

Fig. 2. Structure and clustering of FFAR2 and FFAR3 receptors. a, amino acid alignment of bovine FFAR2 and FFAR3 receptors. Residues identical in both receptors are shown in red, whereas residues differences are shown in black. **b**, Phylogram representing sequence similarities among amino acids of bovine, mice and human FFAR2 and FFAR3 using the TreeView of ClustalW. Accession numbers are as follows: FFAR2 and FFAR3 of bovine, mice and human and their National Center for Biotechnology Information (NCBI) accession numbers (ACM07438.1; ACM07440.1), (Q8VCK6; Q3UFD7) and (O15552; O14843), respectively.

Beside the relevance of ß-adrenergic receptor in lipolysis, there are other energy related genes like GPR109A that effect lipolysis. As mentioned before, in general the concentration of fatty acids, NEFA and BHB increases during negative energy balance (Duske et al., 2009). Elevated levels of ketone bodies (Fig. 3) in the blood may lead to ketosis; the process of ketogenesis was defined in a previous chapter. The putative GPR109A in bovine is also identified as nicotinic acid binding receptor in human placenta cDNA as template (Wise et al., 2003), and is a $G_{i/o}$-coupled seven-transmembrane receptor expressed in adipocytes and activated macrophages (Taggart et al., 2005). It is also known as HM74A or PUMA-G in humans and in mice, respectively (Tunaru et al., 2003), and is activated in pharmacological doses by nicotinic acid (Gille et al., 2008), also known as niacin or vitamin B3. Niacin increases total adiponectin concentrations and decreases lipolysis via GPR109A-dependent and independent pathways in rats (Plaisance et al., 2009). Beta-hydroxybutyrate inhibits lipolysis via activation of GPR109A and thus represents an important negative feedback to adjust lipolytic activity during starvation (Gille et al., 2008). The mechanism of activation is illustrated in Figure. 3. Long-term treatment with extended release formulations of niacin in humans increased insulin sensitivity, adiponectin, C/EBPα, PPARγ and decreased GPR109A mRNA (Linke et al., 2009). The previous reports demonstrate that GPR109A can be activated through the ketone body BHB during starvation in mice (Taggart et al., 2005) and that the SCFA butyrate is an endogenous ligand for this receptor (Thangaraju et al., 2009). As mentioned in a previous chapter, BHB is of relevance to modulate lipolysis in dairy cattle during early lactation when the dairy cattle undergo negative energy balance (Metz et al., 1974). Further reports also showed the presence and extent of change of GPR109A mRNA in dairy cows during the transition period (Lemor et al., 2009). For the SCFA butyrate, Metz et al. (1974) already indicated that it can inhibit lipolysis in bovine AT. Thus the ruminally produced butyrate, might also play a role besides BHB to inhibit lipolysis via GPR109A in cattle, as both factors are known as a ligands for GPR109A (Thangaraju et al., 2009) and during positive energy balance, lipolysis should be down regulated. Less is known about the GPR109A in bovine species.

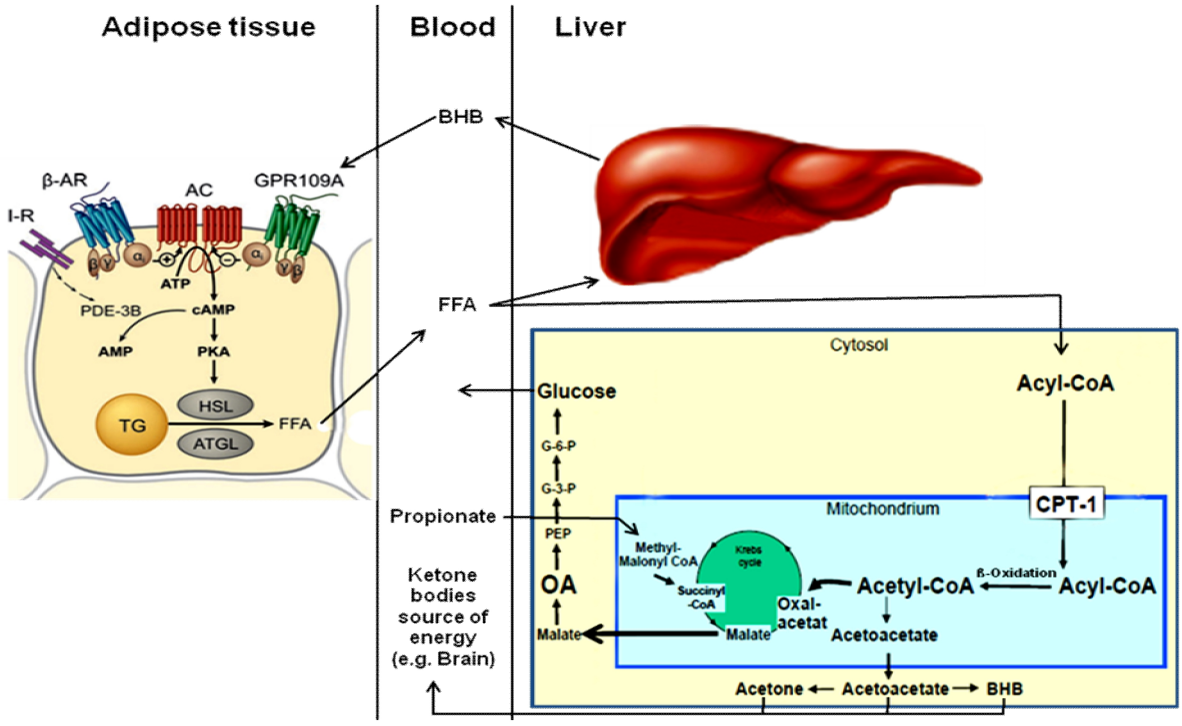

Fig. 3. Potential physiological role of the nicotinic acid receptor GPR109A during starvation. Sympathetic stimulation of adipose cells via β-adrenergic receptors (β-AR) is high and insulin levels are low, resulting in increase of intracellular cAMP levels that stimulate lipolysis. Free fatty acids (FFAs) are released from fat cells and metabolized in the liver see chapter 1.2.) to ketone bodies, including β-hydroxybutyrate (BHB), acetoacetate (AcAc) and acetone. Increased plasma concentrations of BHB activate GPR109A during energy deficient conditions. GPR109A-mediated inhibition of adenylyl cyclase (AC) activity and the resultant decrease in cAMP synthesis would counteract the increased β-AR-mediated cAMP formation and the decreased cAMP degradation by phosphodiesterase 3B (PDE-3B) during negative energy balance. I-R, insulin-receptor. (Modified after Gille et al., 2008 and Rehage, 2010).

1.2.2. Adiponectin and its receptors

Adiponectin, an insulin sensitizing hormone, was discovered in mid 1990s and it is one of the initial investigated adipokines (Scherer et al., 1995). Adiponectin is mainly expressed in adipocytes (Scherer et al., 1995). Adiponectin exerts its effect by decreasing the triglyceride content in insulin-sensitizing tissues and thereby upregulates insulin signalling, increases fatty acid combustion and energy utilization by activating PPARα via adaptor protein containing pleckstrin homology domain (APPL1), and alsop increases fatty acid oxidation and glucose uptake by phosphorylation of AMPK (Deepa et al., 2009). Therefore, the adiponectin system plays an important role in energy homeostasis.

In contrast to leptin, circulating concentrations of adiponectin in obese human and mice are inversely correlated to adipocyte mass (Hu et al., 1996). It should be relalized that adiponectin (μg/ml) and leptin (ng/ml) appear in plasma in a different range of concentration (Bluher et al., 2006). The adiponectin concentrations in lean human are higher than in obese individuals. In obese humans, a drastic reduction of energy intake caused a 33% rise in the average level of adiponectin mRNA, whereas refeeding let to a 32.8% decrease compaired to obese baseline values (Liu et al., 2003). In humans with anorexia nervosa, high plasma adiponectin concentrations were observed and this might be related to the lack of negative feedback exerted by fat mass on adiponectin production and/or to enhanced insulin sensitivity (Delporte et al., 2003). When comparing adiponectin blood concentrations from pre to post-partum, differences were found and the post-partum values were between 12 and 13ng/ml (Raddatz et al., 2008). These concentrations are much lower than those reported for humans (4-20 μg/ml) (Bluher et al., 2006; Liu et al., 2003), and are probably related to the inadequate assay used; validated assays for bovine adiponectin are presently unavailable and the use of human assay systems as done in the quoted study is inappropriate. In addition to its endocrine functions, adiponectin may act in an autocrine or paracrine manner to regulate the function of its receptors in AT (Liu et al., 2008b); the signal transduction pathway will be described in further paragraphs. AdipoR1/R2 were defined as the classical receptors for adiponectin. AdipoR1 is expressed in muscle and appears as a high-affinity receptor for globular adiponectin and a low-affinity receptor for full-length adiponectin, whereas AdipoR2 is abundantly found in liver and serves as an intermediate-affinity receptor for both forms of adiponectin (Kadowaki et al., 2005). Further research showed that both receptors are expressed in muscle, adipocytes (Fasshauer et al., 2004), bovine AT (Lemor et al., 2009) and macrophages (Chinetti et al., 2004). The regulation of both receptors mostly was studied on the mRNA level.

Adiponectin monomers have an amino-terminal collagen-like domain and a carboxy-terminal globular domain that generate trimers, hexamers and high-molecular-weight multimers (Kadowaki et al., 2006). It is also known as AdipoQ, Acrp30, Apm1, or MGC138982 in the bovine species and was characterized by Sato et al. (2001). The bovine adiponectin is a 30kD protein, comprised 240 a.a., with a secretory signal sequence at the *N*-terminal part (a.a.:1–17), a collagenous region (a.a.:43–102) and a globular domain (a.a.:103–240) (UniProtKB/Swiss-Prot: Q3Y5Z3), which are also present in the mouse adiponectin (Scherer et al., 1995). The sequence of the open reading frame in the bovine adiponectin gene is 82.7% (nucleotides, NCBI Acc. No: NM_174742.2) and 83.54% a.a. (NCBI Acc. No:

NP_777167.1) homologous to the human sequence (URL www.genecards.org, (Stelzer et al., 2009)). The genomic DNA (NCBI Acc. No: DQ156120.1) comprises 5460 bp nucleotides, and contains 2 exons.

As mentioned above, AdipoR1/R2 are the classical receptors of adiponectin. AdipoR1/R2 were discovered and cloned from human skeletal muscle cDNA (Yamauchi et al., 2003). The amino acid sequences of both receptors indicated that they are integral membrane proteins, with seven-transmembrane domains, and have an internal *N*-terminus and external *C*-terminus, which is identified to be opposite configured than other reported G-protein coupled receptors. Adiponectin binds to the external *C*-terminus, while the internal *N*-terminus and intracellular domain interacts with a pleckstrin homology domain-containing adaptor protein (APPL1) (Guerre-Millo, 2008). The APPL1 transduces the signalling pathways of AMP kinase (AMPK), PPAR-α and p38 mitogen activated protein kinase (p38-MAPK) (Deepa et al., 2009; Kadowaki et al., 2006). Hexameric and high molecular weight adiponectin also binds to another cell surface protein known as T-cadherin (Hug et al., 2004), with no intracellular domain. It was thought to act as a co-receptor with an unknown binding partner in HMW-adiponectin signalling in specific cell types (Fig. 3). It is abundantly expressed in injured vascular endothelial and smooth muscle cells (Liu et al., 2008a) in atherosclerotic regions, associated with atherosclerosis (Takeuchi et al., 2007). T-cadherin expresion was observed in SC and visceral AT and in muscle, but only muscle mRNA expression was decreased by fasting (Liu et al., 2008a). The bovine AdipoR1 and AdipoR2 comprises 375 and 386 a.a., respectively (AdipoR1, Acc. No.: AAI02260.1: AdipoR2, Acc. No.: AAI10020.1); the multiple alignment (Corpet, 1988) shows 62.12% homology between the a.a. of these two genes. This is comparable to the data known from human and mice with 66.7% homology (Kadowaki et al., 2005).

In dairy cattle restrictive feeding during dry period increased only AdipoR2 mRNA expression in liver after calving (Loor et al., 2006), while the mRNA abundance of both receptors was reduced post-partum in SC AT (Lemor et al., 2009).

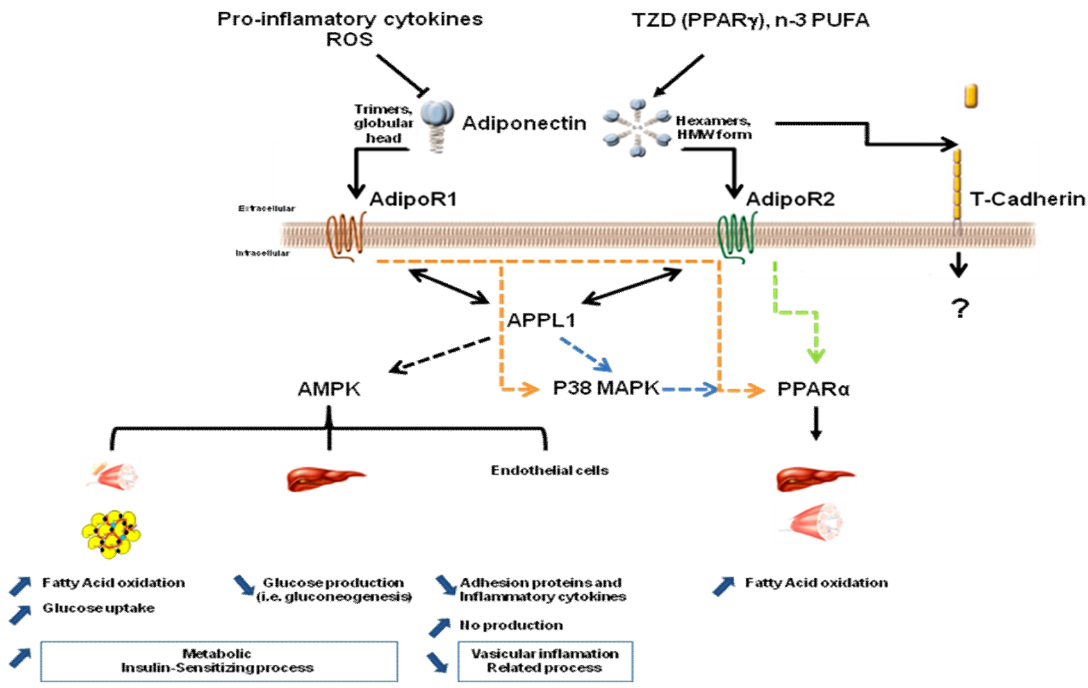

Fig. 4. Schematic representation of adiponectin regulation and currently known mechanisms of metabolic and vascular effects. Abbreviations: ROS: reactive-oxygen species; n-3 PUFA: n-3 polyunsaturated fatty acids; TZD: thiazolidiendiones; NO: nitric oxide; APPL1: a pleckstrin homology domain-containing adaptor protein; AMPK: 5'-AMP-activated kinase; p38 MAPK: p38 mitogen-activated protein kinase (Modified after Deepa et al., 2009; Guerre-Millo, 2008; Kadowaki et al., 2006).

