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Regulation of adiponectin expression by *trans*-cinnamic acid and niacin in murine and bovine adipocytes *in vitro*

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Abstract

Adipose tissue secretes multiple metabolically important proteins known as adipokines. One of the most abundant adipokines in circulation is adiponectin (AdipoQ), which is known as an important modulator of glucose and fat metabolism and a key regulator of insulin sensitivity.It thereby takes part in protection against the development of obesity and type 2 diabetes. Dairy cows undergo various metabolic changes during the transition period. This period from late pregnancy to early lactation is crucial for the potential development of many diseases and metabolic disorders. Energy requirements increase due to the nutrient demand of the fetus and mammary gland and cannot be covered by feed intake only. The negative energy balance is characterized by increased lipolysis and reduced insulin sensitivity in peripheral tissues. Due to its insulin-sensitizing effect, AdipoQ is not only of great interest in humans, but also for dairy cows during the transition period. Hence, strategies to improve synthesis and secretion of AdipoQ are of major interest in various species. Cinnamon is known to exert several beneficial effects by improving insulin sensitivity and lipid profiles and to increase AdipoQ concentrations. In this thesis we focused on the barely explored cinnamon compound *trans*-cinnamic acid (*t*CA) as potential AdipoQ ameliorant. In addition, Niacin (NIA) is known to decrease lipolysis and plasma NEFA levels in lactating cows and to increase AdipoQ concentration in rodents and may therefore have the ability to emend the metabolic situation of dairy cows during the transition period via improving AdipoQ. Since nicotinic acid (NA), a compound of NIA, as well as *t*CA are both ligands of the G-protein-coupled receptor (GPR)109A, not only the effects on AdipoQ were of interest but also the signaling pathway. Therefore, this thesis aimed to investigate the effects of *t*CA on AdipoQ secretion in murine 3T3-L1 adipocytes and to evaluate the changes in AdipoQ secretion and mRNA abundance of selected genes after supplementation with NA in bovine adipocytes. In addition, the involvement of the GPR109A in the signaling pathway towards AdipoQ was examined. Therefore, the murine and bovine adipocytes were pre-incubated with pertussis toxin (PTX), an inhibitor of G_i/G_o protein coupling. Treatment with *t*CA increased AdipoQ concentrations significantly, but incubation with PTX decreased AdipoQ secretion. With this study we identified *t*CA as an influencing variable on AdipoQ, indicating *t*CA as effective compound of cinnamon, with the capability to improve glucose and fat metabolism. Furthermore, an involvement of GPR signaling, induced by *t*CA, was shown. After establishing a bovine primary cell culture model, the potential effect of NA on AdipoQ was tested. Treatment with NA increased both AdipoQ concentrations and the mRNA abundance of GPR109A. Pre-incubation with PTX reduced the AdipoQ response to NA. The NA-stimulated AdipoQ secretion and of GPR109A mRNA expression in bovine adipocytes were suggestive for GPR signaling-dependent improved insulin sensitivity in dairy cows. In addition, in both studies PTX pre-incubation AdipoQ concentrations were significantly decreased, but remained increased when compared to non-treated adipocytes. These observations contrast other findings that observed no increase in AdipoQ secretion after stimulation with NA and PTX in rat adipocytes. Therefore, we suggest, in addition to GPR signaling-dependent pathways, a GPR independent pathway in the regulation of AdipoQ secretion after stimulation with *t*CA and NA. The transcription factor PPARγ is a known stimulator of AdipoQ expression and a regulator of several genes involved in the control of insulin sensitivity, supporting PPARγ as a possible *t*CA and NA-induced stimulator of AdipoQ secretion, independent of GPR109A signaling. This thesis identified two factors improving AdipoQ secretion in murine and bovine adipocytes. Furthermore, participation of GPR signaling as well as another GPR-independent, *t*CA/NA-induced pathway to stimulate AdipoQ was detected. These results may pave the way for further studies to understand and improve the metabolic changes during the transition period in bovine AT *in vitro* and may establish a basis for upcoming *in vivo* studies.

Kurz-Zusammenfassung

Adiponektin (AdipoQ) ist eines der am häufigsten in der Zirkulation vorkommenden Adipokine und ein wichtiger Modulator des Fett- und Glucosestoffwechsels. Des Weiteren gilt AdipoQ als wichtige Einflussgröße in der Verbesserung der Insulinsensitivität und somit als Schutz vor der Entstehung von Übergewicht und Typ-2-Diabetes. Milchkühe sind in der sogenannten Transitionsperiode vielen metabolischen Veränderungen ausgesetzt. Dieser Zeitraum ist kritisch für die mögliche Entstehung verschiedener Erkrankungen wie z.B. Mastits, Metritis oder Ketose. Aufgrund des Nährstoffbedarfs des Fötus und der Milchdrüse steigt der Energiebedarf der Kuh so stark an, dass er die Futteraufnahme übersteigt. Folge dieser negativen Energiebilanz sind gesteigerte Lipolyse und reduzierte Insulinsensitivität in peripheren Geweben. Aufgrund seiner insulin-sensitivierenden Wirkung ist AdipoQ nicht nur für die Human-Forschung interessant, sondern auch für die Gesunderhaltung der Milchkühe während der Transitionsperiode. Daher sind Strategien zur Verbesserung der Synthese und Sekretion von AdipoQ bei verschiedensten Spezies von besonderem Interesse. Zimt ist seit vielen Jahren dafür bekannt, einen positiven Einfluss auf Insulinsensitivität und Lipidstoffwechsel zu nehmen und die AdipoQ-Blut-Konzentration bei Mäusen zu erhöhen. In dieser Arbeit wurde der Fokus auf *trans*-Zimtsäure (*t*CA) als AdipoQ-Stimulator gelegt, ein bis jetzt wenig beachteter Inhaltsstoff von Zimt. Darüber hinaus verringert das Vitamin Niacin (NIA) die Lipolyseaktivität bei laktierenden Kühen und erhöht AdipoQ bei Nagern. Es könnte daher die kritische Situation der Milchkühe während der Transitionsperiode durch eine Erhöhung des AdipoQ-Status verbessern. Da sowohl Nikotinsäure (NA), ein Bestandteil des NIA, als auch *t*CA Liganden des G-Protein-gekoppelten Rezeptors (GPR) 109A sind, war nicht nur der Effekt dieser beiden Substanzen auf AdipoQ von Interesse, sondern auch der Signalweg. Ziel dieser Arbeit war es daher, den Einfluss von *t*CA auf die AdipoQ-Sekretion in murinen 3T3-L1 Adipozyten zu untersuchen und die Veränderungen auf die AdipoQ-Konzentrationen und auf die mRNA-Expression zugehöriger Gene nach Stimulation mit NA in bovinen Adipozyten zu zeigen. Des Weiteren galt es, die Beteiligung des GPR109A als Signalweg zu charakterisieren. Dazu wurden die Adipozyten mit Pertussis-Toxin (PTX) vorinkubiert. Dieses katalysiert die Bindung des inaktiven G-Proteins im Rezeptor und führt somit zu einer Unterbrechung in der Signalweiterleitung. Die Stimulation mit *t*CA steigerte signifikant die AdipoQ-Sekretion, die Behandlung mit PTX führte zu einer verringerten AdipoQ-Konzentration. Mit dieser Studie konnte *t*CA als Einflussgröße auf AdipoQ nachgewiesen werden. Somit wurde *t*CA nicht nur als aktiver Bestandteil von Zimt identifiziert, sondern auch dessen Fähigkeit zur Verbesserung des Glucose- und Fettstoffwechsels gezeigt. Des Weiteren konnte eine *t*CA-induzierte Signalweiterleitung durch GPRs gezeigt werden. In einer bovinen Adipozytenzellkultur wurde der potentielle Effekt von NA auf AdipoQ getestet. Stimulation mit NA steigerte sowohl die Sekretion von AdipoQ als auch die mRNA-Expression von GPR109A signifikant. Inkubation mit PTX verringerte die AdipoQ-Antwort auf NA. Diese NA-stimulierte Sekretion von AdipoQ und GPR109A-mRNA-Expression in bovinen Adipozyten deuten auf eine GPR-abhängige Verbesserung der Insulinsensitivität bei Milchkühen hin. Interessanterweise führte die Inkubation mit PTX in beiden Studien zu signifikant verringerten AdipoQ-Konzentrationen, jedoch immer noch erhöht im Vergleich zu nicht behandelten Adipozyten. Daher unterstellen wir neben einem GPR-abhängigen Signalweg zusätzlich eine GPR-unabhängige Signalweiterleitung bei der Regulation von AdipoQ. Der Transkriptsionsfaktor PPARγ ist ein bekannter Stimulator der Expression von AdipoQ und Regulator verschiedener Gene, die in die Kontrolle der Insulinsensitivität involviert sind und stellt daher einen möglichen *t*CA/NA-induzierten Stimulator von AdipoQ, unabhängig vom GPR109A dar. Diese Arbeit stellt zwei Faktoren vor, welche die Sekretion von AdipoQ in murinen und bovinen Adipozyten verbessern. Des Weiteren wurde sowohl die Beteiligung des GPR-Signalweges als auch eines GPR-unabhängigen, *t*CA/NA-induzierten Pfades zur Stimulation von AdipoQ gezeigt. Diese Ergebnisse könnten den Weg für weitere Studien zum Verständnis und zur Verbesserung der metabolischen Veränderungen während der Transitionsperiode im bovinen Fettgewebe *in vitro* ebnen und somit eine Grundlage für künftige *in vivo* Studien schaffen.