1.2.3. IL-6 as an inflammatory cytokine

In association with other adipokines, IL-6, a multifunctional proinflammatory cytokine, is closely related with both carbohydrate and lipid metabolism (García-Escobar et al., 2010). Effects of IL-6 were observed as early as 1921 (Richards et al., 1995). It was termed B cell differentiation factor in the early 1970's, as it was detected to be responsible for the final maturation of B cells (Schreiber et al., 1995). Approximately 30% of the IL-6 detected in plasma are attributed to the production from white AT; however, most of the adipose derived IL-6 comes from cells of the AT stromal vascular fraction (Galic et al., 2010). Beside skeletal muscle, in which IL-6 stimulated during physical activity (Ara et al., 2010) many other types of cells like monocytes, macrophages, T cells, B cells, mast cells, fibroblast cell and endothelial cells produce IL-6 during acute or chronic inflammation, infection and injury (Ara et al., 2010; Fain, 2010), are another main source of IL-6 (Gustafson et al., 2007). The release of IL-6 by omental AT is higher than by SC AT but the gene expression of IL-6 was higher in

freshly isolated SC AT (Fain et al., 2004). The visceral depots also drain into the portal circulation, therefore the metabolic effects of IL-6 on the liver become important (Pittas et al., 2004). As mentioned above it should be realized that RP blood drains to the systemic circulation via the inferior vena cava (He et al., 2008). The rest of the IL-6 is released from different cell types, including immune cells, fibroblasts, endothelial cells, myocytes, and a variety of endocrine cells (Fain, 2010). In monogastric species like man, the IL-6 mRNA was expressed in heart, brain, lymph nodes, ovaries, breast, and AT (URL www.genecards.org, (Stelzer et al., 2009)). However, IL-6 may also affect fatty acid metabolism in an auto- or paracrine manner, or induce tissue insulin resistance in other peripheral organs (Spranger et al., 2003) thus demonstrating the role of IL-6 and inflammation in metabolism.

The secretion of IL-6 is regulated by several physiological factors, including hormones, cytokines, physical activity, stress, and diet (Ara et al., 2010), and is positively correlated to adipocyte cell size (Gustafson et al., 2007). In AT IL-6 reduces insulin-dependent glycogen synthesis, decreases glucose uptake, increases triglyceride release, and down-regulates lipoprotein lipase, thus promoting obesity, insulin resistant type 2 diabetes and atherosclerosis in humans (Ara et al., 2010; Eder et al., 2009) (Fig. 4). The impaired insulin signalling in mature adipocytes by IL-6 is mediated through decreased tyrosine phosphorylation of key signalling molecules, increased inhibitory serine phosphorylation, and downregulation of the expression of several proteins in the insulin signalling pathway. These effects lead to insulin resistance, increase lipolysis and reduce glucose uptake by the AT (Gustafson et al., 2007), and turn to impair insulin-induced insulin receptor and IRS-1 phosphorylation in adipocytes and hepatocytes of monogastrics (Galic et al., 2010). Thus IL-6 promotes a local insulin resistance including an impaired inhibitory effect of insulin on FFA, release by antagonizing cytokine-induced activation of STAT signalling (Gustafson et al., 2007). Less data is known about IL-6 in cattle. Recently it was shown that IL-6 is expressed at low abundance in adipose depots of cows compared to other inflammatory markers, whereby regulation of IL-6 by a proinflammatory stimulus, i.e. lipopolysaccharide, was shown (Mukesh et al., 2009).

The family of cytokines is characterized through the common receptor subunit gp130 and a four-ahelix bundle structure (Heinrich et al., 1998). IL-6, one of the members of this family, signals through a cell-surface class I cytokine receptor complex consisting of the ligand-binding a subunit (IL-6Ra/gp80), which binds the soluble ligand IL-6 and 2b subunits (gp130), which, through their cytoplasmic domain, function as the signal-transducing component of the complex. Gp130 is the common signal transducer for several cytokines and is al-

most ubiquitously expressed in most tissues. In contrast, the expression of IL-6Ra/gp80 is restricted to certain tissue. IL-6Ra/gp80 also exists in a soluble form designated sIL-6R, and it stabilizes IL-6, promotes the formation of a functional multimolecular complex with gp130, and enhances signalling. This is in contrast to most of the other soluble receptors that trap the ligand and act as antagonists. As IL-6 interacts with its receptor, it triggers the gp130 and IL-6R proteins to form a complex, thus activating the receptor. These complexes bring together the intracellular regions of gp130 to initiate a signal transduction cascade through certain transcription factors, Janus family of kinases (JAK1, JAK2, and TYK2), and signal transducer and activator of transcription (STAT)-3. Binding of STAT-3 to a specific DNA domain promotes the expression of a large variety of genes that affect survival, proliferation, differentiation, osteogenesis/ osteolysis, angiogenesis, and immune modulation. IL-6 activates three different signalling pathways, STAT-3, Erk1/2, and PkB/Akt (Ara et al., 2010). It inhibits insulin signalling by insulin-dependent IRS-1 tyrosine phosphorylation, phosphatidylinositol 3-kinase (PI3K) in association with IRS-1, and protein kinase B activation/AKT (PkB/Akt) (Eder et al., 2009). The full length IL-6 protein are made of 208 a.a with 65, 53, 42 and 42% homology to sequences of porcine, human, mouse and rat IL-6, respectively (Droogmans et al., 1992).

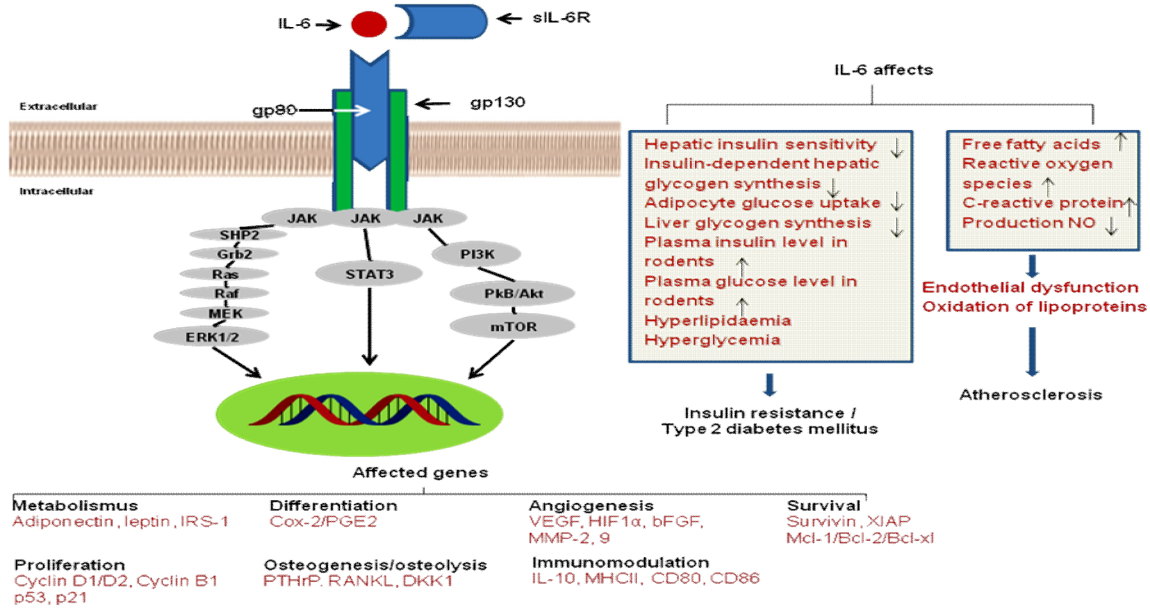

Fig. 5. IL-6-mediated signalling, gene expression, and its cellular effects. IL-6 activates three pathways, STAT-3, Erk1/2, and PkB/Akt, which result in upregulation of a number of genes that affect metabolism, survival, proliferation, differentiation, osteogenesis/osteolysis, angiogenesis and immune modulation, in a variety of target cells and affect insulin sensitivity and accelerate atherosclerosis in humans (modified after Ara et al., 2010; Eder et al., 2009).

1.2.4. PPARγ2 a key regulator of adipogenesis

PPARγ belongs to a class of ligand-dependant nuclear receptor transcription factors and it is the most intensively studied PPAR isoform. The receptor participates in biological pathways of intense basic and clinical interest, such as differentiation, insulin sensitivity, type 2 diabetes, atherosclerosis, and cancer (Rosen et al., 2001). It was shown that PPARγ is involved in energy homeostasis during the feeding-fasting nutritional transition in lean rats (Caimari et al., 2010). It is therapeutically targeted for the prevention and treatment of AT expansion and its associated clinical disorders, including hyperlipemia, hypertension, and type 2 diabetes. Recently, it has been discovered that the PPARs play a central role in the transcriptional control of genes encoding proteins involved in the above processes. Three homologous PPARs, PPARα, PPARβ/δ, and PPARγ are differentially expressed, among which PPARγ displays a dynamic and specific regulation during the differentiation of an adipocytes cell (fibroblast, adipofibroblast or preadipocyte).

PPAR*γ* has the highest expression levels in AT compared with other metabolic organs, such as skeletal muscle, liver, and pancreas. PPAR*γ* activation through insulin sensitizing compounds (thiazolidinediones, (TZDs)) in type 2 diabetic patients improves whole-body insulin sensitivity, leading to reduced insulin and glucose plasma levels in human. The mechanisms of PPAR*γ*-mediated insulin sensitization are complex and are thought to involve specific effects on fat, skeletal muscle, and liver, even though AT appears to be the major target of TZD-mediated effects on insulin sensitivity. The PPARγ includes 4 mRNA isoforms, PPARγ1, 2, 3, 4. The PPARγ2 is the one of the isoforms which functions in adipocyte differentiation through gene regulation. It is highly expressed in AT (Fernyhough et al., 2007), and was cloned and characterized in the bovine species by Sundvold et al. (1997). The highest expression was detected in AT with equal amounts for PPARγ1/2. PPARγ1 was expressed at relatively high levels in bovine spleen and lung and to a lower extent in ovary, mammary gland, and small intestine. The amount of PPARγ2 was apparently lower than that of PPARγ1 in spleen, lung, and ovary. The common cDNA sequence of bovine PPARγ1/γ2 showed strong conservation compared to hamster, human and mouse PPARγ cDNAs, with 98 % a.a. identity. A comparison of the 30 a.a., found in the *N*-terminal extension of the bovine PPARγ2, with the corresponding murine and human sequences showed about 60% identity. The degree of sequence conservation in the bovine PPARγ2 *N*-terminal specific extension

showed 64% identity between the respective human and mouse a.a. sequences (Sundvold et al., 1997).

Apart from its insulin sensitizing effect, PPARγ regulates transcription and translation of genes like GLUT4, IRS-1 (Fernyhough et al., 2007). In this way it plays an important role in regulationg energy balance in AT. In mice, the effect of C3 on FFAR2 expression as an energy balance related gene in association with increased PPARγ2 was addressed (Hong et al., 2005). Previous reports indicated also the close intracellular cross-talk between PPARγ, C/EBP and SREBP1, which affects lipid and fatty acid metabolism in the body and maintenance of AT. PPARγ was shown also to activate the expression of several adipocyte marker proteins, such as FABP4 (Tontonoz et al., 1994) (Fig. 5). To our knowledge no information is available about the PPARγ2 and its relation with energy status in lactating cows.

1.2.5. C/EBPα and its transcriptional effect

One of the putative regulators of lipogenesis and gluconeogenesis is C/EBPs. This family of transcription factors is important in the regulation of genes involved in energy metabolism (Breed et al., 1997) such as leptin, GLUT4 and PPARγ (Fernyhough et al., 2007). The expression of the C/EBPs is regulated under several physiological and pathophysiological conditions through the actions of a number of factors, including cytokines, mitogens, hormones, nutrients and agents that cause cellular stress. They play pivotal roles in a number of processes, including differentiation, the inflammatory response, liver regeneration, metabolism and numerous other cellular responses (Ramji et al., 2002). The therapeutic usage of this family is known. For instance, targeted suppression of one of the members of C/EBP in pancreatic β-cells has been recently proposed for preventing both type 1 and type 2 diabetes (Ramji et al., 2002). The C/EBP family belongs to the large family of basic leucine zipper (bZip) transcription factors. All members of the C/EBP family have a *C*-terminal leucine zipper domain for dimerization and a basic domain for DNA binding, respectively. C/EBPα is one of six members of the C/EBP family of transcription factors, and plays an important role in lipid deposition and adipocyte differentiation (Ramji et al., 2002; Taniguchi et al., 1996). Expression of C/EBPα was observed at high levels in the AT, liver, intestine, lung, adrenal gland, peripheral-blood mononuclear cells and placenta. In the AT and in liver, the highest levels of mRNA are present in the terminally differentiated cells. The expression of C/EBPα transcriptionally affects FABP4, GLUT4 and leptin in adipocytes. The cross regulation between PPARγ and

C/EBPα and their transcriptional effect on adipogenesis and insulin sensitivity was indicated by several studies (Fernyhough et al., 2007; Wu et al., 1999). The bovine C/EBPα gene, like the rat gene, is intron-free and was characterized by Taniguchi et al. (1996). An open reading frame (nucleotide 169-1,230) in the bovine C/EBPα gene encodes a protein of 353 a.a. residues (Taniguchi et al., 1996). The sequence of the open reading frame in the bovine C/EBPα gene is 89.9% (nucleotides) and 92.5% (a.a.) homologous to rat (Taniguchi et al., 1996) and is 94.43% (nucleotides, NM_176784.2) and 95.18% (a.a., NP_789741.2) homologous to human (URL www.gene cards.org, (Stelzer et al., 2009)). Less is known about the regulation of C/EBP family under the different physiological conduction in dairy cows.

1.2.6. SREBP1 and its effect on PPARγ

SREBPs are a family of transcription factors that regulate lipid metabolism (Ma et al., 2010). The SREBP gene in human has two isoforms SREBP1 and SREBP2 with a nucleotide specificity that precisely matches the requirement for sterol-regulated transcription (Wang et al., 1993). SREBP2 is involved in cholesterol synthesis, as opposed to fatty acid synthesis, in liver and AT of mice (Horton et al., 1998b). Three alternatively spliced forms of human SREBP1 mRNA (designated SREBPla, SREBPlb, and SREBPlc) were identified (Yokoyama et al., 1993). In AT of humans the mRNA of SPEBP1c was increased after weight reduction (Kolehmainen et al., 2001). In dairy cattle, the dietary supplementation with unsaturated fatty acids like from sunflower seeds leads to increased milk fat content in spite of decreased de novo milk fat synthesis as supported by a reduction in lipogenic genes like SREBP1 in mammary gland (Møller et al., 2010). Between the various effectors of insulin, the SREBP1 pathway is considered as a system that induces the liver to prepare the carbohydrate availability by increasing the glucose-phosphorylating activity in the liver. The lipogenic capacity in the liver is maintained by SREBP1c. In liver glucose will be transformed to lipids and mainly stored in AT, allowing survival during even longer periods of energy deficit. This gene has allowed species survival in the context of successive food availability and restriction. In association with metabolic disease it should also be considered from an opposite view. On the one hand it acts as transcription factor to the genomic actions of insulin on carbohydrate and lipid metabolism, and on the other hand, a loss of function leads to impaired insulin sensitivity (Foufelle et al., 2002). Therefore, it plays a central role in energy homeostasis by promoting glycolysis, lipogenesis, and adipogenesis (Hoashi et al., 2007), and it is affected dramatically

upon nutritional status (Caimari et al., 2010); this parallels closely the regulation of two adipocyte genes that are crucial in energy homeostasis, fatty acid synthetase (FAS) and leptin (Kim et al., 1998). Under different physiological situations, the expression of SREBP1 affects PPARγ, a key regulator of energy homeostasis (Fig. 5).

In humans, SREBP1a and 1c transcripts are produced through the use of alternative transcription sites and differ in their first exon (exon 1a and exon 1c) and their last two exons (exon 18a and 19a or exon 18c and 19c) (Hoashi et al., 2007). The tissue expression pattern of SREBP1a and SREBP1c expression differs; SREBP1c is the predominant isoform *in vivo*, and is expressed in liver, adrenal gland, AT, brain, kidney, muscle and pancreas, with highest expression in liver and AT (Shimomura et al., 1997). SREBP1a is the predominant isoform in proliferative tissues such as spleen, testis and ileum. As SREBP1a has higher transcriptional activity compared with SREBP1c, the baseline ratio of SREBP1a and SREBP1c may be particularly important in regulation of gene transcription (Hagen et al., 2010).

The SREBPs are endodoplasmatic reticulum membrane-bound transcription factors of the basic-helix-loop-helix-leucine zipper family that were shown to regulate gene expression of several enzymes implicated in cholesterol, lipid, and glucose metabolism (Foufelle et al., 2002; Osborne, 2000). The bovine SREBP1 gene was characterized by Hoashi et al. (2007), and its a.a. sequence has high homology with those of human SREBP1a (82.9%) and 1c (71.9%), as well as mice (78.5%) and rats (76.5%). During the early stages of differentiation, SREBP1 affects the transcriptional activity of PPARγ (Fig. 5), and also the insulin signalling and modulation of insulin sensitivity is affected via PPARγ (Leonardini et al., 2009). The feeding-fasting nutritional transition affects SREBP1 mRNA abundance in liver and SC AT of mice and obese humans in a time dependent manner, respectively (Horton et al., 1998a; Kolehmainen et al., 2001). Energy balance related genes such as SREBP1, PPARγ in SC and retroperitoneal AT are rarely studied in bovine, except for perimuscular preadipocytes during differentiation (Taniguchi et al., 2008). Several recent reports indicated the repression of SREBP1 after feeding supplementary unsaturated fatty acids and the stimulated expression during the lactation period in mammary tissue of dairy cattle (Møller et al., 2010; Ren et al., 2010).

1.2.7. IRS-1 and GLUT4 and their relation with insulin sensitivity

Dietary carbohydrates are mostly converted to volatile fatty acids in the rumen of ruminants, however, only a little amount of glucose is absorbed. The most important substrate for glucose synthesis in ruminants is propionate. Ruminal propionate may account for more than half of the substrate used for glucose synthesis in fed animals. In cattle, propionate may account for 50–60% of the glucose and 11–35% of the lactate production. In fasted animals less propionate is available, and the glucose producing organs must find endogenous sources as substrate for gluconeogenesis. In this situation, glycerol produced from lipolysis becomes a more important glucose precursor; its contribution may reach 40% during fasting. The organs that release glucose into the blood are liver, gut, skeletal muscle and kidney, among which the liver is the most important glucose-producing organ (85–90%) in the ruminant. Different organs of ruminants like sheep utilize glucouse in different rates (muscle, 20–40%; tail fat pad, 10%; uterus, 8% to 30%; mammary gland, 25–50% depending to the stage of pregnancy or milk) according to their blood flow (Brockman, 2005). Insulin appears to increase glucose uptake by the help of two elements (IRS-1 and GLUT4), which will be discussed in details in the further paragraph.

Insulin acts by binding to its cell surface receptor and phosphorylates intracellular substrates, which include the insulin receptor substrates (IRS) family and Shc (a common substrate of tyrosine kinase receptors). The IRS protein family is characterized by *C*-terminal pleckstrin-homology and phosphotyrosine-binding domains, which are required for high-efficiency coupling to the activated insulin receptor, and an *N*-terminal region with multiple sites of tyrosine phosphorylation (Myers et al., 1996). Tyrosine phosphorylation of IRS-1 and Shc on the insulin receptor activates phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling. The combination of these pathways regulates glucose transport by GLUT4 translocation at the plasma membrane (Khan et al., 2002), glycogen synthesis via protein kinase B-mediated inhibitory phosphorylation of glycogen synthase kinase-3, which negatively regulates glycogen syntheses (Cross et al., 1995), lipogenesis via up-regulation of the expression of the fatty acid synthase gene (Bourlier et al., 2008) and protein metabolism, and in general controls the gene expression patterns (Fig. 5).

As mentioned in the previous chapters, insulin resistance is defined as a pathologic state of decreased responsiveness of target tissues to normal circulating levels of insulin. The pathophysiology of insulin resistance involves the complex network of signalling pathways, acti-

vated by the insulin receptor, which regulates intermediary metabolism in monogastrics. In ruminants, the secretion of insulin follows the infusion of C3 (Lee et al., 2002), however insulin secretion showns divergent response to acetate (Harmon, 1992). Insulin also increases leptin mRNA, one of the energy balance related factors, in bovine AT (Houseknecht et al., 2000). The skeletal muscle is the principal site of glucose uptake under insulin-stimulated conditions in human i*n vivo* studies, whereas less glucose is metabolized by AT (Sesti, 2006). As mentioned above, the insulin signal transduction depends on IRS-1 and GLUT4 in monogastric species. The IRS-1 is one of the best characterized and known members of four intracellular IRS proteins in monogastrics (White, 1998). Analogous information is not entirely available for cattle although the whole genomic DNA was recently identified by Zimin et al. (2009) in the bovine species. It is known that in adipose cells of rats, IRS-1 mediates the stimulatory effect of insulin on GLUT4 (Im et al., 2006) and GLUT4 in turn regulates glucose transport by translocation to the plasma membrane (Khan et al., 2002). In coming paragraphs, the family of facilitative glucose transporters will be discussed in detail. In general, gluconeogenesis in liver is the main source of glucose in ruminants, and GLUT4 is an insulin dependent glucose transporter. Reports indicated that the mechanisms underlying an increase in insulin sensitivity are related to adiponectin increased total GLUT4 expression and the number of GLUT4 transporters acutely recruited to the plasma membrane in response to insulin (Fu et al., 2005). Activation of the insulin receptor evokes increased transcription of SREBP and PPAR (Oliver et al., 2010). Observatory reports indicated that although GLUT4 mRNA is affected by insulin in monogastrics *in vivo*, cultured adipocytes have to be stimulated with dexamethasone in addition to insulin (Hajduch et al., 1995); the effect of insulin could be nevertheless be different *in vivo*. However, in ruminants, 6 h hyperinsulinemia did not show any effect on GLUT4 protein abundance in caprine AT or skeletal muscles (Balage et al., 1998). GLUT4 mRNA was detected in skeletal muscle, heart, and AT, but not in liver, kidney, lung, brain, or spleen, which is essentially the same as in humans and rodents.

The family of facilitative glucose transporters (GLUTs) composed of five isoforms (GLUT1-4 and GLUTX1), was described to clear glucose from the bloodstream into cells of target tissues like muscle and adipose (Watson et al., 2001). GLUT4 is the major insulin-dependent glucose transporter in adipocytes, muscle and heart, whereby glucose uptake by AT is less than in skeletal muscle (Olefsky, 1999). The bovine GLUT4 was cloned and characterized by Abe et al. (1997), and is expressed in muscle and AT. Bovine GLUT4 is composed of 509 a.a. and is 64% and 92% identical with bovine GLUT1 and rats GLUT4, respectively.

Less is known about the role of insulin signalling and GLUT4 in the AT of ruminants like bovine, but it might be relevant for glycogen synthesis in monogastric species. In these species the insulin signalling leads to the activation of glycogen synthase, which might be also relevant for bovine. Therefore, higher blood concentration of glucose in AT of ruminants after feeding might be relevant for glycogen synthesis in AT (Fig. 5), in which glycogen metabolism might function as a metabolic switch between feeding and fasting and/or regulation of lipogenesis and lipolysis (Markan et al., 2010).

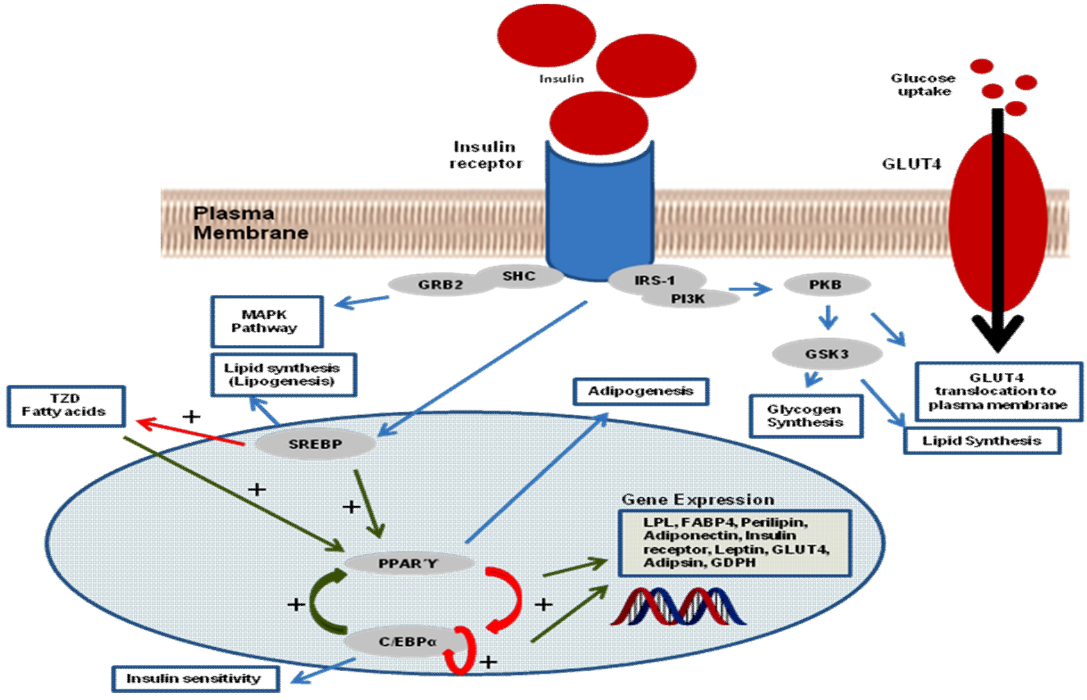

Fig. 6. Activation of the insulin receptor evokes increased transcription of SREBP and PPARγ. Tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and SHC on the insulin receptor activate phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling. Growth factor receptorbound protein-2 (GRB2), protein kinase B (PKB), glycogen synthase kinase-3 (GSK3), signalling factors that both directly and indirectly regulate PPARγ. Note that PPARγ is an indirect regulator of itself through its action on C/EBPα. Green arrows indicate direct regulation; red arrows indicate indirect regulation. (Modified after Fernyhough et al., 2007; Oliver et al., 2010; Rosen et al., 2002).

Less known about the regulation of the aforementioned energy balance related genes discussed in adipose tissue in relation to energy status in ruminant like dairy cattle. Some of the studies discussed in the present thesis demonstrated the genes in view of type 2 diabetis in human and rodents in detail. However, these studies cannot be conveyed to dairy cattle.

In modern animal husbandry, milk and beef production in association with improved welfare of dairy cattle are of special interest. To maintain milk production and gain the highest economical benefit, the pregnancy of cow is necessary. During the transition from pregnancy to lactation, particularly in high-yielding dairy cows, the energy balance is of importance for health, production, and profitability. As discussed before, under different physiological states the AT as the energy storage and endocrine organ plays a crucial role. The optimal expansion of AT is also of importance for beef cattle industry.

Therefore understanding the transcriptional pattern of the aforementioned energy related genes in AT under dynamic physiological regulation is highly relevant. However, it is important to know that the interpretation of the gene regulation in ruminant according to the monogastric gene regulation under different physiological conditions is not possible due to the differences between species (Hishikawa et al., 2005). Therefore, herein we aimed to study:

1. The effect of C3 and BHB on genes of interest (GOI) in bovine SC and RP AT, establishing an AT explant model.
2. To obtain accurate results concering the differential effect of C3 and BHB on GOI, by optimizing the evaluation of qPCR data through selecting appropriate RGs in these explants.

The goal of the first approach was to improve the knowledge about insulin dependent and independent effects of C3 and BHB on the genes of interest. The goal of the second approach was to improve the reliability of qPCR data performing accurate normalization. In addition, to address the cell type expressing the genes C/EBPα, FFAR2, FFAR3, FABP4 and PPARγ2 increasing during differentiation, we established a bovine primary preadipocyte culture.

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Putative reference genes for gene expression studies in propionate and ß-hydroxybutyrate treated bovine adipose tissue explants

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Abstract

Accurate gene expression normalization using a stable reference gene (RG) improves the reliability of quantitative real-time PCR (qPCR) results. Therefore, a validation of RGs should be done before their use. Only few studies on RGs have been done in cattle, and none in bovine adipose tissue (AT) explants, therefore, we characterize the effects of an *in vitro* treatment with propionate and ß-hydroxybutyric acid (BHB) on the mRNA content of these RGs comparing the output data from the geNorm™ and the Normfinder© program. geNorm™ and Normfinder© estimated the most stable RGs in the following sequence for subcutaneous and for retroperitoneal AT explants treated with propionate: low density lipoprotein receptor-related protein 10 (LRP10) > hippocalcin-like 1 (HPCAL1) > glyceraldehyde-phosphate-dehydrogen- ase (GAPDH) > ribosomal protein S9 (RPS9) > RNA polymerase II (Pol II) > beta2 actin (ACTB) > 18S ribosomal RNA (18S rRNA). BHB treated AT explants yielded a different stability ranking for RGs using geNorm™: HPCAL1, GAPDH > Pol II > LRP10 > ACTB > RPS9 > 18S rRNA. Normfinder© estimated a different stability ranking for the RGs as shown in the following sequence for subcutaneous and retroperitoneal AT explants treated with BHB: HPCAL1 > Pol II > GAPDH > ACTB > LRP10 > RPS9 > 18S rRNA. Subsequent pairwise analysis of variation of RGs using geNorm™ suggested that LRP10, HPCAL1 and GAPDH should be used for accurate normalization of subcutaneous and retroperitoneal AT explants treated with propionate, while HPCAL1, GAPDH and Pol II should be used for BHB treatment.

Key words: reference gene, adipose tissue explants, propionate, ß-hydroxybutyrate

Introduction

Adipose tissue (AT) and its functions both as a storage organ and as an endocrine gland is increasingly recognized as being of major importance for the adaptational capability of dairy animals to the needs of early lactation. Aiming to characterize the regulatory role of nutrients for gene expression in bovine AT, we have established a tissue explant system in which several variables can be tested. However, we realized that the standardization of mRNA expression data commonly done by using the abundance of one so called reference gene (RG) might be inappropriate at least in this explant model and we therefore did the study reported herein.

Different studies evidence the role of SCFA like propionate on the abundance of mRNAs encoding regulatory relevant genes in ruminant AT *in vivo* and *in vitro* (Soliman et al. 2007; Mielenz et al. 2008). In addition, BHB inhibits lipolysis in cattle (Metz et al. 1974) and affects lipid metabolism (Bjorntorp and Schersten 1967). Quantification of specific mRNAs has been extensively applied for both *in vivo* and *in vitro* adipocytokine expression studies in ruminant AT explants stimulated with growth hormone (Houseknecht et al. 2000), or in adipocytes stimulated with SCFA (Soliman et al. 2007). qPCR provides good sensitivity, easy determination of efficiency and excellent reproducibility. Critical to the application of qPCR is normalization of target gene expression. Until now some studies in bovine mammary gland identified stable RGs using only one software (Piantoni et al. 2008; Kadegowda et al. 2009) but not for bovine AT or bovine AT explants. Universally, a RG is considered as optimal for normalization when it is constitutively expressed at a constant level (Vandesompele et al. 2002). However, Tricarico et al. (2002) demonstrated that no RG can be purported to be suitable for any given set of conditions. The current consensus is that multiple stably expressed RGs are required for reliable normalization and are particularly recommended for measuring differences of expression in situations in which a single suitable gene could not be found (Vandesompele et al. 2002).