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List of abbreviations

a.p.	antepartum
AC	adenylyl cyclase
AcAc	acetoacetat
ACC	acetyl coenzyme A carboxylase
AdipoQ	adiponectin
AdipoR1	adiponectin receptor 1
AdipoR2	adiponectin receptor 2
AMP	adenosine monophosphate
AMPK	5` adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
APPL1	adaptor protein containing containing a pleckstrin homology domain, a phosphotyrosine domain and a leucine zipper motif 1
AT	adipose tissue
ATGL	adipose triglyceride lipase
ATP	adenosine triphosphate
BHB	beta-hydroxybutyrate
BSA	bovine serum albumin
C/EBP	CAAT-enhancer binding protein
CaMKII	Ca^{2+}/calmodulin-dependant protein kinase
cAMP	intracellular cyclic adenosine monophosphate
cDNA	copy desoxyribonucleic acid
CN	cinnamon
CoA	acetyl coenzyme A
DMEM	Dulbecco’s modified eagle’s medium high glucose
DMEM-LG	Dulbecco’s modified Eagle’s medium low glucose
DNA	desoxyribonucleic acid

DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
EIF3K	eukaryotic translation initiation factor 3, subunit K
ELISA	enzyme-linked immunosorbent assay
EMD	emerin
ER	endoplasmatical reticulum
FABP4	fatty acid binding protein 4
FCS	fetal calf serum
FFA	free fatty acids
FoxO1	forkhead box O1
gAd	globular adiponectin
GDP	guanosine diphosphate
Glut4	glucose transporter 4
GPR	G-protein-coupled receptor
GTP	guanosine triphosphate
HCA_2	hydroxycarboxylic acid receptor 2
HDL	high density lipoprotein
HMW	high molecular weight
HSL	hormone sensitive lipase
IBMX	3-isobutyl-methylxanthine
IL-18	interleukin 18
IL-6	interleukin 6
LDL	low density lipoprotein
LKB1	liver kinase B 1
LMW	low molecular weight
LRP10	lipoprotein receptor-related protein 10
MARVELD1	marvel domain containing 1
MMW	middle molecular weight

mRNA	messenger ribonucleic acid
MW	molecular weight
NA	nicotinic acid
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NEFA	non-esterified fatty acids
NIA	niacin
p.p.	postpartum
p38MAPK	p38 mitogen-activated kinase
pAMPK	phosphorylated 5` adenosine monophosphate-activated protein kinase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pen/strep	penicillin/streptomycin
PKA	protein kinase A
POLII	RNA polymerase II
PPARα	peroxisome proliferator-activated receptor α
PPARγ	peroxisome proliferator-activated receptor γ
PTX	pertussis toxin
PVDF	polyvinylidene difluoride membrane
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rp	retroperitoneal
rpm	rounds per minute
RT	room temperature
sc	subcutaneous
SEM	standard error of mean
SREBP	sterol regulatory element-binding protein

TBST	tris-buffered saline containing tween
*t*CA	*trans*-cinnamic acid
TG	triglyceride
Thr172	threonine 172
TNFα	tumor necrosis factor α
vc	visceral
VLDL	very low density lipoprotein
β-AR	beta-adrenoreceptors

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CHAPTER I: GENERAL INTRODUCTION

1. Introduction

Adipose tissue (AT) was regarded for a long time solely as energy storage or to provide thermal and mechanical insulation. Nowadays AT is known to be a dynamic, endocrine organ. A variety of bioactive molecules, known as adipokines are released from AT and play a major role not only in regulation of lipid and glucose metabolism but also in inflammation and immunity [Tilg & Moschen, 2006]. One of the most abundant adipokines is adiponectin (AdipoQ), that exerts insulin-sensitizing effects in liver and skeletal muscle and thereby takes part in protection against the development of obesity and type 2 diabetes [Tishinsky et al., 2012]. The role of AdipoQ is not only of great interest in humans, but due to its insulin-sensitizing effect also for dairy cows during transition period. The time from late pregnancy to early lactation is crucial for the potential development of many diseases and metabolic disorders, such as mastitis and metritis or ketosis [Drackley, 1999]. Energy requirements increase due to the nutrient demand of the fetus and the initiation of lactation and cannot be compensated by feed intake only. The negative energy balance is characterized by increased lipolysis and reduced insulin sensitivity in peripheral tissues, such as AT and muscle. Hence, strategies to improve synthesis and secretion of AdipoQ are of major interest in various species.

2. Literature review

2.1. The role of adipokines

Adipose tissue presents a loose connective tissue composed of mature adipocytes, pre-adipocytes (adipocytes not yet loaded with lipids), endothelial cells, fibroblasts, immune cells like leukocytes and macrophages and various other cell types. Numerous bioactive molecules, including inflammtory cytokines, chemokines, acute phase proteins and complement-like factors [Karastergiou & Mohamed-Ali 2010], secreted by AT have been identified and are generally referred to as adipokines (Table 1 lists some of them). Through their autocrine, paracrine and endocrine functions, adipokines influence the regulation of appetite and satiety, fat distribution, insulin sensitivity and insulin secretion, energy expenditure, inflammation, blood pressure, and endothelial function [Blüher 2014]. An imbalance of these adipokines

(e.g., during starvation or obesity) can lead to profound metabolic changes (e.g., increased inflammation) and subsequently to the development of diseases (e.g., insulin resistance).

Table 1: Exemplary adipokines

Characteristics	Adipokine	Factors directly affecting metabolism	Pro-inflammatory factors	Effects on other adipokines
Complement-like factors	Adiponectin[1]	X		TNFα ↓, IL-6↓
Cytokines	Leptin[2]	X	X	TNFα↑, IL-6↑, Resistin↓, Adiponectin↑
	IL-6[3]/ TNFα[4]		X/X	Leptin↑, Resistin↑, Visvatin↑, Adiponectin↓
Chemokines	Chemerin[5]	X		TNFα↓, IL-6↓, Adiponectin↑
Acute phase proteins	Haptoglobin[6]		X	unknown
Growth factors	Visfatin[7]	X		TNFα↑, IL-6↑
Others	Resistin[8]	X		TNFα↑, IL-6↑
	Vaspin[9]	X		Leptin↓, Resistin↓, TNFα↓, IL-6↓, Adiponectin↑

[1] Hu et al., 1996; Maeda et al., 1996; Scherer et al., 1995
[2] Klein et al., 1996; Zhang et al., 1994
[3] Mohamed-Ali et al., 1997
[4] Hotamisligil et al., 1993; Mohamed-Ali et al., 1999
[5] Goralski et al., 2007
[6] Friedrichs et al., 1995
[7] Fukuhara et al., 2005
[8] Steppan et al., 2001
[9] Hida et al., 2005
Abbreviations: IL: Interleukin; TNF: Tumor necrosis factor

2.2. Adiponectin

Adiponectin was first recovered by Scherer et al. [1995] in 3T3-L1 adipocytes, followed by the detection in human cells and the isolation of bovine AdipoQ by Sato et al. [2001]. Adiponectin is mainly secreted by adipocytes [Hu et al., 1996], but also expressed in cells like osteoblasts [Berner et al., 2004] or in placental tissue [Chen et al., 2006]. With a concentration of 5 - 30 µg/mL, AdipoQ is the most abundant adipokine present in human serum. The concentration of AdipoQ in serum is inversely correlated with adipocyte mass and obesity [Hu et al., 1996], which is in contrast to other adipokines like Leptin. High levels of AdipoQ promote fatty acid oxidation as well as glucose uptake in myocytes and adipocytes, and lower gluconeogenesis in liver, altogether improving insulin sensitivity. Reduced AdipoQ

expression contributes to the pathogenesis of insulin resistance and type 2 diabetes [Yamamoto et al., 2004].

2.2.1. Adiponectin structure and expression

Adiponectin structurally belongs to the complement 1q family and consists of multiple domains: a signalling peptide at the N-terminus, a species-specific variable region, followed by a collagenous domain and a C-terminal globular domain, which comprises the same amino acid sequence as the complement factor C1q. Bovine AdipoQ is a 30 kDa protein, consisting of 240 amino acids, and shows 82% homology with the murine and 92% with human AdipoQ, respectively [Sato et al., 2001] (Figure 1).

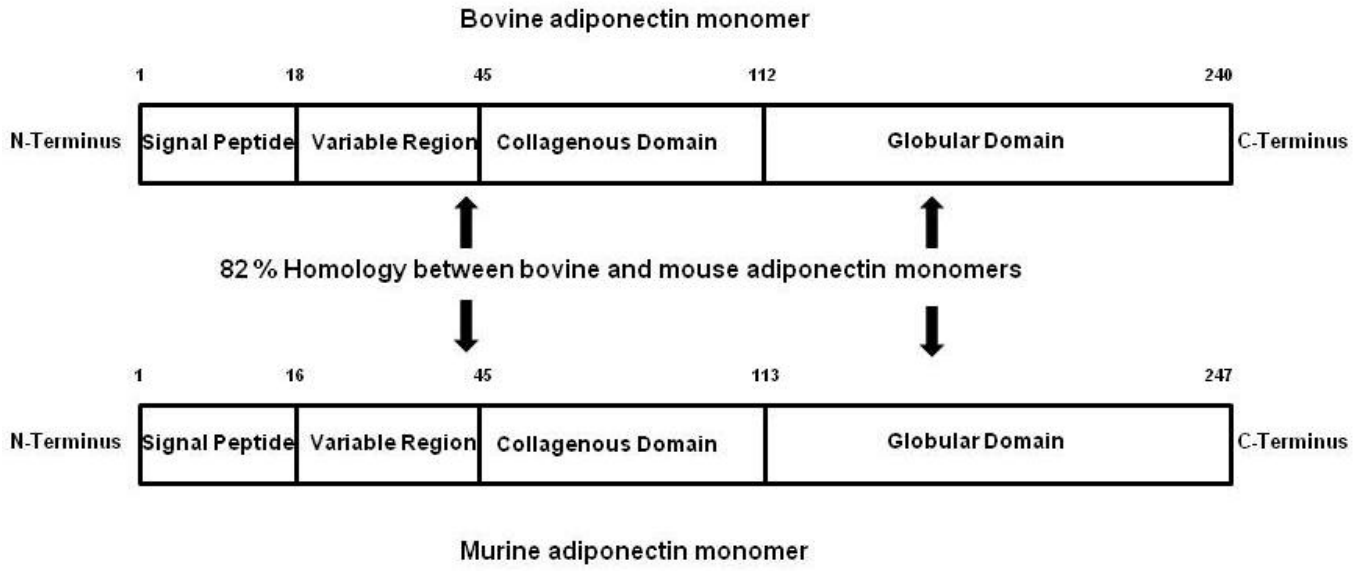

Figure 1: Domains and structure of bovine and murine adiponectin**.** Numbers indicate the positions of the amino acids (modified according to Sato et al., 2001 and Thundyil et al., 2012).

Adiponectin is synthesized as a monomer and undergoes several post-translational modifications to form isoforms of different molecular weight (MW) (Figure 2). Trimers are formed through disulphide bond linkage in the collagenous domains [Pajvani et al., 2003]. This form can undergo further post-translational modifications like hydroxylation and glycosylation of proline and lysine residues in the collagenous domain or disulphide bond linkage between cysteine residues in the variable region to oligomerize into hexamers and larger complexes [Tsao et al., 2003, Pajvani et al., 2003]. These isoforms are released into circulation as low molecular weight (trimers) (LMW), middle molecular weight (hexamers) (MMW) or high molecular weight (multimers of 18-mers or above) (HMW). In addition, a

small globular fraction of AdipoQ (gAd) exists, generated by proteolytic cleavage of the monomer, that presents less than 1% of total AdipoQ [Fruebis et al., 2001]. The HMW AdipoQ is the most abundant form in the serum and also possesses the most potent insulin-sensitizing activity in humans [Pajvani et al., 2003, Wang et al., 2008]. Giesy et al. [2012] also detected the HMW form as predominantly in early and late lactating cows, suggesting the MW distribution as one factor for decreasing insulin sensitivity in periparturient dairy cattle.

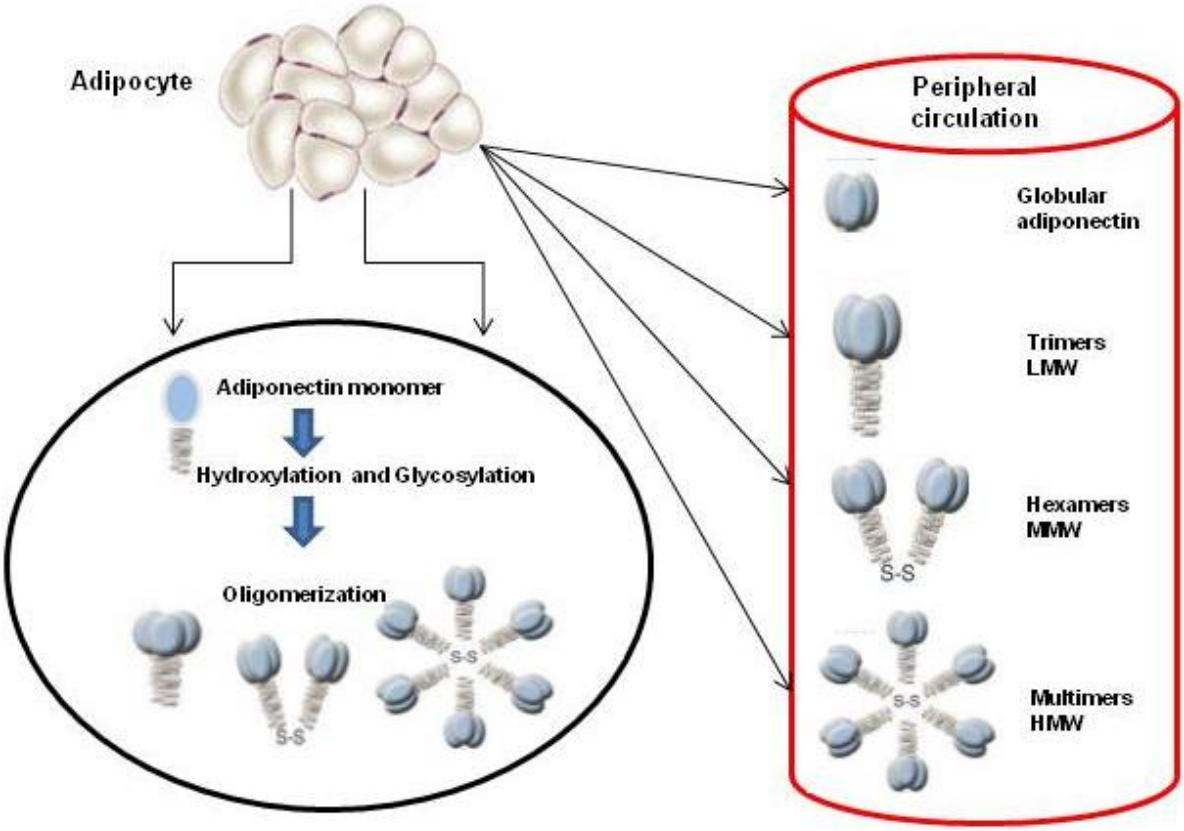

Figure 2: Synthesis, multimerisation and secretion of adiponectin. After transcription the adiponectin monomer is released into the endoplasmatic reticulum and then undergoes various post-translational modifications, like disulfide bonds (S-S), to form different isoforms. Adiponectin is released into the peripheral circulation as trimers (LMW: low molecular weight), hexamers (MMW: middle molecular weight) and multimers (HMW: high molecular weight) or after proteolytic cleavage of trimers as globular adiponectin (modified according to Kadowaki & Yamauchi, 2005, Thundyil et al., 2012).