Accordingly, a number of statistical programs, delivering potentially different results has been developed to assess the appropriateness of RGs. For instance, geNorm™ determines the expression stability of non-normalized control genes by assigning for each gene a gene stability measure (*M*). Another program, Normfinder©, uses a model based approach to rank all RGs based on inter- and intra-group expression variations (Andersen et al. 2004). In addition, pairwise analysis of variation is recommended by geNorm™. As there is no universally accepted method to analyze the applicability of common stable RGs, the present study aimed to

validate the stability of two novel (LRP10 and HPCAL1) and five frequently used RGs in the bovine species comparing the output of these two programs for samples from an experiment in which RNA was extracted from subcutaneous and retroperitoneal AT explants derived from a typical dairy breed and treated with either propionate or BHB. Furthermore, we will demonstrate that the analysis of a limited data set from a preliminary test is sufficient to validate the larger data set for selecting the stable RGs.

Materials and methods

Subcutaneous and retroperitoneal AT was obtained from sixteen Holstein–Friesian cows showing comparable depots sizes at the local abbatoir. The incubations were performed in duplicate with 4.5 mL of DMEM/Ham's F-12 with L-Glutamine (PAA, Colbe, Germany), supplemented with 100 µg/mL of streptomycin and 50 µg/mL of gentamicin as a basal medium, medium with insulin (100 nM), media with different propionate (Sigma-Aldrich Chemie, Taufkirchen, Germany) contents (0.5, 1, 2, and 3 mM) and media with different DL-ß-hydroxybutyric acid sodium salt (Sigma-Aldrich Chemie) concentrations (0.5, 3 and 10 mM) for 4 h, at 37°C and 5% CO_2. Afterwards, tissue explants (200 mg) were frozen in liquid nitrogen. Tissue homogenization was performed after adding 1 mL of Trizol (Invitrogen, Karlsruhe, Germany) using Precellys® homogenizator (Peqlab, Erlangen, Germany). Total RNA extraction was done. Subsequently, total RNA was purified using RNeasy Mini Kit (Qiagen, Hilden, Germany) after DNase digestion using RNase-free DNase set (Qiagen) in solution. RNA integrity based on the ratio of 28S to 18S rRNA was assessed by SYBR Green II (Invitrogen) RNA denaturing gel electrophoresis according to the instruction of manufacture. RNA quantification was performed using the RiboGreen® RNA Assay Kit (Invitrogen) in a Mx3000P-cycler (Stratagene, Amsterdam, Netherlands) and Nanodrop ND-1000 (Peqlab) based on the manufacture instruction. First strand cDNA was synthesized from 200 ng RNA using Revert Aid reverse transcriptase (Fermentas, St. Leon-Rot, Germany) and 250 pmol of random hexamer primer in a final volume of 20 µL. No-RT controls were performed by omitting reverse transcription, and no template controls were conducted by adding nuclease free water. The reverse transcribed cDNAs were diluted 1:4 using ultra pure H_2O except the cDNA which was used for 18S rRNA analysis (1:400); and kept at -20°C for further use. qPCR was conducted in a Mx3000P-cycler (Stratagene). Quantification of mRNA was accomplished through PCR amplicon standard curves for the purified and sequenced RGs (18S

rRNA, ACTB, GAPDH, HPCAL1, Pol II, LRP10 and RPS9) using SYBR Green JumpStart™ (Sigma-Aldrich Chemie) in total volume of 10 µL per reaction. A melting curve was produced to confirm the specificity and purity of the PCR product. In addition, a pooled cDNA was used as an inter-run calibrator. Primer design was conducted for ACTB, GAPDH, HPCAL1, LRP10, and RPS9 using Primer3 (v. 0.4.0) software (Rozen and Skaletsky 2000). 18S rRNA and Pol II primers were derived from Thielen et al. (2007) and Murani et al. (2007), respectively. qPCR cycling conditions were the same for all the primer pairs except annealing temperatures and cycles number: initial denaturation at 95°C for 10' followed by different cycle numbers (Table 1), denaturation at 95°C for 30", annealing temperature (Table 1) for 30" and extension at 72°C for 30".

Statistical analyses were carried out using geNorm™ (Vandesompele et al. 2002) and Normfinder© (Andersen et al. 2004) to determine the most stable genes out of seven analyzed RGs in AT explants from four animals treated with propionate (n=24) and BHB (n=20). In detail, geNorm™ provides an algorithm which calculates all possible pairwise combinations of expression ratios for each gene versus all other genes in each sample as a gene instability measure (*M*), and genes with the highest *M* value (i.e. least stable expression) are progressively eliminated until the two most stably expressed genes remain, regardless of the experimental conditions or cell type. Moreover, normalization using geNorm™ can also be complemented by limited sample input amounts. Afterwards, normalization factors based on the geometric mean of the expression levels of the best "*n*" RGs are calculated (Vandesompele et al. 2002). In addition, analysis of pairwise variations of the most stable RGs (i.e. LRP10, HPCAL1 and GAPDH for propionate; HPCAL1, GAPDH and Pol II for BHB) was performed in the data set used for validation. The additional seven (propionate, n=84) and five (BHB, n=50) animals were analyzed for the set of samples to reinforce the pairwise variations results obtained from the four animals in the panel of seven analyzed RGs. In contrast to geNorm™, Normfinder© automatically calculates the stability value for all candidate normalization genes tested on a sample set containing any number of samples organized in any given number of groups. In addition, nonparametric Spearman rank correlation coefficients (correlations with $P < 0.01$ were considered significant) were calculated as recommended earlier (Vandesompele et al. 2002) using SPSS 14.0 (SPSS Science Software, Erkrath, Germany).

Results

Internal gene stability measure, *M* was determined as the average pairwise variation of a particular gene with all other RGs using the geNorm™. The program estimated *M* values of the most stable RGs for subcutaneous and retroperitoneal AT explants treated with propionate, as shown in Fig. 1a. RGs with the lowest *M* value indicate the most stable expression, and vice versa. The stepwise exclusion of the RGs with the least stable expression showed that LRP10 and HPCAL1 (M=0.47) were the most stably expressed RGs in the samples analyzed. The conventionally used RG 18S rRNA proved to be quite unstable in AT explants treated with different concentrations of propionate.

In contrast to propionate treated AT explants, BHB treatment of subcutaneous and retroperitoneal AT explants yielded different ranking stability values. Fig. 1c shows stability ranking of RGs estimated by geNorm™. 18S rRNA remained the least stable, whereas GAPDH and HPCAL1 (M=0.34) were the ones with the lowest *M*, indicating that they were most stably expressed in BHB treated bovine subcutaneous and retroperitoneal AT explants. The pairwise variations (Vn/ Vn+1) were calculated between two sequential normalization factors (NFn and NFn+1), reflecting the effect of adding an $(n+1)^{th}$ RG (Vandesompele et al. 2002). The pairwise determination of variation V2/3 and V3/4 equaled 0.193 and 0.168 for propionate, and 0.198 and 0.170 for BHB treated bovine AT explants (Fig. 2a, 2b), while the pairwise variation V2/3 equaled 0.198 for propionate and 0.191 for BHB treated AT explants in the data set used for validation. Spearman rank correlation coefficients (r) were calculated for NF2 and NF3 values (r=0.85, r=0.90) and for NF3 and NF4 values (r=0.93, r=0.76), for propionate and BHB treated AT explants, respectively. Furthermore, r values equaled 0.97 for propionate and 0.94 for BHB bovine AT explants for NF2 and NF3 in the data set used for validation.

Normfinder© estimated the most stable RGs in the same sequence for propionate treated bovine subcutaneous and retroperitoneal AT explants, among which the top ranked candidate was LRP10 (Fig. 1b). Normfinder© estimated values of the most stable RGs for subcutaneous and retroperitoneal AT explants treated with BHB in a different ranking sequence compared to propionate treatment (Fig. 1d).

Discussion

The current study was carried out using a set of reliable RGs which differ depending on the stimulant. Applying different statistical methods might yield different results thereby potentially confusing the users. Our results from gene expression stability analyses using geNorm™ and Normfinder© in propionate treated AT explants showed that 18S rRNA was the least stable RG. However, the data about the gene expression stability from different studies are controversial (De Ketelaere et al. 2006; Johnson et al. 1995). geNorm™ estimated LRP10 and HPCAL1 to be the most stable RGs in AT explants treated with propionate. Normfinder© also estimated LRP10 as the most stable gene, with the same sequence of the following RGs, as found with geNorm™. However, only the best RG is recommended by this program as the one to be used for normalization. In agreement with our results, LRP10 and HPCAL1 were identified as being most stably expressed in human AT (Gabrielsson et al. 2005). Comparing the model based approach using Normfinder© (Andersen et al. 2004) with the pairwise comparison approach by geNorm™ (Vandesompele et al. 2002), we believe that both programs are well applicable for precisely analyzing the qPCR data as to define a set of adequate RGs for data normalization in the used model. Similar to previous studies (Skovgaard et al. 2007), and also supported by our analysis of data, we emphasize that multiple stably expressed RGs should be used for reliable normalization in such a model as the AT explants treated with propionate.

For determining the optimal number of RG, pairwise variation analysis was recommended and performed by the integrated analysis of geNorm™ (Vandesompele et al. 2002). The analysis showed that V2/3 and V3/4 equaled 0.193 and 0.168, respectively. Pairwise variation (V) of 0.15 has been recommended as an arbitrary cut-off point below which the inclusion of additional RGs expression is not required (Vandesompele et al. 2002). This arbitrary degree of RG expression stability could not be achieved for AT in our and in others´ studies (De Ketelaere et al. 2006; Ayers et al. 2007), which increased the recommended cut-off point for pairwise variation (Vn/ Vn+1) to 0.2 for AT and bovine polymorphonuclear leukocytes. Our study reached a cut-off point V2/3=0.193 using three most stable RGs LRP10, GAPDH, and HPCAL1 for propionate treated AT explants. This is in contrast with Ayers et al. (2007) who recommended to use eight RGs for reliable normalization in canine AT which might depend on the gene panel they used or on their specific experimental model. We found no improvement regarding to the defined cut-off point of 0.2 by including a fourth RG which was in ac-

cordance to the theoretical performance proposed by Vandesompele et al. (2002). In addition, by analyzing the effect of adding a fourth RG in propionate treated AT by Spearman rank correlation, we observed only a marginal difference between the *r* values (*r*=0.85 for NF2 and NF3 vs. *r*=0.93 for NF3 and NF4), showing again that the use of a fourth RG would not significantly improve the quality of the normalization. Different from the stimulation with propionate, geNorm™ estimated that in the BHB treated AT explants, GAPDH and HPCAL1 were the most stable genes, whereas 18S rRNA still was the most variable one. In contrast to the results of geNorm™, Normfinder© estimated HPCAL1 as the most stable RG in BHB treated AT explants, with 18S rRNA as the most variable one as well. Interestingly, the result from the model-based approach by Normfinder© and from the pairwise comparison approach by geNorm™ indicated different rankings for the stability of expression in BHB-treated AT explants.

Pairwise analysis of variation was performed for BHB treated AT explants. The results indicated that V2/3 and V3/4 equaled 0.198 and 0.170, respectively, which is below the recommended cut-off point 0.2 (De Ketelaere et al. 2006; Ayers et al. 2007), below which the inclusion of additional RGs expression is not required (Vandesompele et al. 2002). Our study reached a cut-off point (V2/3=0.198) using the three most stable RGs, i.e. GAPDH, HPCAL1 and Pol II for BHB treated AT explants. As discussed before, the inclusion of the fourth RG (LRP10) had no significant effect for the calculation of a reliable normalization factor. This estimation was reinforced by Spearman rank correlation showing a higher correlation for NF2 and NF3 (*r*=0.90) than for NF3 and NF4 (*r*=0.76). Therefore, in accordance to the suggestions of Vandesompele et al. (2002), we decided to use only three RGs.

The validation of the data set with an enlarged number of animals (n=7 for propionate; n=5 for BHB) in comparison to two animals per treatment showed that there is no difference between the results. Therefore, as a result from our study the adequacy of using a smaller subset of samples for identifying suitable RGs from a larger assortment of potential RGs was confirmed. Besides its validity, this approach implies efficient use of resources available for research.

Comparing the statistical base and the results obtained by the model-based approach from Normfinder© (Andersen et al. 2004) versus the pairwise comparison approach by geNorm™ (Vandesompele et al. 2002) for propionate and BHB treated AT explants, we believe, similar to others (Skovgaard et al. 2007), that normalization based on a single top ranked gene admit-

tedly is less labor intensive but may also introduce more variation than using the geometric mean of three well ranked genes as provided by geNorm^{TM}.

In summary, we compared two treatments in bovine AT *in vitro* in which the SCFA propionate represents a situation of positive energy balance, and the ketone body BHB, as an indicator of lipolysis, increases during negative energy balance and concomitantly adjust lipolytic activity. The outputs of two programs for the selection of RGs were compared. We found that cautious selection of a panel of RGs is necessary for all the experimental setups. Expression stabilities of RGs differ depending on different nutritional conditions that we liked to demonstrate by our treatments.

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Table 1: Primer sequences and conditions

Name	Acc. No.	Forward Primer Sequence [5′-3′] Reverse Primer Sequence [5′-3′]	Len. (bp)	Ta. (°C)	Ct.
18S rRNA	AF176811	GAGAAACGGCTACCACATCC CACCAGACTTGCCCTCCA	170	58	16.81
ACTB	AY141970	CTCTTCCAGCCTTCCTTCCT GGGCAGTGATCTCTTTCTGC	178	60	21.18
GAPDH	U85042	AATGGAAAGGCCATCACCATC GTGGTTCACGCCCATCACA	204	59	21.63
HPCAL1	NM_001098964	CCATCGACTTCAGGGAGTTC CGTCGAGGTCATACATGCTG	99	60	28.22
Pol II	X63564	GAAGGGGGAGAGACAAACTG GGGAGGAAGAAGAAAAAGGG	86	58	21
LRP10	BC149232	CCAGAGGATGAGGACGATGT ATAGGGTTGCTGTCCCTGTG	139	61	23.35
RPS9	NM_001101152	GTGAGGTCTGGAGGGTCAAA GGGCATTACCTTCGAACAGA	108	62	25.86

Fragment length (Len.), annealing temperature (Ta), median cycle threshold (Ct.), accession number (Acc. No)

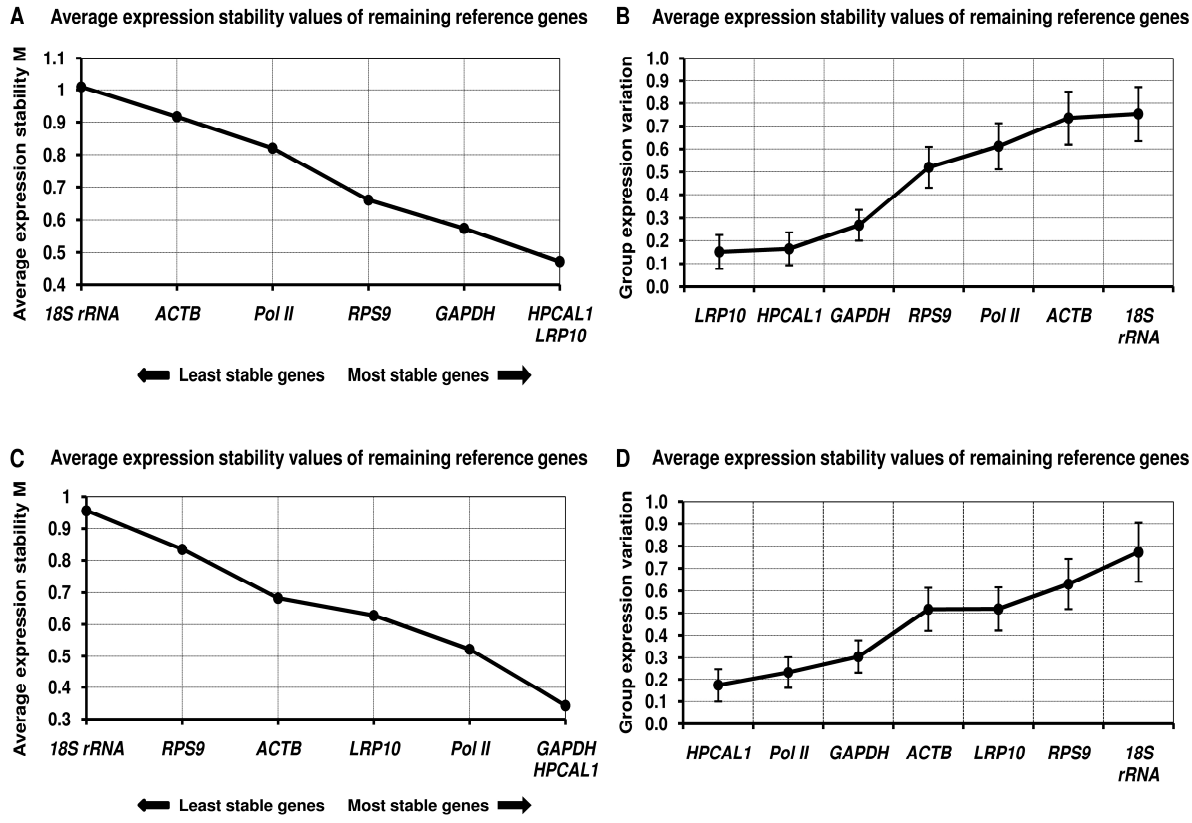

Fig. 1. Average expression stability value (*M*) of seven RGs calculated by geNorm™ and Normfinder©. Average expression stability ranking (*M*) estimated by geNorm™ for propionate (a) and BHB (c) treated adipose tissue explants, inter- and intra-group expression variations calculated by Normfinder© for propionate (b) and BHB (d) treated adipose tissue explants. RGs with lower average stability expression (*M*) and inter- and intra-group expression variation (shown as group expression variation) indicate higher stability expression.