2.2.2. Adiponectin receptors and signaling

Adiponectin exerts its biolocigal effects by binding three receptors that show differences in structure, expression, signaling and their binding affinities for the various AdipoQ multimers. The two main receptors, Adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) are seven-transmembrane proteins with an intracellular amino terminus and an extracellular carboxyl terminus. The AdipoR1 is expressed ubiquitously but most abundantly in skeletal muscle and

has a high affinity for gAd. The AdipoR2 is mostly expressed in liver [Yamauchi et al., 2003] and has an intermediate binding affinity for both gAd and full-length AdipoQ. These different binding abilities suggest that gAd exerts its effects mainly in liver, contrary to full-length AdipoQ, that acts predominately in skeletal muscle [Yamauchi et al., 2003]. The third receptor for AdipoQ is T-Cadherin, a cell surface-protein with no intracellular domain that is mainly expressed on endothelial and smooth muscle cells and has a binding affinity for hexameric and HMW AdipoQ [Hug et al., 2004]. Since T-Cadherin lacks an intracellular domain and is not expressed in primary target tissues of AdipoQ , like liver, it is discussed as being rather an AdipoQ binding protein than a functional receptor [Guerre-Millo, 2008, Thundyil et al., 2012] (Figure 3).

Upon binding of free AdipoQ to the extracellular carboxyl terminus of a receptor, an adapter protein containing a pleckstrin homology domain, a phosphotyrosine domain and a leucine zipper motif (APPL1) binds to the intracellular receptor domain, acting as a link between the receptor und the following downstream signaling molecules [Mao et al., 2006]. Hence, the APPL1 transduces the downstream signaling pathway of p38 mitogen-activated kinase (p38MAPK), peroxisome proliferator-activated receptor α (PPARα) and the 5` adenosine monophosphate-activated protein kinase (AMPK) [Matsuzawa, 2005], whereas the latter acts as the major downstream component of AdipoQ signaling [Thundyil et al., 2012].

The AMPK is a serine/threonine kinase, present in most tissues and exists as a heterotrimeric complex of catalytic α and regulatory β and γ subunits, with several isoforms of these subunits. Besides activation through AdipoQ or other adipocytokines like leptin or ghrelin, a decrease in the energy status of a cell, reflected by an increase of the intracellular AMP/ATP ratio, caused by hypoxia or glucose deprivation, also activates AMPK [Hardie & Sakamoto, 2006]. Activity of AMPK is regulated by allosteric, as well as covalent mechanisms. Binding of AMP in the γ subunit activates the enzyme up to 5-fold, but more importantly the AMP binding promotes subsequently the phosphorylation of a threonine residue (Thr^{172}) in the α subunit (pAMPK) by different upstream kinases, such as Ca^{2+}/calmodulin-dependant protein kinase (CaMKII) or the tumor supressor LKB1 [Jensen et al., 2009]. This phosphorylation is essential for full activation and produces at least the 100-fold kinase activity [Towler & Hardie, 2007]. Upon activation, AMPK phosphorylates various proteins to switch on catabolic pathways and inhibiting anabolic processes. In skeletal muscle, AMPK inhibits after AdipoQ-activation acetyl coenzyme A (CoA) carboxylase (ACC) by phosphorylation of $Serine^{79}$, leading to an decrease in malonyl CoA followed by an increase in carnitine palmitoyltransferase 1 and thus in fatty acid oxidation. Furthermore, AdipoQ stimulates

glucose uptake in skeletal muscle by glucose transporter 4 (Glut4) translocation [Ceddia et al., 2005]. Adiponectin-induced activation of AMPK in liver decreases gluconeogenesis [Yamauchi et al., 2002]. Stimulation with gAd increased glucose uptake and fatty acid oxidation in isolated adipocytes [Wu et al., 2003].

As mentioned above, p38-MAPK and PPARα are both further signalling molecules activated by AdipoQ. The PPARα, a transcription factor, predominately expressed in liver (and to a lesser extent in skeletal muscle and bone) is a major regulator of lipid metabolism in the liver. It is activated directly through AdipoQ-APPL1 binding and/or sequentially by p38MAPK phosphorylation and leads to increased fatty acid oxidation in myocytes and hepatocytes [Yamauchi et al., 2003, Deepa & Dong, 2009]. The p38MAPK regulates various cellular processes such as inflammation, cell-growth, differentiation or death.

The two AdipoQ receptors differ not only in expression and binging affinities, but also in AdipoQ signaling pathways. Whereas AdipoR1 mainly activates the AMPK pathway, the AdipoR2 acts primarily via the PPAR pathway, collaboratively regulating glucose homeostasis and lipid metabolism and hence increasing insulin sensitivity [Yamauchi et al., 2007].

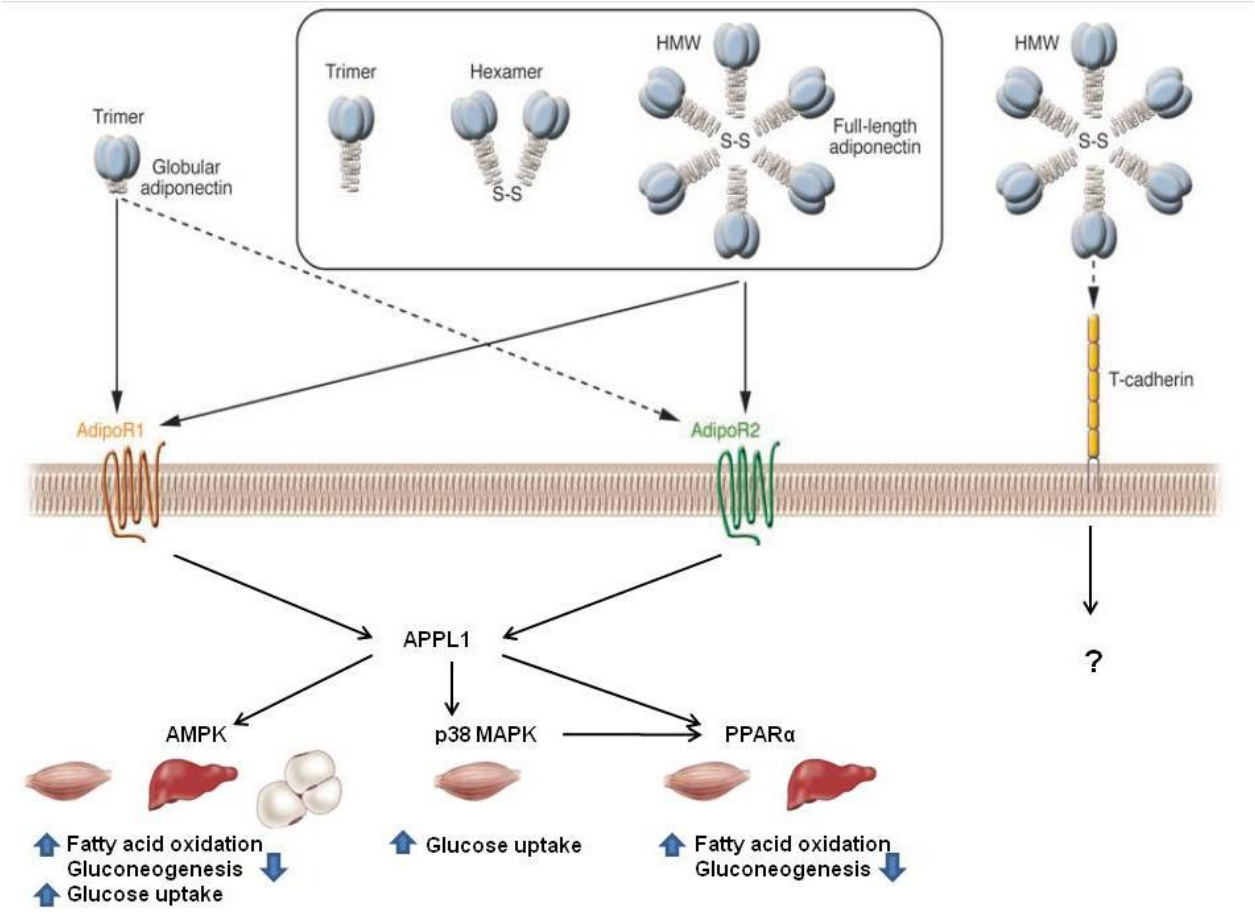

Figure 3: Schematic representation of adiponectin receptor activation and signaling. Different isoforms bind extracellular adiponectin receptor 1 and/or 2 (AdipoR1/R2), the intracellular N-terminus interacts with a pleckstrin homology domain-containing adaptor protein (APPL1) and transduces the signalling pathways of adenosine monophosphate-activated protein kinase (AMPK), peroxisome-proliferator-activated receptor α (PPAR α) and p38mitogen-activated protein kinase (p38 MAPK) (modified according to Kadowaki & Yamauchi, 2005, Deepa & Dong, 2009).

2.2.3. Regulation of adiponectin

Since AdipoQ is known to play a major role in glucose and lipid metabolism, compounds able to increase AdipoQ secretion are of great interest to improve insulin sensitivity and other metabolic diseases. Various AdipoQ regulators have been disclosed such as hormones, other adipokines or transcription factors. A short overview is given in Table 2.

Table 2: Regulation of adiponectin mRNA and protein expression in adipose tissue

Compound	Effect on AdipoQ expression	Adipocyte species/model	Reference
Transcriptionfactors			
PPARγ	mRNA↑	humans	Maeda et al., 2001;
		rodents	Iwaki et al., 2003
C/EBPs	mRNA↑	3T3-L1	Segawa et al., 2009
		mice	Gustafson et al., 2003
SREBPs	mRNA↑	humans	Kita et al., 2005
FoxO1	mRNA↑	mice	Nakae et al., 2008
			Qiao & Shao, 2006
Adipokines			
TNFα	mRNA↓	humans	Liu & Liu, 2009
		3T3-L1	Fasshauer et al., 2002
	protein↓	humans	Degawa-Yamauchi et al., 2005
IL-6	mRNA↓	humans	Liu & Liu, 2009
IL-18	mRNA↓	humans	Liu & Liu, 2009
		3T3-L1	Chandrasekar et al., 2008
Hormones			
Testosteron	mRNA↓ protein ↓	3T3-L1	Nishizawa et al., 2002
	mRNA, protein NE	mice	
Estrogens	mRNA↓, protein ↓	3T3-L1	Combs et al., 2003
Glucocorticoids	mRNA↓	3T3-L1	Fasshauer et al., 2001
			Fasshauer et al., 2002
	mRNA↓ protein ↓	humans	Degawa-Yamauchi et al., 2005
Growth hormone	mRNA↓	3T3-L1	Fasshauer et al., 2002
	mRNA↑, protein↑	3T3-L1	Xu et al., 2004
			Wölfing et al., 2008
Insulin	mRNA↓	3T3-L1	Fasshauer et al., 2002
	protein↑	humans	Motoshima et al., 2002

Abbreviations: PPAR: Peroxisome-proliferator-activated receptor, C/EBP: CAAT-enhancer binding protein; SREBP: Sterol regulatory element-binding protein; FoxO1: Forkhead box O1; TNF: Tumor necrosis factor; IL: Interleukin; NE: no effect; 3T3-L1: murine preadipocyte cell line

2.3. The importance of adiponectin in cattle during the transition period

The transition period is defined as 3 wk antepartum (a.p.) until 3 wk postpartum (p.p.). In this time period, dairy cows undergo various metabolic changes, mainly due to the nutrient demands of the fetus and the initiation of milk production [Bauman & Currie, 1980]. The increased consumption of energy and of the major nutrients, like glucose, amino and fatty acids, cannot be covered with increased dietary intake, resulting in a negative energy balance. Energy stores need to be mobilized through increased lipolysis [McNamara, 1989], and non-esterified fatty acids (NEFA) are released from adipose tissue. Exaggerated plasma NEFA concentrations lead to fat accumulation in the liver, and thus the prevalence of ketosis and fatty liver are enhanced [Grummer, 1993, Drackely, 1999]. In addition, the lack of glucose leads to reduced insulin sensitivity in peripheral tissues like adipose tissue or muscle [Bell, 1995], further increasing NEFA concentrations and may result in increased prevalence of metabolic disorder [Pires et al., 2007]. The NEFA are oxidated to acetyl-CoA, the first step to generate energy via the tricarboxylic acid cycle. The limiting step of this reaction is the supply of oxalacetate and its precursor, pyruvate (the product of glucose degradation in glycolysis). During starvation the circulating NEFA are oxidated to acetyl-CoA, but due to the lack of glucose, acetyl-CoA cannot enter the citric acid cyclus. Ketone bodies like acetoacetate (AcAc) or beta-hydroxybutyrate (BHB) are formed to supply energy for brain or muscle in the absence of glucose. Therefore, BHB is used as an indicator lipolysis in dairy cows.

Information about AdipoQ during the transition period is very little. Singh et al. [2014] reported that plasma AdipoQ decreased from wk 3 a.p. and reached the lowest concentrations at the time of parturition; thereafter, the concentration increased gradually and approached a.p. values within 3 wk of lactation. This is in line with Giesy et al. [2012] who showed a maximal reduction of 45% of AdipoQ on the day after parturition and a progressive return to late pregnancy values by d 56 of lactation. Komatsu et al. [2007] demonstrated an increase in AT AdipoQ mRNA abundance between peak lactation and non lactating cows. In addition, our group [Lemor et al., 2009] showed a reduction in the mRNA abundance of AdipoR1/2, as well as Koltes & Spurlock [2012] in the AdipoQ mRNA abundance during transition period both in subcutaneous (sc) AT. In summary, decreased values of the AdipoQ system may support insulin sensitivity from late pregnancy to early lactating in dairy cows. Therefore, support to increase AdipoQ synthesis and release is of great interest.

2.4. Nutritional impact on the expression of adiponectin

Type 2 diabetes, obesity, and the metabolic syndrome are often associated with insulin resistance in humans. Due to its insulin-sensitizing effect, AdipoQ is a potential target in treatment of these diseases. Besides hormonal regulation, the influence of nutrition is more and more discussed to improve AdipoQ values. Some of the nutrients considered relevant in this context will be reviewed below. Several studies showed that the pattern of fatty acids in the diet affects the AdipoQ system. Diets rich in ω-3 polyunsaturated fatty acids increased AdipoQ concentrations in mice [Fukumitsu et al., 2008]. Similar results were observed by the partial replacement of vegetable oil by eicosapentaenoic acid and docosahexaenoic acid [Flachs et al., 2006]. Mullen at al. [2010] reported that AdipoQ resistance in the soleus muscle of rats caused by a diet with saturated fat was prevented by the replacement with polyunsaturated fatty acids. Besides the effect of fatty acids, supplementation with Vitamin E increased AdipoQ mRNA and protein expression *in vivo* and *in vitro* in rodents as well as in humans [Vincente et al., 2009]. Other antioxidants failed to induce this effect, pointing to an antioxidative-independant induction of AdipoQ expression by Vitamin E. A number of spices and herbs have a long tradition in treating elevated blood glucose and serum lipid levels and impaired insulin response. The main catechin in green tea, (-)- epigallochatechin gallate, increased plasma AdipoQ concentrations and insulin response in humans [De Oliveira et al., 2012], similar to (-)-catechin that increased expression and secretion of AdipoQ in 3T3-L1 [Cho et al., 2007].