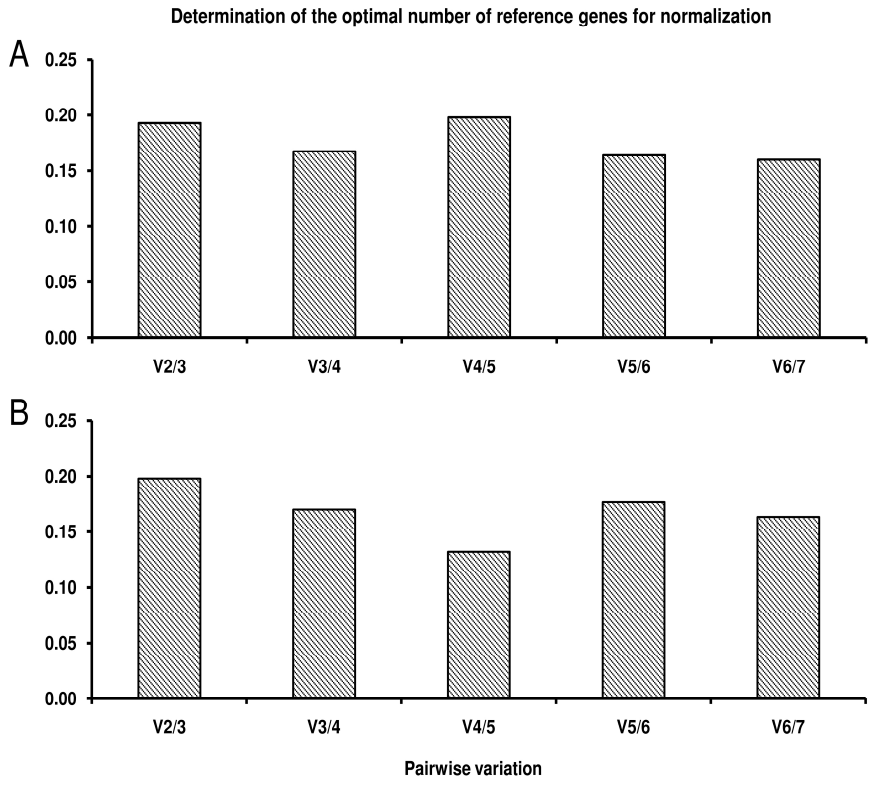

Fig. 2. Determination of the optimal number of RGs for normalization using geNorm™. Ratio of pairwise variations (Vn/ Vn+1) for every series of normalization factors to determine the optimal number of RGs for accurate normalization in propionate (a) and BHB (b) bovine adipose tissue explants.

3. Manuscript 2 (to be submitted)

Expression analysis of key genes related to nutrient sensing and insulin sensitivity in bovine white adipose tissue explants from subcutaneous and visceral depots stimulated with propionate or ß-hydroxybutyrate and in differentiating bovine preadipocytes

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Abstract

Ruminants rely on short chain fatty acids (SCFA) as principal energy source. Adipose tissue (AT) is classically known as an energy storage organ; above that, it gains increasing interest as a regulatory active gland secreting multiple endocrine and auto/paracrine hormones. Herein, we compared the effects of propionate (C3), β-hydroxybutyrate (BHB) and insulin on mRNA abundance of energy balance related genes by short term incubation (4 h) in bovine AT explants from one subcutaneous (SC) and one visceral fat depot *in vitro*. Propionate increased the mRNA abundance of adiponectin receptor 1 (AdipoR1), AdipoR2 and PPARγ2 in treated samples versus controls (Treat. vs. Con.) in SC AT explants, while it suppressed mRNA abundance of insulin receptor substrate 1 (IRS-1) in retroperitoneal (RP) AT. A trend ($P \leq 0.15$) for an increase of mRNA abundance of AdipoR1, AdipoR2, C/EBPα, PPARγ2, facilitated glucose transporter 4 (GLUT4), and sterol regulatory element-binding protein 1 (SREBP1) in response to C3 was exclusively observed in SC AT. In RP AT, C3 showed a trend for increasing mRNA abundance of AdipoR1, C/EBPα, GLUT4 and for decreasing of IL-6. With BHB treatment, there was a trend for decreased mRNA abundance of adiponectin and AdipoR1 in SC AT, and for increasing mRNA abundance of adiponectin in RP AT. The insulin-induced alterations were limited to the mRNAs of free fatty acid receptor 3 (FFAR3) and IL-6 in SC and RP AT, respectively, for which a trend for increased abundances was recorded. To address the cell type mainly expressing the genes of our interest, we established a bovine primary preadipocyte culture and demonstrated an increase in mRNA abundance of C/EBPα, FFAR2, FFAR3, fatty acid binding protein (FABP4) and PPARγ2 during differentiation. Our results show that *in vitro* stimulation of AT explants with different concentrations of C3, BHB or insulin during short-term treatment exerts divergent effects in both depots on the mRNA abundance of energy balance related genes. Adiponectin receptor mRNA was less affected by BHB than by C3 in the bovine explant model, indicating that the bovine adiponectin system might be more sensitive to C3 than to BHB. In contrast to other studies, we demonstrated the presence of FFAR2 and FFAR3 mRNA not only in both AT depots but also in differentiated preadipocytes isolated from bovine SC AT.

Key words: Propionate, β-hydroxybutyrate, cattle, energy balance related genes, nutrient sensing receptors

1. **Introduction**

Besides storage of energy, adipose tissue (AT) is also considered also as an endocrine organ, secreting different factors, the so-called adipokines. Adipokines regulate insulin signalling, food intake, energy expenditure and inflammatory processes [1]. Short chain fatty acids (SCFA), i.e. acetate, propionate (C3) and butyrate, are the main energy source for ruminants, whereby C3 from the portal vein is efficiently extracted (80 – 85%) by the liver [2], and provides the main substrate for hepatic gluconeogenesis. Propionate stimulates insulin secretion directly [3]; about 20% of the absorbed C3 reach the circulation; the peripheral C3 serum concentrations reportedly range between 0.06 – 0.08 mM [4]. Using C3 as *in vitro* stimulus in tissue explants or cell culture allows for dissecting C3 and insulin effects. Short chain fatty acids like C3 affect the abundance of mRNAs encoding genes related to energy metabolism in ruminant AT *in vivo* and *in vitro* [5,6]. Nevertheless, the transcriptional response of subcutaneous (SC) and retroperitoneal (RP) AT depots in relation to energy signalling responsiveness in dairy cattle is largely unknown. AT composed of adipocytes, stromal vascular cells including preadipocytes and the AT matrix, produces various proteins which influence insulin sensitivity and inflammation in both an endocrine, and an auto/paracrine manner [7]. Different factors affect the adipocyte and matrix regulatory mechanisms of genes involved in energy utilization and expenditure *in vivo* or *in vitro*. Depot-dependent differences in mRNA expression of energy balance related genes in AT (e.g. adiponectin system related genes) were addressed [8]. Short chain fatty acids are ligands for the human G protein-coupled receptors 41/43, alternatively called free fatty acid binding receptors 3/2 (FFAR2/3). Activation of FFAR2 and FFAR3 by SCFA stimulates different signalling pathways, and both receptors have tissue specific expression patterns [9]. Infusion of C3 *in vivo* augments the mRNA abundance of FFAR3 in SC but not in perirenal caprine AT [6], which might be a direct C3 effect or an indirect one mediated by the C3 stimulated insulin release [3]. Activation of FFAR2 in adipocytes will lead to inhibition of lipolysis [10], and both receptors are involved in monitoring energy storage. We previously observed that FFAR3 but not FFAR2 mRNA in SC AT increases in dairy cows during the transition from late pregnancy to early lactation [11]. Adiponectin signals through adiponectin receptors 1 and 2 (AdipoR1/R2) and different pathways, it is involved in energy metabolism and increases insulin sensitivity [12,13]. In dairy cattle, restrictive feeding during the dry period increased AdipoR2 mRNA expression in liver after calving [14], but mRNA abundance of both receptors was reduced post-partum in SC AT in

ad libitum fed cows [11]. In AT of monogastric species, the AT matrix cross-talk between the energy balance related factors affects insulin signal transduction, leading to adipogenesis via PPARγ and C/EBPα. Both of them transcriptionally activate a large number of adipocyte-specific genes including fatty acid binding protein (FABP4) [15], known as a differentiation marker.

In dairy cattle, the circulating concentrations of NEFA and of the ketone body β-hydroxybutyrate (BHB) often increase as result of intensive lipolysis in the energy deficient state of early lactation. Beta-hydroxybutyrate and butyrate inhibit adipocyte lipolysis in bovine AT *in vitro* [16]. It was shown that both compounds activate and bind the nicotinic acid receptor [17]. A putative G protein-coupled receptor 109A (GPR109A) mRNA is expressed and regulated during the transition period in dairy cattle [11]. Less information is available in cattle about the regulation of energy balance related genes, in association to indicators of insulin sensitivity. Therefore, we established an explant model for SC and RP adipose depots to study transcription of energy balance related genes (i.e. adiponectin, AdipoR1/R2, C/EBPα, FABP4, FFAR2, FFAR3, facilitated glucose transporter 4: GLUT4, GPR109A, IL-6, insulin receptor substrate 1: IRS-1, PPARγ2, sterol regulatory element-binding protein 1: SREBP1) in response to C3 or BHB, and to insulin in bovine AT *in vitro*. The AT explants comprise not only adipocytes but also other stromal vascular cells, and we therefore established a primary cell culture model from bovine SC AT to investigate some of the genes listed above in isolated differentiating preadipocytes *in vitro.*

2. **Materials and methods**

2.1 *Animals, tissue collection, explant culture and treatments*

Subcutaneous and RP AT were obtained from 14 Holstein–Friesian dairy cows (n=7 for C3 and BHB treatment each) from a local abattoir. The cows were not fed for at least 6 hours before slaughter. Immediately after removal of the skin and opening of the abdominal cavity, samples from the subcutaneous fat depot around the sternum and from the retroperitoneal depot (near the kidney) were dissected. About 3 g tissue were placed in sterile sodium chloride solution (0.9%) to remove the excess blood and connective tissue. The remaining tissue was immediately transferred to a 50 mL tube containing DMEM/Ham's F-12 with L-Glutamine (PAA, Colbe, Germany) supplemented with 100 µg/mL of streptomycin and 50 µg/mL of gentamicin (PAA), as basal medium. The tube was immersed in 37°C water in a thermo-flask

dewar and transported to the laboratory. Two hundred mg of the SC and RP AT were dissected and cut to 10 small pieces under the clean bench. The incubations were performed at 37°C and 5% CO_2 in duplicate with 4.5 mL of basal medium, basal medium with 100 nM insulin (Sigma-Aldrich Chemie, Taufkirchen, Germany), basal media with different propionate concentrations, pH adjusted to pH 7 by NaOH (Sigma-Aldrich; 0.5, 1, 2, and 3 mM), and basal media with different DL-ß-hydroxybutyric acid sodium salt concentrations (Sigma-Aldrich; 0.5, 3 and 10 mM) for 4 h, Afterwards, tissue explants (200 mg) were collected, immediately frozen in liquid nitrogen and stored at -80°C.

2.2 *Isolation of stromal vascular cells*

Subcutaneous AT was obtained from 3 additional Holstein–Friesian dairy cows at slaughter. The stromal-vascular cell fraction (SVC) was isolated under sterile conditions using the method of Grant et al. [18] with minor modifications. The mixture of the isolated SVC of three cows was seeded at 37°C and 5% CO_2 in 25 cm^2 cell culture flask (Sarstedt, Nümbrecht, Germany) at 4000 cells/cm^2. The cells were exposed to differentiation for 13 d and were subsequently stained using Oil Red O to specify intracellular lipid as described by Hirai et al [19]. Sampling of differentiated preadipocytes was performed for time-course analyses at the time points 0, 2, 4, 6, 8, 10, 12, and 13 d.

2.3 *RNA extraction*

Tissue homogenization was performed after adding 1 mL of Trizol (Invitrogen, Karlsruhe, Germany) using a Precellys® homogenizator (Peqlab, Erlangen, Germany) for AT explants, while preadipocytes homogenization was performed using a cell scraper (Sarstedt) and inversion pipetting of Trizol (Invitrogen) in the culture flask (Sarstedt) during sampling for time-course analyses. Total RNA was extracted according to the protocol of the manufacturer. Subsequently, total RNA was purified using RNeasy Mini Kit (Qiagen, Hilden, Germany) after DNase digestion in solution using RNase-free DNase set (Qiagen), following the manufacturer's instructions. Messenger ribonucleic acid integrity, based on the ratio of 28S to 18S rRNA, was assessed by SYBR Green II (Invitrogen) RNA denaturing gel and ethidium bromide stained formaldehyde gel electrophoresis for AT explants and isolated preadipocytes, respectively. Messenger RNA quantification was performed using the RiboGreen RNA Assay Kit (Invitrogen) in a Mx3000P-cycler (Stratagene, Amsterdam, Netherlands) or Nanodrop

ND-1000 (Peqlab). The analysis was performed for corresponding SC and RP depots from the same cow in C3 and BHB treated explants. Due to technical reasons, the number of analyzed genes (n=4) and animals varied in BHB treated samples (SC, n=7; RP, n=5).

2.4 *cDNA synthesis*

First strand cDNA was synthesized from 200 ng (AT explants) and 350 ng (differentiated preadipocytes) RNA using Revert Aid reverse transcriptase (Fermentas, St. Leon-Rot, Germany) and 250 pmol of random hexamer primers (Sigma-Aldrich) in a final volume of 20 µL. The amount of total RNA used for cDNA synthesis for AT explants and preadipocytes varied dependent of the established assay. Adipose tissue explants had lower yield of total RNA extracted from 200 mg of cultured tissue explants in comparison to the amount of total RNA extracted from one culture flask with differentiated preadipocytes. The No-RT controls were performed by omitting reverse transcription, and no template controls were conducted by adding nuclease free water. The reverse transcribed cDNAs were diluted 1:4 using ultra pure H_2O and kept at -20°C for further use.