This thesis will focus on two other nutrients, *trans*-cinnamic acid (*t*CA) and niacin (NIA) as well as their potential role in the AdipoQ system. Since both nutrients, or rather one of their compounds, are ligands of the G-protein-coupled receptor (GPR) 109A, information about their signal transduction should be gathered since modulation of the glucose and lipid metabolism by AdipoQ might act via this pathway.

2.4.1 The G-protein-coupled receptor 109A

The GPR109A, recently known as hydroxycarboxylic acid receptor 2 (HCA2), is mainly expressed in adipocytes and immune cells, such as monocyctes, macrophages and dendritic cells [Gille et al., 2008]. The GPR109A belongs to the seven-transmembrane receptor family with an extracellular amino-terminal segment and an intracellular carboxy-terminal tail. The coupled G-protein exists as a heterotrimeric complex of Gα and Gβ/γ subunits and belongs, in case of GPR109A, to the G_i family. Upon activation by a ligand, the Gα subunit undergoes a

conformational change and replaces Guanosine diphosphate (GDP) with Guanosine triphosphate (GTP), which initiates the translocation of Gα and Gβ/α subunits (Figure 4).

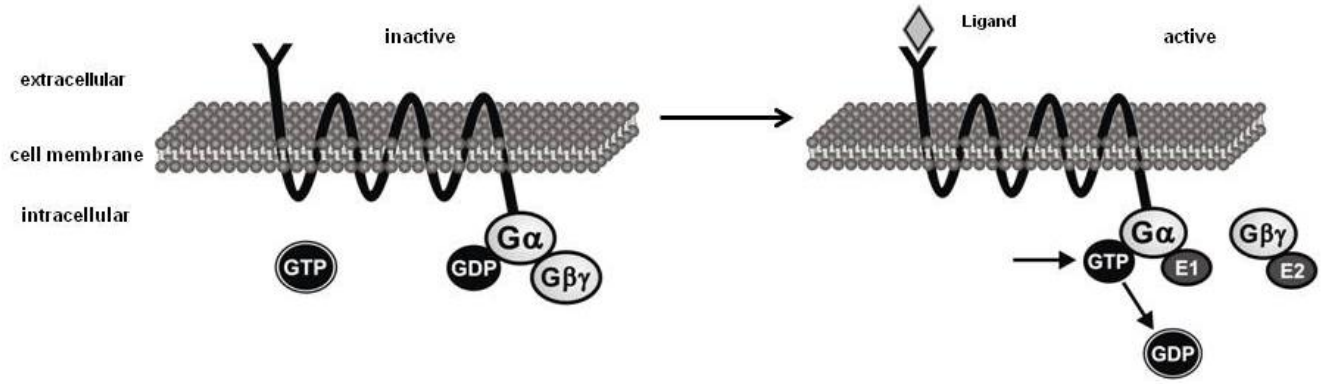

Figure 4: Signal transduction of G-protein-coupled receptor. The seven transmembran receptor is associated with a heterotrimeric G-protein with Gα and Gβγ subunits, and a bound Guanosine diphosphate (GDP). After activation of the receptor by its ligand, the Gα translocates from the other subunits and replaces GDP with Guanosine triphosphate (GTP). Both subunits (Gα and Gβγ) have own effectors (E1/E2) to transmit signals and thereby initiate cellular responses (modified according to Tuteja, 2009).

Both subunits interact with their downstream effectors to continue differential signal transduction depending on the cell type. Nicotinic acid as a compound of NIA is a known ligand of the GPR109A. In adipocytes, activation of the GPR109A by NA inhibits the adenylyl cyclase (AC), resulting in a decrease of intracellular cyclic adenosine monophosphate (cAMP) level and the subsequent inactivation of protein kinase A (PKA) and hormone sensitive lipase (HSL) leading to reduced trigyceride hydrolysis, thereby decreasing liopolysis and the release of free fatty acids (FFA) into the circulation (Figure 5A) [Gille et al., 2008, Wanders et al., 2012, Tuteja, 2009, Tunaru et al., 2003]. After signal transduction, the GTP is hydrolyzed to GTP and the inactive Gα re-associates with Gβγ to the original heterotrimeric complex. As mentioned above, ketone bodies like BHB accumulate due to the metabolization of NEFA during starvation to supply energy for brain or muscle in the absence of glucose. Beta-hydroxybutyrate is the endogenous ligand of GPR109A [Offermanns et al., 2011, Taggart et al., 2005] and is known since the early 1970`s to inhibit lipolysis in bovine adipose tissue *in vitro* [Metz et al., 1974]. Through activation of its receptor and the following signal transduction, BHB is part of a negative feedback loop to adjust lipolytic activity under starvation (Figure 5B) [Gille et al., 2008].

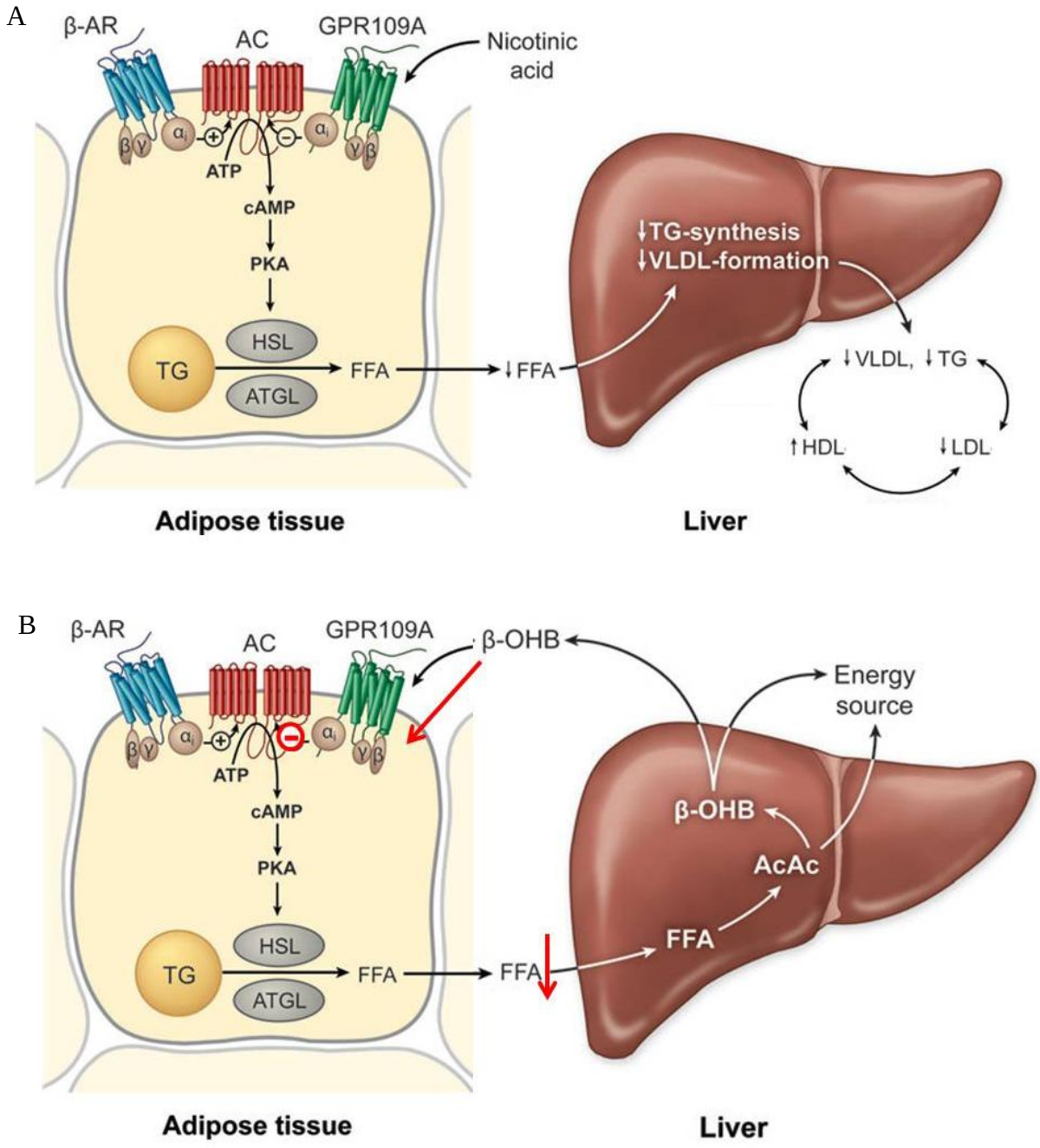

Figure 5: Metabolic effects of G-protein-coupled receptor 109A (GPR109A) binding . A: Binding of nicotinic acid to GPR109A inhibits the adenylyl cyclase (AC) and leads to decreased levels of cyclic adenosine monophosphate (cAMP) and the subsequent inhibition of protein kinase A (PKA) and hormone sensitive lipase (HSL). The amount of free fatty acids (FFA) decreases, so that less triglyceride (TG) and very low density lipoproteins (VLDL) can be synthesized in the liver. Abbreviations: high density lipoproteins (HDL), low density lipoproteins (LDL), adipose triglyceride lipase (ATGL) B: During starvation or negative energy balance, sympathetic stimulation of adipose cells via beta-adrenoceptors (β-AR) is high and insulin is low resulting in increased levels of cAMP. Lipolysis is stimulated via PKA and HSL and FFA are released from adipose tissue. In the liver the FFA are metabolized to ketone bodies such as beta-hydroxybutyrate (here abbreviated as β-OHB) or acetoacetate (AcAc) to act as energy source for brain or muscle. The amount of β-OHB increases and binds as endogenous ligand to GPR109A. After binding and induction of signal transduction the AC is inhibited, the activity of PKA and ATGL is reduced and the levels of FFA decrease (signal transduction via β-OHB is depicted in red color). This receptor-ligand binding is part of negative feedback loop to adjust lipolytic activity during starvation (modified according to Gille et al., 2008).

2.4.2 Niacin

Niacin (NIA), also known as Vitamin B3, is a collective term for both nicotinic acid (NA) and nicotinamide [Wanders et al., 2012]. Niacin and NA are often used synonymously, even though only NA is a ligand for the GPR109A. To be as precisely as possible, the term NIA will be used in this thesis with respect to both common information about NIA and complementary literature. The term NA will be used referring to our own results. Nicotinic acid and nicotinamide are both precursors of the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) and are essential for oxidative phosphorylation in energy metabolism [Jacobson et al., 2012]. Furthermore, NIA is used for more than 50 years as an antidyslipidemic drug in humans, since pharmacological doses of NIA reduce triglycerides, lipoprotein A and low-density lipoprotein lipoproteins (LDL), as well as increase high-density lipoprotein (HDL) lipoproteins [Lukasova et al., 2011]. These antilipolytic effects are mediated through the GPR109A [Tunaru et al., 2003]. Besides the effect on lipid profiles, several studies demonstrated that NIA stimulates the expression and secretion of AdipoQ [Plaisance et al., 2009, Wanders et al., 2013, Digby et al., 2010, Westphal et al., 2007] in humans and rodents. Linke et al. [2008] showed not only an increase of HDL and AdipoQ protein, but also of AdipoQ, C/EBP and PPARγ gene expression during treatment with extended-release NIA in humans. Furthermore, improved insulin sensitivity was observed, supporting the AdipoQ stimulating effect of NIA. As mentioned above, the GPR109A has been demonstrated as the molecular target for the antilipolytic actions of NIA. In addition, Plaisance et al. [2009] reported that the secretion of AdipoQ is inhibited by pertussis toxin (PTX), a G_i/G_o-protein-un-coupling compound, during NIA treatment in 3T3-L1 adipocytes. Similar results have been obtained in GPR109A knockout mice; these findings support the involvement of GPR-signaling in NIA-induced AdipoQ stimulation [Plaisance et al., 2009].

Not only in rodents and humans, but also in cattle the antilipolytic effect of NIA has been demonstrated. Several studies showed a decrease of lipolysis in lactating cows after abomasal infusion [Pires & Grummer, 2007, Pires et al., 2007, Pescara et al., 2010] or when used as feed supplement [Jaster & Ward, 1990]. In addition, feeding of encapsulated NIA to Holstein cows in the transition period was followed by a decrease in the plasma NEFA level [Morey et al., 2011]. Recently, NIA was demonstrated to decrease isoproterenol-stimulated lipolysis in adipose tissue explants of dairy cows by reducing the phosphorylation of hormone-sensitive lipase, suggesting the presence of a GPR109A-mediated anti-lipolytic pathway in dairy cows [Kenez et al., 2014]. However, information about the effects of NIA on AdipoQ and the

linked signalling pathway in cattle is still lacking, although the improvement of lipid profiles and insulin response could be of great significance for dairy cows especially during the transition period.

2.4.3 *Trans*-cinnamic acid

Cinnamon (CN) is the bark of trees from the genus *Cinnamomum* and contains several active compounds such as cinnamaldehyde and *trans*-cinnamic acid (*t*CA). Cinnamon is known to improve insulin sensitivity and lipid profiles: in 3T3-L1 adipocytes glucose uptake, glycogen synthesis and activated glycogen synthase were stimulated by cinnamon [Jarvill-Taylor et al., 2001]. *In vivo* studies presented decreased fasting blood glucose, TG, LDL cholesterol and total cholesterol after supplementation with cinnamon in humans [Ziegenfuss et al., 2006, Khan et al., 2003, Mang et al., 2006]. Furthermore, increased levels of AdipoQ and PPARγ mRNA abundance (in liver and AT) as well as improved insulin sensitivity were reported after the administration of cinnamon bark extracts in mice [Kim & Choung, 2010]. Huang et al. [2011] showed in 3T3-L1 cells *in vitro*, as well as in murine adipose tissue *in vivo*, an increased AMPK activation after supplementation with cinnamaldehyde. However, Chen at al. [2009] reported that in case of oral uptake, cinnamaldehyde is partially metabolized to *t*CA in stomach and small intestine and completely in liver, before released into the circulation of rodents. This points to *t*CA as the active compound of cinnamon, responsible for the insulin-sensitizing effect. Based on this hypothesis, Hafizura et al. [2015] showed that *t*CA and not cinnamaldehyde decreased blood glucose and improved glucose tolerance *in vitro* and stimulated insulin secretion *in vivo*, suggesting *t*CA also as influencing variable for the AdipoQ system. Although the beneficial effects of cinnamon and its compounds were proven in several studies, little was known about its signaling pathway. Interestingly, Ren et al. [2009] identified *t*CA as a ligand of GPR109A and thus, *t*CA, like other ligands, might improve insulin sensitivity through stimulation of AdipoQ and the associated molecules like AMPK.

3. Objectives

Adiponectin is known to improve insulin sensitivity and lipid metabolism. Therefore, the examination of new factors to increase AdipoQ synthesis and secretion is of great importance to ameliorate metabolic diseases like insulin resistance and Type II diabetes. Due to the harmful metabolic changes during the transition period, knowledge on the regulation of AdipoQ is of special interest in cattle. Therefore, the development of a quick and uncomplicated bovine specific adipocyte cell culture model was crucial to gather basic information on the cellular level on synthesis and secretion of bovine AdipoQ.

First steps in cell culture on adipocytes were done using 3T3-L1cells differentiated to adipocytes. In this system, *t*CA, a barely explored compound of cinnamon, was examined.

Afterwards, a primary cell culture model consisting of differentiated bovine adipocytes was developed that can be used to test the influence of potential effectors on the bovine AdipoQ synthesis and secretion *in vitro*. Niacin is known to decrease lipolysis and plasma NEFA levels in lactating cows and to increase AdipoQ concentration in rodents. Due to its potential ability to improve the metabolic situation of dairy cows during the transition period, NIA, i.e., its compound NA, was tested in this bovine cell culture model. In addition to the effects of NA on AdipoQ also the pathway of signal transduction should be taken into account. Therefore, the present research study has been designed with the following objectives:

1. to investigate effects of *t*CA on the secretion of AdipoQ and the activation of AMPK in murine adipoyctes,
2. to evaluate the changes of AdipoQ secretion and mRNA abundance of selected genes after supplementation with NA in bovine adipocytes,
3. to characterize the involvement of the GPR109A in the signaling pathway to AdipoQ.