2.5 *Quantification of mRNA*

The mRNA of the different genes of interest (GOI) was quantified using quantitative real-time PCR (qPCR) in a Mx3000P-cycler (Stratagene). Quantification of mRNA was accomplished using PCR amplicon standard curves for the purified and sequenced GOI. Primer design was conducted for the GOI using Primer3 (v. 0.4.0) software [20], except for the GOI FFAR3 which was designed and obtained from Tib MolBiol GmbH (Berlin, Germany) for hydrolysis probe qPCR assay (Table 1). SYBR Green I based qPCR was performed in total volume of 10 µL per reaction comprising 2 µL of template, 1 µL of the assay specific primer mix, 5 µL of the SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich Chemie) and 2 µL water. Melting curve analysis confirmed the presence of a single product for each qPCR assay. Hydrolysis probe qPCR was performed in total volume of 20 µL per reaction comprising 2 µL of template, 2 µL of the assay specific primer mix (final concentration 0.5 µM each), 5 µL of the qPCR MasterMix Plus (Eurogentec, Köln, Germany), 0.5 µL of the probe (final concentration 0.25 µM; FAM as the fluorescent reporter dye and BlackBerry as quencher), and 3 µL water. In addition, a pooled cDNA was used as an inter-run calibrator. Hydrolysis probe qPCR was conducted at 95°C for 10 min followed by 50 cycle numbers, denaturation at

95°C for 10 s and annealing at 60°C for 60 s. SYBR Green qPCR cycling conditions were the same for the rest of primer pairs except annealing temperatures and cycles number: initial denaturation at 95°C for 10 min followed by different cycle numbers (Table 1), denaturation at 95°C for 30 s, annealing temperature (Table 1) for 30 s and extension at 72°C for 30 s. The geometric mean of the reference genes LRP10, HPCAL1 and GAPDH was used for accurate normalization of SC and RP AT explants treated with propionate, while the geometric mean of the genes HPCAL1, GAPDH and Pol II was used for BHB treatment normalization, based on our previous studies [21] using the geNorm software [22]. The ratio of the GOI to the geometric mean of the corresponding reference genes was calculated, and data are presented as percentage value relative to the controls (means ± SEM).

2.6 *Statistical Analyses*

Statistical analyses were performed with the software package SPSS 17.0 (SPSS Science Software, Erkrath, Germany). In view of the exploratory context of our study we considered not only significant ($P \leq 0.05$) results but also present and discuss trends ($P \leq 0.15$). Data were analyzed using the paired-samples t-test for the treated samples versus control comparable to other studies [23] for all the GOI.

3. **Results**

3.1 *Effects of propionate in AT explants*

Short-term C3 treatment increased rather than decreased the mRNA abundance of 6 from the 10 GOI investigated in SC AT as summarized in Fig. 1a. The presentation of the differences observed between controls and treatments comprises both significances ($P \leq 0.05$) and trends ($P \leq 0.15$); for the sake of clarity, we renounce in the following text sections on detailed differentiation between significance and trend. In RP AT, 5 of the GOI were affected, from which IRS-1 and IL-6 were decreased, whereas C/EBPα, GLUT4, and AdipoR1 mRNA were increased by C3 (Fig. 1b). The GOI solely affected in SC explants by C3 were AdipoR2, PPARγ2, and SREBP1, for AdipoR1, GLUT4 and C/EBPα increases were observed in explants from both depots (Fig. 1 a, b). For AdipoR1, the results from SC and RP explants are exemplarily presented as bar graphs (Fig. 1 c, d), respectively.

3.2 *Effects of ß-hydroxybuyrate in AT explants*

For testing the effects of BHB, the GOI were limited to the adiponectin system (adiponectin, and its receptors) and GPR109A, encoding the receptor for niacin and BHB, respectively. Differences with untreated controls were established only as trends and were limited to adiponectin (3 nM BHB) and AdipoR1 (10 nM BHB) in SC AT, and to adiponectin (0.5 mM BHB) in RP explants (Fig. 2 a, b). A more detailed presentation is exemplarily shown for adiponectin (Fig. 2 c, d).

3.3 *Effect of insulin on AT explants*

Short-term insulin stimulation (100 nM) increased the mRNA abundance of IL-6 and FFAR3 as a trend only in RP and SC AT, respectively, whereas the mRNA of adiponectin, AdipoR1, AdipoR2, PPARγ2, IRS-1, GLUT4, C/EBPα, GPR109A, and SREBP1 remained unchanged (Fig. 1). The lack of an effect of insulin was confirmed in the second set of cow samples (Fig. 2)**.**

3.4 *Differentiating preadipocytes*

The timely changes of the mRNA abundances from the GOI in differentiating preadipocytes are presented in Fig. 3. Data are shown as percentage values, whereby the undifferentiated cells at d 0 were considered 100%. On d 2, a first increase was noted for the mRNAs of C/EBPα (253%), FFAR3 (252%), PPARγ2 (326%), and, most pronounced of FABP4 (124,000%). After 13 d differentiation, all mRNAs had reached between 801 (FFAR2) and 1.19e+06 (FABP4) fold higher abundances than on d 0.

4. **Discussion**

Short chain fatty acids are a primary source of energy, regulate insulin and glucagon secretion, and other physiological processes in ruminants. Energy balance related genes, produced by AT and liver, such as the inflammatory cytokine IL-6, affect gluconeogenesis, ß-oxidation and insulin sensitivity. Adipose tissue and macrophages within AT function in endocrine and paracrine manner in monogastrics to promote inflammation and affect insulin sensitivity [7], but less is known in cattle. Herein we used an explant model which we consider as advantageous to investigate insulin dependent or insulin independent effects of C3 and BHB on tran-

scriptional regulation of energy balance related genes in AT of different localizations. In contrast to *in vivo* studies, the effects of insulin and of SCFA on gene regulatory mechanisms can be dissected in the explant model. In addition, it preserves AT composition, which includes other cells besides mature adipocytes. We observed a similar degree of interindividual variance of mRNA abundance as reported in previous studies for human AT [24]; to report the responses in a descriptive manner, we herein also report trends ($P \leq 0.15$).

Recently it was shown that the expression of IL-6 mRNA and other inflammatory markers in cattle AT explants is depot dependent [25]. In our present study, we demonstrate differential effects of C3 and BHB on the mRNA abundance of genes linked to adiposity like IL-6 and AdipoR1/2 in bovine SC and RP AT explants *in vitro*. In our study we used C3 and not acetate; both SCFAs activate the same receptor, i.e FFAR3 [26], thus allowing to investigate direct SCFA-mediated, insulin independent effects. Using physiological C3 concentrations of 0.5 mM and 1 mM C3 in cows, only a numerical increase in intracellular Ca^{2+} was observed [27], but Soliman et al. [5] demonstrated a significant increase of leptin mRNA using 1 mM C3 and acetate in bovine differentiated adipocytes *in vitro*.

In mice, C3 initiates signal transduction of FFAR3 *in vitro* and stimulates leptin mRNA *in vivo* [28]; analogous observations were made with bovine adipocytes *in vitro* [5]. Activation of FFAR2 by C3 and acetate inhibits lipolysis and stimulates adipogenesis [10], and stimulation of FFAR3 by propionate increases leptin production in mice [28]. In our previous study [11] the mRNA expression of SCFA receptors FFAR2 and FFAR3 in SC AT during the transition period in dairy cows was demonstrated. These results differ from the observations of Wang el al. [26], who reported that AT is the only bovine tissue examined, which does not express FFAR3 or FFAR2 mRNA. Herein we provide further evidence for the presence of both receptor mRNAs in bovine isolated preadipocytes of SC origin during a 13 d differentiation period; moreover, we demonstrate a differentially regulated expression of FFAR2 and FFAR3 mRNA in SC and RP AT explants *in vitro.* In the present short-time experiments, neither C3 nor BHB seem to be relevant regulators of both FFARs; for insulin a stimulatory trend for FFAR3 mRNA was limited to SC AT. This result might explain for the effect of short-term C3 infusion in goats, where an effect of a C3 infusion on FFAR3 was observed only in SC AT [6]. In addition, insulin stimulated IL-6 mRNA in RT AT but not in SC AT during short-term treatment in the explant model. Other energy balance related genes were not influenced in short-term manner by insulin. Recently we reported that FFAR3 mRNA but not FFAR2 mRNA in SC AT increases post-partum compared to prepartum in dairy cows [11]. In

comparison to our current results, we suggest that long-term exposure and/or other factors besides insulin are relevant for the post-partum extent of change of FFAR3 mRNA, since short-term insulin effects were observed as a trend for increased FFAR3 mRNA abundance.

In future, the physiological relevance of SCFA receptors in AT metabolism should be clarified in more detail, not only at the mRNA level but also at the protein level. At least their mRNAs are differentially regulated but it has to be taken into account, that physiological SCFA concentrations are relatively low to activate the receptors *in vivo* [26], and effects at least on leptin *in vivo* are rather limited [29]. Nevertheless, other hormones than leptin might be influenced by C3 *in vivo* and Yonezawa et al. [27] observed at least a numerical change (about 80 fold) of intracellular Ca^{2+} concentrations even at 0.5 µM C3.

We observed higher mRNA abundance of FFAR3 than of FFAR2 in isolated preadipocytes. A putative GPR109A is regulated in bovine SC AT during transition period in cattle [11]. Because of the fact that BHB, a ketone body indicating negative energy balance, and butyrate inhibit lipolysis in bovine AT *in vitro* [16], we expected an effect of BHB, on lipolysis in AT explants *in vitro*. Surprisingly, an effect of BHB on the adiponectin system- and GPR109A mRNA in AT explants was not observed, which might be related to short-term treatment and therefore may differ from previous observations during the transition period in dairy cow [11].

Energy homoeostasis is related to the adiponectin system, which increases insulin sensitivity [12]. Adiponectin acts as an autocrine/paracrine factor, is involved in adipocyte lipid accumulation and differentiation, involves C/EBPα and SREBP1 expression [30], and regulates the expression of its receptors in AT [31]. We recognized depot dependent effects of C3 on AdipoR1/R2 mRNA abundance. The short-term effect of C3 on adiponectin mRNA was not investigated, as adiponectin appears to reflect only long-term changes in body energy balance with little evidence for short-term regulatory influence at least in porcine differentiated adipocytes [31]. Previous studies indicated that niacin affects adiponectin secretion via GPR109A in short-term [32]. We observed that adiponectin mRNA abundance decreased in response to BHB in SC AT explants during short-term stimulation after at least 6 hours restrictive feeding in slaughterhouse, while the mRNA of this gene was increased in RP AT. Desensitization in SC AT and improved inhibition of lipolysis in RP AT might be seen in relation to functional aspects of the different depots, whereby visceral AT is of higher relevance for immediate mobilization of fatty acids [33]. Furthermore, decreased AdipoR1 mRNA abundance in SC AT using BHB stimulus might be relevant to the situation, in which post-partum reduction of

AdipoR1 mRNA abundance in dairy cattle during the transition period was observed [11], where increasing amounts of NEFA and increased BHB concentrations as indicators for negative energy balance are often observed [34].

In humans, RP AT is one of the main sources for IL-6 [35], which is elevated in obesity. Interleukin 6 affects AT metabolism in an autocrine/paracrine manner in AT explants [36]. In cattle, IL-6 mRNA is expressed at low abundance in AT compared to other inflammatory markers, whereby regulation of IL-6 by a proinflammatory stimulus, i.e. lipopolysaccharide, was shown [25]. Short chain fatty acids increased the mRNA abundance of IL-6 in intestinal samples of piglets after long-term infusion [37]. Propionate decreased IL-6 mRNA abundance exclusively in RP AT. Similarly to our results, C3 dose-dependently suppressed IL-6 mRNA abundance and protein release from colon organ cultures in mice [38], in which concentration-dependent silencing by demethylation of IL-6 mRNA expression might be of relevance too [39]. García-Escobar et al. [40] also observed a decrease of IL-6 in short-term depending on the fatty acid pattern of dietary fat. Hunger increases IL-6 release, whereby IL-6 can act locally to increase lipolysis [36]. Nutritional supply in short-term may reduce IL-6 and might thus reduce its effects on lipolysis in an autocrine/paracrine manner in situations of positive energy balance, which are different when compared to over nutrition during obesity.

Hong et al. [10] showed that C3 elevates the FFAR2 expression and up-regulates PPARγ*2*, which is a key regulator of preadipocyte differentiation, regulates SREBP1 and C/EBP, and effects lipid and fatty acid metabolism. Peroxisome proliferator-activated receptor γ modulates indirectly insulin-signalling through the up-regulation of IRS-1 and 2 of the signalling cascade for GLUT-4 [15]. To our knowledge, no data were available about the IRS-1 and SREBP1 mRNA abundance in bovine SC and RP AT, except for perimuscular preadipocytes during differentiation [41], in which the time-course reported differs from our observation in peadipocytes isolated from SC AT. Like for FFARs, our study using isolated preadipocytes indicates the increase of mRNA abundance of these energy balance related genes during differentiation. We observed a pronounced increase of PPARγ2 and C/EBPα as well as FABP4 mRNA abundance in preadipocytes after 10 d of differentiation. Our results suggest that potential *in vitro* effects of short-term C3 treatment on PPARγ2 mRNA abundance are limited to SC AT, while the potential of C3 to increase C/EBPα mRNA abundance might be limited to SC and RP AT only in higher doses. In view of the data known from monogastric species, increased PPARγ2 mRNA in presence of C3 might thus increase insulin sensitivity, and in parallel may improve lipogenesis in SC AT but not in RP AT in the bovine species.

Hepatic SREBP1 mRNA abundance was decreased in short-term after 3 h fasting and increased after refeeding in mice [42], while in obese humans short term fasting (12 h) was associated with increased SREBP1 mRNA expression in SC AT, which is regulated in an insulin-dependent manner [43]. In cattle, we observed insulin independent effects of C3 on SREBP1 and IRS-1 mRNA in SC and RP AT, respectively. Our results indicate that potential *in vitro* effects of short-term C3 treatment on SREBP1 mRNA abundance are limited to SC AT, whereas C3 seemed to decrease IRS-1 mRNA abundance only in RP AT *in vitro*. Thus, SREBP1 might increase fatty acid synthesis under increased energy load in the presence of C3 in SC AT in short-term, whereas decreasing IRS-1 mRNA abundance in bovine RP AT under higher energy load might indicate reduced insulin sensitivity. Interestingly we observed no direct insulin effect on adiponectin, AdipoR1, AdipoR2, PPARγ2, IRS-1, GLUT4, C/EBPα, GPR109A, and SREBP1 in our explant model, which is different to the *in vivo* situation. In monogastrics, partially contradicting results about insulin sensitivity and insulin resistance in liver via increased SREBP1c and IRS-2 but not IRS-1 are reported [44]. We suspect that an effect by the model, the tissue or the species cannot be ruled out.

The insulin dependent glucose transporter GLUT 4 is expressed tissue-dependently, whereby glucose uptake by AT is less than by skeletal muscle [45]. The mechanisms underlying the adiponectin stimulated increase in insulin sensitivity are probably due to the increase of total GLUT4 expression and number of GLUT4 transporters acutely recruited to the plasma membrane in response to insulin [30]. Ruminants rely on endogenous gluconeogenesis for their glucose demand [46]. Glucose is the principal precursor for the synthesis of lactose (a major osmotic agent in milk), and participates in determining the milk volume produced [47]. In contrast to monogastric animals, insulin did not alter GLUT4 mRNA abundance in the present explant model. However, the insulin effect on adipocytes from monogastric species might be different in lactating animals; moreover, it requires co-incubation with dexamethasone [48]. In goats 6 h hyperinsulinemia did not affect GLUT4 protein abundance in AT or skeletal muscles [49], suggesting that insulin might not be the prime factor involved in the short-term regulation of GLUT4 in ruminants. Our data indicate that C3 affects GLUT4 mRNA abundance in SC and RP AT during short-term treatment. An increase in GLUT4 mRNA by C3 might be seen in addition to stimulation of gluconeogenesis by SCFA in liver, higher blood glucose concentrations after feeding and increased glucose uptake for glycogen synthesis in AT; glycogen metabolism in AT might function as a metabolic switch between feeding and fasting and/or regulation of lipogenesis and lipolysis [50].

In conclusion, we compared the effects of C3, BHB and insulin in bovine SC and RP AT explants *in vitro*, and observed divergent effects in both depots on the mRNA abundance of energy balance related genes. Our study is the first report highlighting the effect of C3 or BHB on the aforementioned energy status related genes in SC and RP AT of dairy cattle *in vitro*. The analyzed energy balance related genes in adipose tissue were regulated specifically trough C3 and BHB in a both depot specific and insulin-independent manner. Interestingly, our data indicate that IL-6 as well as FFAR3 mRNA might be insulin-dependently regulated in RP and SC AT during short-term stimulation, respectively. Adiponectin receptor 1/2 were more influenced by C3 than by BHB in the bovine explant model indicating, that the adiponectin system in cattle might be more sensitive to C3 than to BHB. Insulin sensitivity of AT, represented by the differential regulation of energy balance related genes might be influenced by C3 and BHB directly on the level of the AT. Differential regulation between different AT depots is evident in cattle.

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Table 1:
Primer sequences and qPCR conditions

Name	Acc. No.	Forward Primer Sequence [5´-3´] Reverse Primer Sequence [5´-3´]	Len. (bp)	Ta. (°C)	Ct.
Adiponectin	NM_174742	CTGGAGAGAAGGGAGAGAAAG TGGGTACATTGGGAACAGTG	204	60	25.73
Adiponectin receptor 1	NM_001034055	GCTGAAGTGAGAGGAAGAGTC GAGGGAATGGAGTTTATTGCC	118	60	22.3
Adiponectin receptor 2	NM_001040499	GGCAACATCTGGACACATC CTGGAGACCCCTTCTGAG	200	60	24.94
C/EBPα	BC149006	TGGACAAGAACAGCAACGAG TTGTCACTGGTCAGCTCCAG	130	60	22.4
FABP4	X89244	CATCTTGCTGAAAGCTGCAC AGCCACTTTCCTGGTAGCAA	160	56	19.42
FFAR2	NM_001163784	CGCTCCTTAATTTCCTGCTG CAAAGGACCTGCGTACGACT	174	60	30.89
FFAR3	NM_001145233	AAAGCAGCAGTGGCCATGA GAGGTTTAGCAAGAGCACGTCC	189	60	39.94
	Probe:	6FAM-TCTTCTCCGTGTACCTCTTCACCTTCCTC--BBQ			
GAPDH	U85042	AATGGAAAGGCCATCACCATC GTGGTTCACGCCCATCACA	204	59	21.63
GLUT4	AY458600	ACCTTATGGCCACTCCTCCT CTCAGCCAACACCTCAGACA	180	60	31.3
GPR109A	XR_028237	GGACAGCGGGCATCATCTC CCAGCGGAAGGCATCACAG	140	61	21.53
HPCAL1	NM_001098964	CCATCGACTTCAGGGAGTTC CGTCGAGGTCATACATGCTG	99	60	28.22
IL-6	BC123577	TGCAGTCTTCAAACGAGTGG TAAGTTGTGTGCCCAGTGGA	182	60	20.6
IRS-1	XM_581382	CAAGACCATCAGCTTCGTGA GTCCACCTGCATCCAGAACT	157	59	26.48
LRP10	BC149232	CCAGAGGATGAGGACGATGT ATAGGGTTGCTGTCCCTGTG	139	61	23.35
Pol II	X63564	GAAGGGGGAGAGACAAACTG GGGAGGAAGAAGAAAAAGGG	86	60	21
PPARγ2	Y12420	AGGATGGGGTCCTCATATCC GCGTTGAACTTCACAGCAAA	121	60	20.75
SREBP1	AB355703	ACCGCTCTTCCATCAATGAC GCTGAAGGAAGCGGATGTAG	120	60	22.29

6-carboxyfluorescein (FAM), accession number (Acc. No), annealing temperature (Ta), blackberry quencher (BBQ), fragment length (Len.), and median cycle threshold (Ct.)

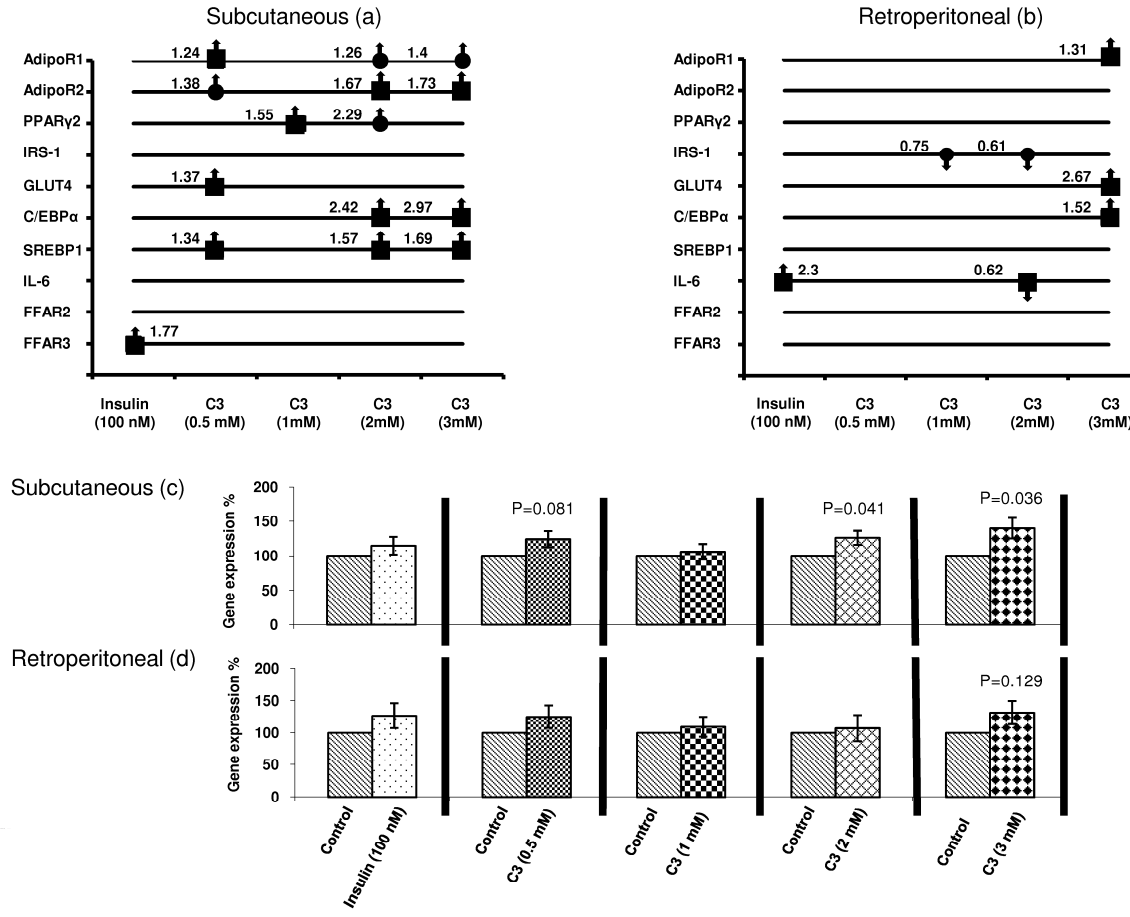

Fig. 1. Summarized presentation of the effects of insulin and four different doses of propionate (C3) on mRNA abundance of ten energy balance related genes in bovine subcutaneous (a) and retroperitoneal (b) adipose tissue explants. Differences between the treatments and the controls are depicted as symbols on the line of each gene of interest. The arrows at the symbols indicate the direction of change: arrows facing upwards indicate increases and vice versa. The corresponding numbers beneath the symbols give the fold change compared to the controls. For significant ($P \leq 0.05$) differences, circle symbols (●) and for trends ($P \leq 0.15$) square symbols (■) are used, respectively. Exemplarily, AdipR1 mRNA abundance data (means ± SEM) are shown for subcutaneous (c) and retroperitoneal (d) adipose tissue explants in treatments versus controls.

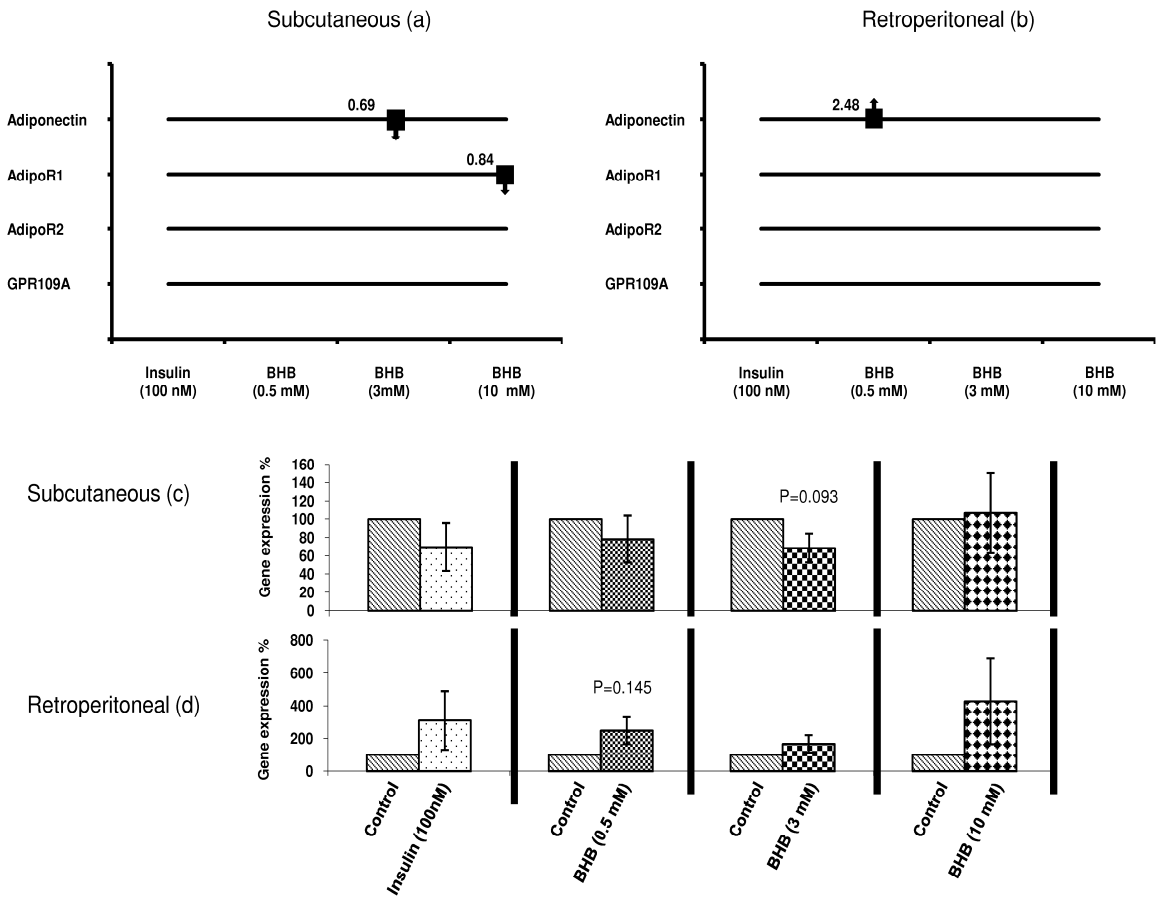

Fig. 2. Summarized presentation of the effects of insulin and three different doses of ß-hydroxybutyrate (BHB) on mRNA abundance of ten energy balance related genes in bovine subcutaneous (a) and retroperitoneal (b) adipose tissue explants. Differences between the treatments and the controls are depicted as symbols on the line of each gene of interest. The arrows at the symbols indicate the direction of change: arrows facing upwards indicate increases and vice versa. The corresponding numbers beneath the symbols give the fold change compared to the controls. For trends ($P \leq 0.15$) differences, square symbols (■) are used. Exemplarily, adiponectin mRNA abundance data (means ± SEM) are shown for subcutaneous (c) and retroperitoneal (d) adipose tissue explants in treatments versus controls.

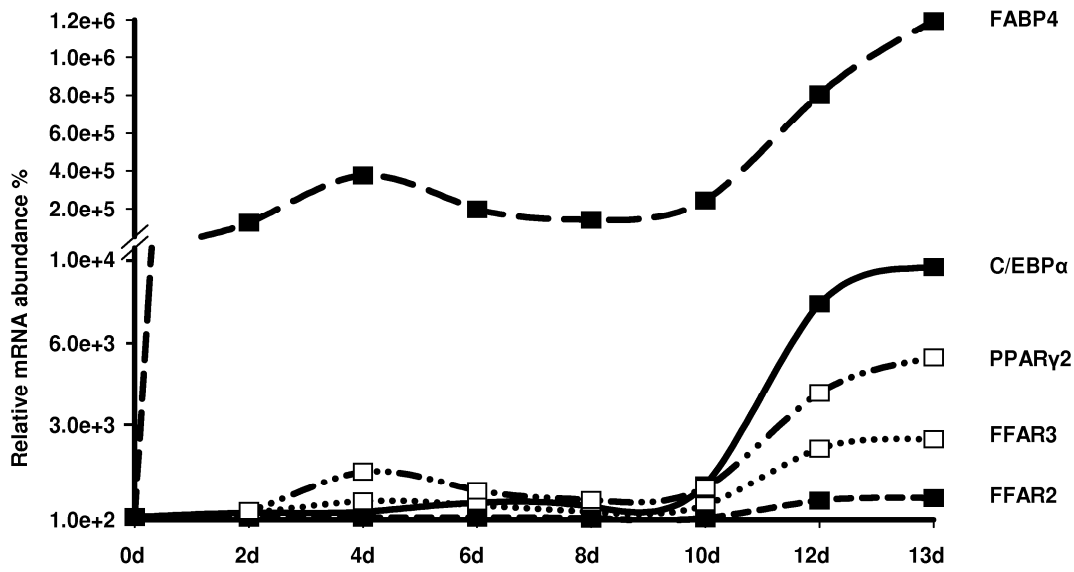

Fig. 3. Timely changes of the mRNA abundance of five genes of interest in bovine preadipocytes differentiating for 13 d. The mRNA quantities of target genes were normalized to the geometric mean of two reference genes (GAPDH and LRP10) and are presented as fold increase in comparison to undifferentiated cells at d 0 (=100%).

4. Conclusions

Herein, we compared the short-term effects of C3, BHB and insulin on energy balance related genes' mRNA abundance in bovine SC and RP AT *in vitro*. To obtain the accurate results concering the differential effects of C3 and BHB on these genes, we evaluated the stability of seven different RGs (i.e. 18S rRNA, ACTB, GAPDH, HPCAL1, LRP10, Pol II and RPS9) in bovine SC and RP AT explants to quantify the mRNA of 13 energy balance related genes in AT (i.e. adiponectin, AdipoR1/R2, C/EBPα, FABP4, FFAR2, FFAR3, GLUT4, GPR109A, IL-6, IRS-1, PPARγ2 and SREBP1) in explants from both AT depots; moreover the presence and increased mRNA abundance of FFAR2, FFAR3 and other energy balance related genes like C/EBPα, FABP4 and PPARγ2 in bovine SC isolated preadipocytes during differentiation was shown. All in all, in contrast to other studies, the presence of FFAR2 and FFAR3 mRNA not only in both AT depots but also in differentiated preadipocytes isolated from bovine SC AT was demonstrated. The lack of the presence of FFAR2 and FFAR3 in AT seems to be due to technical aspects of the detection method applied by this groups (Wang et al., 2009).
Using two normalization programs to select highly stable RGs, different expression stabilities of the RGs depending on the experimental conditions (i.e. C3 and BHB treatments) in bovine SC and RP AT *in vitro* were obtained. Thus, the requirement and necessity for a cautious selection of a panel of RGs for all the experimental setups is highly important. Exploratory data analysis of C3 and BHB effects on AT explants indicated insulin independent and depot specific effects of both stimuli on most of the demonstrated energy balance related genes. The lack of C3 and BHB effects on FFAR2/3 and GPR109A, respectively in the presented model does not rule out *in vivo* effects of both stimuli; as the *in vivo* situation differs from the *in vitro* situation (Machinal et al., 1999). AdipoR1/2 were more influenced by C3 than by BHB in the bovine explant model indicating, that in cattle the adiponectin system might be more sensitive to C3 than to BHB in short-term stimulation. Based on the results of short-term C3 treatment on bovine SC and RP AT *in vitro*, improved insulin sensitivity, increase glucose uptake and lipid metabolism or lipid accumulation by C3 treatment were supposed. It is known that adiponectin acts as an autocrine/paracrine factor which is involved in adipocyte lipid accumulation and differentiation which involves C/EBPα and SREBP1 expression (Fu et al., 2005). Insulin sensitivity of AT, represented by the differential regulation of energy balance related genes might be influenced by C3 and BHB directly on the AT. Adiponectin as

well as PPARγ activate AMPK, which is an important regulator of energy homeostasis and improves insulin sensitivity (Lim et al., 2010). In this context, further studies are necessary to investigate the linkage between the mRNA abundance and protein expression of adiponectin in bovine SC and RP AT in relation to AMPK phosphorylation as no data is available for this interaction in the bovine species. Surprisingly, in our study, IL-6 as well as FFAR3 mRNA seems to be insulin-dependently regulated in RP and SC AT, respectively, during short-term stimulation. Our observation of FFAR2 and FFAR3 mediated signalling by C3, at least for IL-6, might be related to SCFA concentration dependent silencing by demethylation in cattle and likewise in monogastrics, might repress IL-6 mRNA abundance (Benjamin et al., 2001) as it was shown for mouse leptin promoter activity (Yokomori et al., 2002). Therefore, to elucidate the exact influence of C3 on IL-6 in association with the insulin signalling pathways in ruminants like bovine, further studies are necessary at the protein level.

The results of this study about the mRNA of the aforementioned energy balance related genes in bovine AT explants *in vitro* treated by C3, BHB and insulin might open new aspects for further studies to improve understanding of the crucial time like transition period, during which the dairy cattle undergoes negative energy balance. Our study of mRNA of energy balance related genes indicates differential regulation between different AT depots in cattle in relation to C3 and BHB treatments. Less is known about the cross-talk of energy balance related genes in bovine AT; therefore, further studies are necessary to investigate this interaction between the energy balance related genes not only at the level of the transcriptome but also of the proteome.

5. Summary

Expression of genes related to energy balance in adipose tissue of dairy cattle: effects of SCFA on mRNA abundance as quantified by qPCR and relevance of appropriate selection of reference genes

Ruminants rely on SCFA as principal energy source. Energy balance is defined as the difference between the consumed and the required energy. States of negative energy balance may lead to metabolic disturbances in dairy cows but are in fact inevitable during early lactation. Energy deficits are associated with immunosuppression, in contrast, excess energy surplus with increased body condition scores is also associated with inflammatory reactions. In high performing dairy cattle both aspects are highly relevant; negative energy balance leads to lipolysis during early lactation, during which the circulating concentrations of NEFA and the ketone body BHB increase. The energy deficit in consequence might cause metabolic diseases and reproductive disorders, and indirectly lead to infectious diseases. Besides its role as an energy storage organ, AT is also considered as an endocrine organ. It produces various factors, among which are transcriptional factors, summarized in this study as energy balance related genes, affecting several bodily functions. Herein, we demonstrated depot-specific, insulin dependent and independent effects of propionate and ß-hydroxybutyrate on the transcriptional regulation of energy balance related genes in SC and RP AT in a bovine explant model. For the mRNA quantifications using qPCR method, adequate protocols were established. To obtain accurate results for qPCR data, a validation of RGs was performed using the geNorm™ and the Normfinder© program before their use. The quantified energy balance related genes were adiponectin, AdipoR1/R2, C/EBPα, FFAR2, FFAR3, GLUT4, GPR109A, IL-6, IRS-1, PPARγ2 and SREBP1. Furthermore, to address the cell type expressing the genes C/EBPα, FFAR2, FFAR3, FABP4 and PPARγ2, we established a bovine primary preadipocyte culture model, thus focusing on one important cell type present amongst others in AT.
To investigate the effect of C3, BHB or insulin on AT explants, the SC fat depot around the sternum and the visceral, i.e. RP depot near the kidney of dairy cows were obtained to check for differences between visceral and subcutaneous adipose depots. The incubations were performed in basal medium, basal medium with 100 nM insulin, basal medium with different C3 concentrations (0.5, 1, 2, and 3 mM) or with different BHB concentrations (0.5, 3 and 10

mM) for 4 h; the advantage of our studied model is the possibility to differentiate between the C3, BHB and insulin effects which is not possible in *in vivo* approaches.

Comparing the two programs for optimizing RG use, similar stability rankings for the C3 treatment were obtained: LRP10 > HPCAL1 > GAPDH > RPS9 > Pol II > ACTB > 18S rRNA. BHB treated AT explants yielded a different stability ranking for RGs using geNorm™: HPCAL1, GAPDH > Pol II > LRP10 > ACTB > RPS9 > 18S rRNA than Normfinder© which yielded the following stability ranking in SC and RP AT explants treated with BHB: HPCAL1 > Pol II > GAPDH > ACTB > LRP10 > RPS9 > 18S rRNA. Subsequent pairwise analysis of variation of RGs using geNorm™ suggested that LRP10, HPCAL1 and GAPDH should be used for accurate normalization of SC and RP AT explants treated with C3, while HPCAL1, GAPDH and Pol II should be used for BHB treatment.

Propionate increased the mRNA abundance of AdipoR1, AdipoR2 and PPARγ2 in treated samples vs. control in SC AT explants, while it suppressed the mRNA abundance of IRS-1 in RP AT. A trend ($P \leq 0.15$) for an increase of the mRNA abundance of AdipoR1, AdipoR2, C/EBPα, PPARγ2, GLUT4, and SREBP1 in response to C3 was exclusively observed in SC AT. In RP AT, C3 showed a trend for increasing AdipoR1, C/EBPα, GLUT4 mRNA abundance and for decreasing concentrations of IL-6 mRNA. With BHB treatment, there was a trend for decreased mRNA abundance of adiponectin and AdipoR1 in SC AT, and for increasing mRNA abundance of adiponectin in RP AT. The insulin-induced alterations were limited to the mRNAs of FFAR3 and IL-6 in SC and RP AT, respectively, for which a trend for increased abundances was recorded. The results show that *in vitro* stimulation of AT explants with different concentrations of C3, BHB or insulin during short-term treatment exerts divergent effects in both depots on the mRNA abundance of energy balance related genes. AdipoR1/2 were more influenced by C3 than by BHB in the bovine explant model indicating that the bovine adiponectin system might be more sensitive to C3 than to BHB. Herein we have addressed one of the major cell types in AT by establishing a bovine primary preadipocyte culture for which increased mRNA abundance of C/EBPα, FFAR2, FFAR3, FABP4 and PPARγ2 were observed during differentiation; in contrast to other studies we demonstrated the presence of FFAR2 and FFAR3 mRNA not only in both AT depots but also in differentiated preadipocytes isolated from bovine SC AT.

The present thesis serves as a basis for further studies showing divergent mRNA abundance of several energy balance related genes using C3, BHB or insulin in bovine SC and RP AT *in vitro* in insulin dependent and independent manners. At the mRNA level, several of the ener-

gy balance related genes analyzed herein, were differentially regulated between different adipose depots; considering C3 and BHB as indicators for a positive and a negative energy balance at the cellular level, these results might be extrapolated to the AT response in cows undergoing deficient or balanced energy supply. In future, the physiological relevance of SCFA receptors as well as of the adiponectin system in AT metabolism should be clarified in more detail, not only at the mRNA level but also at the protein level.

6. Zusammenfassung

Expression von Energie-Bilanz-assoziierten Genen im Fettgewebe von Milchkühen: Einfluss von SCFA auf die mRNA-Expression, quantifiziert mittels qPCR sowie Relevanz der Auswahl geeigneter Referenzgene

Kurzkettige Fettsäuren (SCFA) bilden die hauptsächliche Energiequelle bei Wiederkäuern. Die Energie-Bilanz ist definiert als die Differenz zwischen der aufgenommenen und der verbrauchten Energie. Eine negative Energie-Bilanz kann zu metabolischen Störungen bei Milchkühen führen, ist aber besonders zu Beginn der Laktation unumgänglich. Ein Energiedefizit ist mit Immunsuppression assoziiert, ein Energieüberschuss mit entsprechend erhöhter Körperkondition steht hingegen mit Entzündungsreaktionen in Zusammenhang. Bei hochleistenden Milchkühen sind beide Aspekte relevant: Eine negative Energie-Bilanz führt zur erhöhten Lipolyse während der Frühlaktation und damit oft zu erhöhten NEFA- und BHB-Konzentrationen im Blut. In Folge können metabolische Erkrankungen und reproduktive Störungen, sowie indirekt infektiöse Krankheiten stehen. Neben seiner Funktion als Energiespeicher wird das Fettgewebe auch als endokrines Organ angesehen. Es exprimiert verschiedene Faktoren, u.a. auch Transkriptionsfaktoren, die im Körper pleiotrope Wirkungen ausüben. Diese Faktoren werden hier als Energie-Bilanz-assoziierte Gene zusammengefasst, Wir konnten Depot-spezifische, Insulin-abhängige und -unabhängige Wirkungen von C3 und BHB auf die Transkriptionsregulierung von Genen, die mit der Energie-Homöostase in Zusammenhang stehen, im bovinen SC und RP Fettgewebe-Explantat-Modell nachweisen. Für die mRNA-Quantifizierungen mit der qPCR-Methode wurden entsprechende Protokolle entwickelt. Um genaue Ergebnisse für die qPCR-Daten zu erhalten, wurde eine Validierung der RGs vor ihrem Gebrauch unter Verwendung des geNorm^{TM}- und des Normfinder$^{©}$ -Programms durchgeführt. Die quantifizierten Zielgene waren Adiponectin, AdipoR1/R2, C/EBPα, FFAR2, FFAR3, GLUT4, GPR109A, IL-6, IRS-1, PPARγ2 und SREBP1. Außerdem haben wir eine bovine primäre Praeadipozytenkultur etabliert, um die mRNA-Expression von C/EBPα, FFAR2, FFAR3, FABP4 und PPARγ2 während der Differenzierung zu untersuchen.

Um die Wirkung von C3, BHB oder Insulin auf das SC und viszerale Fettdepot zu untersuchen, wurden Gewebe-Explantate aus der Sternumregion (SC) und aus dem Fett um die Nieren (RP, viszeral) entnommen. Inkubiert wurde in basalem Medium, basalem Medium mit 100 nM Insulin, sowie basalem Medium mit unterschiedlichen Konzentrationen von C3 (0,5,

1, 2 und 3 mM) bzw. BHB (0,5, 3 und 10 mM) für 4 Stunden. Das Explantatmodells bietet den Vorteil die C3-, BHB- und Insulin-Wirkungen separat zu betrachten, was *in vivo* nicht möglich ist.

Der Vergleich der verschiedenen RGs in den beiden verwendeten Programmen ergab eine ähnliche Reihenfolge der Stabilität für die C3-Behandlung: LRP10 > HPCAL1 > GAPDH > RPS9 > Pol II > ACTB > 18S rRNA. BHB behandelte Fettgewebsexplantate zeigten unterschiedliche Stabilitäten für RGs mit geNorm^{TM}: HPCAL1, GAPDH > Pol II > LRP10 > ACTB > RPS9 > 18S rRNA im Vergleich zu Normfinder$^{©}$ in dem für die RGs in SC und RP Explantaten die folgende Stabilitätsreihung gefunden wurde: HPCAL1 > Pol II > GAPDH > ACTB > LRP10 > RPS9 > 18S rRNA. Die paarweise Analyse der Schwankungen der RGs mit geNorm^{TM} legt nahe, dass LRP10, HPCAL1 und GAPDH für eine optimierte Normalisierung der SC und RP Fettgewebe-Explantate verwendet werden sollten, jedoch für die BHB-Behandlung HPCAL1, GAPDH und Pol II.

Propionat erhöhte die mRNA-Menge von AdipoR1, AdipoR2 und PPARγ2 in SC Fettgewebe in den behandelten Proben verglichen mit den Kontrollen, während es die mRNA-Menge von IRS-1 in RP Fettgewebe reduzierte. Eine Tendenz ($P \leq 0{,}15$) zur Erhöhung der mRNA-Menge an AdipoR1, AdipoR2, C/EBPα, PPARγ2, GLUT4, und SREBP1 als Antwort auf C3-Behandlung wurde ausschließlich in SC Fettgewebe beobachtet. In RP Fettgewebe zeigte C3 eine Tendenz zur Erhöhung der mRNA-Abundanz von AdipoR1, C/EBPα, GLUT4 und einer sinkenden Abundanz von IL-6. Unter der BHB-Behandlung ergab sich ein Trend für sinkende mRNA-Abundanzen bei Adiponectin und AdipoR1 in SC AT, und für steigende Werte bei Adiponectin in RP AT. Die Insulin-induzierten Veränderungen waren jeweils auf die mRNAs von FFAR3 im SC und IL-6 im RP Fettgewebe beschränkt, für die eine Tendenz zur Erhöhung der mRNA-Menge beobachtet wurde. Unsere Ergebnisse zeigen, dass *in vitro* Stimulation von Fettgewebe-Explantaten mit verschiedenen Konzentrationen von C3, BHB bzw. Insulin während kurzfristiger Behandlung divergente Effekte in beiden Depots auf die mRNA-Abundanz von Energie-Bilanz assoziierten Genen ausübt. AdipoR1/2 wurden stärker von C3 beeinflusst als von BHB. Das bovine Explantatmodell deutet darauf hin, dass das Adiponectin-System von Rindern eventuell sensitiver gegenüber C3 als gegenüber BHB ist. Zudem haben wir in einer selbstetablierten primären Präadipozytenkultur während der Differenzierung erhöhte mRNA-Mengen der Gene C/EBPα, FABP4 und PPARγ2 nachgewiesen. Im Gegensatz zu anderen Studien konnten wir außerdem die Anwesenheit von FFAR2 und FFAR3 demonstrieren.

Die vorliegende Arbeit dient als Grundlage für weitere Studien; die mRNA-Abundanz von mehreren Energie-Bilanz assoziierten Genen in SC und RP Fettgewebe von Rindern erwies sich *in vitro* als divergent in Respons auf C3, BHB bzw. Insulin reguliert, wobei insulinabhängige oder –unabhängige Effekte auftraten. Die Ergebnisse zeigen, dass die verschiedenen mRNAs in den verschiedenen Depots unterschiedlich reguliert werden, und somit auf die Wirkungen eines ausgeglichenen bzw. unausgeglichenen Energiestatus bei der Milchkuh schließen lassen.

In Zukunft sollten Untersuchungen zur physiologische Relevanz von sowohl SCFA-Rezeptoren als auch des Adiponectin-Systems im Fettgewebestoffwechsel auf die Protein-Ebene erweitert werden untersucht werden.

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