CHAPTER II: MANUSCRIPT 1

Trans-Cinnamic Acid Increases Adiponectin and the Phosphorylation of AMP-Activated Protein Kinase through G-Protein-Coupled Receptor Signaling in 3T3-L1 Adipocytes

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Abstract: Adiponectin and intracellular 5'adenosine monophosphate-activated protein kinase (AMPK) are important modulators of glucose and fat metabolism. Cinnamon exerts beneficial effects by improving insulin sensitivity and blood lipids, e.g., through increasing adiponectin concentrations and AMPK activation. The underlying mechanism is unknown. The G_i/G_o-protein-coupled receptor (GPR) 109A stimulates adiponectin secretion after binding its ligand niacin. *Trans*-cinnamic acid (*t*CA), a compound of cinnamon is another ligand. We hypothesize whether AMPK activation and adiponectin secretion by *t*CA is transmitted by GPR signaling. Differentiated 3T3-L1 cells were incubated with pertussis toxin (PTX), an inhibitor of G_i/G_o-protein-coupling, and treated with different *t*CA concentrations. Treatment with *t*CA increased adiponectin and the pAMPK/AMPK ratio ($p \leq 0.001$). PTX incubation abolished the increased pAMPK/AMPK ratio and adiponectin secretion. The latter remained increased compared to controls ($p \leq 0.002$). *t*CA treatment stimulated adiponectin secretion and AMPK activation; the inhibitory effect of PTX suggests GPR is involved in *t*CA stimulated signaling.

Keywords: *trans*-cinnamic acid; Adiponectin; 5'adenosine monophosphate-activated protein kinase; G-protein-coupled receptor 109A

1. Introduction

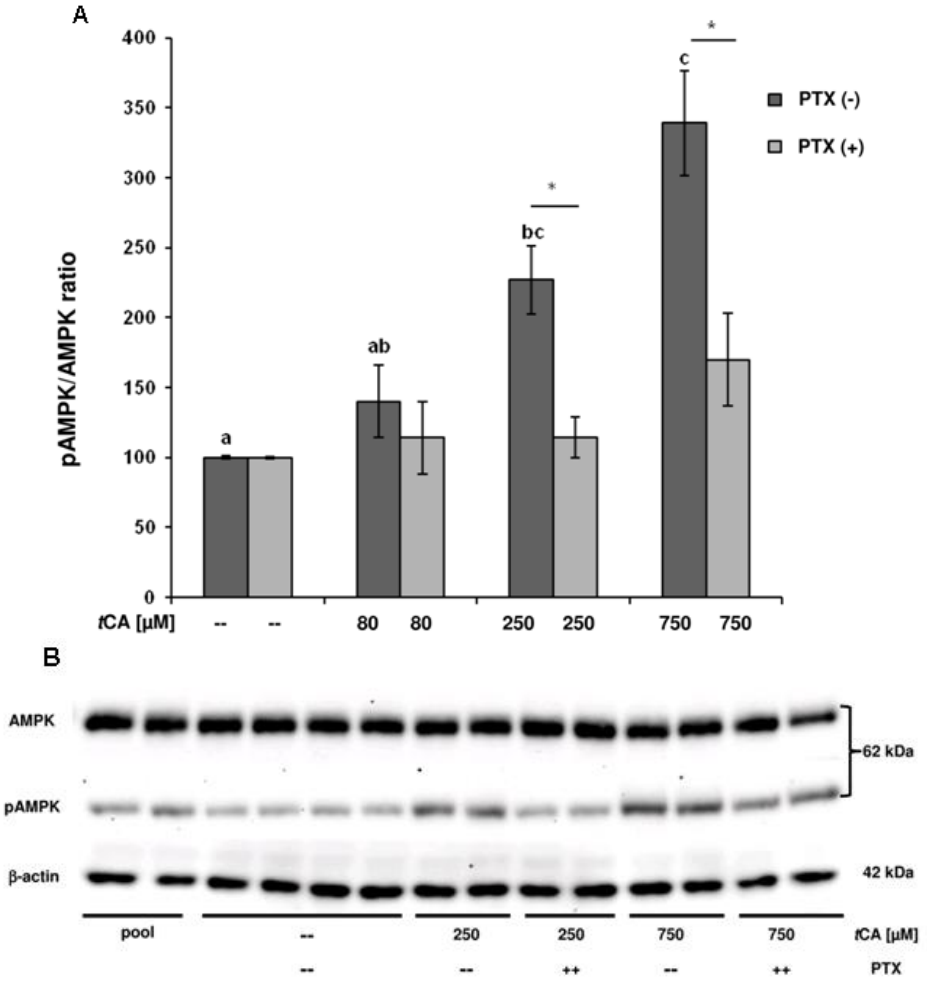
Cinnamon (CN) is known to exert several beneficial effects by improving insulin sensitivity and lipid profiles. Enhanced glucose uptake and glycogen synthesis were reported after stimulation of 3T3-L1 adipocytes with hydroxychalcone, a compound of cinnamon [1]. Khan *et al*. [2] demonstrated that supplementation with cinnamon reduces fasting serum glucose and improves blood lipid profiles in patients with type 2 diabetes. In mice treated with an extract of cinnamon bark, the concentration of the adipokine adiponectin (AdipoQ) was increased [3]. Adiponectin is mainly expressed in adipocytes [4] and is important for modulating glucose and fat metabolism in insulin-sensitive tissues like skeletal muscle and liver. Adiponectin exerts its effects via binding to its receptors AdipoR1/R2 and activation of peroxisome proliferator-activated receptor α (PPARα) and 5'adenosine monophosphate-activated protein kinase (AMPK) [5]. The AMPK is a heterotrimeric kinase complex, consisting of a catalytic α subunit and regulatory β and γ subunits [6]. Multiple isoforms of these subunits have been identified [7], and the α_1-subunit represents the predominant isoform in adipose tissue [8] as well as in cultured 3T3-L1 cells [9]. Besides AdipoQ, metabolic active hormones like leptin or insulin, and an increased cellular AMP/ATP ratio activate AMPK through phosphorylation (pAMPK) of threonine 172 in the α_1-subunit. Huang *et al*. [10] showed in 3T3-L1 cells *in vitro*, as well as in murine adipose tissue *in vivo*, an increased AMPK activation after supplementation with cinnamaldehyde, one compound of CN. Upon activation, AMPK switches on catabolic pathways (e.g., fatty-acid oxidation and glycolysis) and inhibits anabolic processes like cholesterol, glycogen, and protein synthesis in liver and muscle. The AMPK acts as an intra-cellular energy sensor and hence improves insulin sensitivity in insulin-sensitive tissues like adipose tissue, but here the data about AMPK and its effect remain poorly distinguished [11]. The effect of various ingredients of CN on AMPK and AdipoQ is reported, but the underlying mechanism is not characterized. *Tran*s-cinnamic acid (*t*CA), another isolated compound of cinnamon, was recently identified as a ligand of the G-protein-coupled receptor (GPR) 109A [12]. The seven transmembrane GPR109A, a member of the recently deorphanized hydroxycarboxylic acid receptor family, which is also known as HCA_2 [13], is expressed in activated macrophages and in adipocytes [14]. The binding of GPR109A agonists like niacin and its endogenous ligand β-hydroxybutyrate has been shown to activate this receptor and stimulate AdipoQ secretion in adipose tissue [15]. Therefore, we hypothesized that *trans*-cinnamic acid, as compound of CA, stimulates AdipoQ and AMPK also through G-protein-coupled receptor signaling.

To verify this hypothesis, we investigated the changes in AdipoQ secretion and the prevalence of the phosphorylated form of AMPK in differentiated 3T3-L1 adipocytes stimulated with different concentrations of the recent characterized GPR109A ligand *t*CA. To prove signaling by G-protein-coupled receptors, the adipocytes were additionally pre-incubated with pertussis toxin (PTX), an inhibitor of G_i/G_o protein coupling.

2. Results

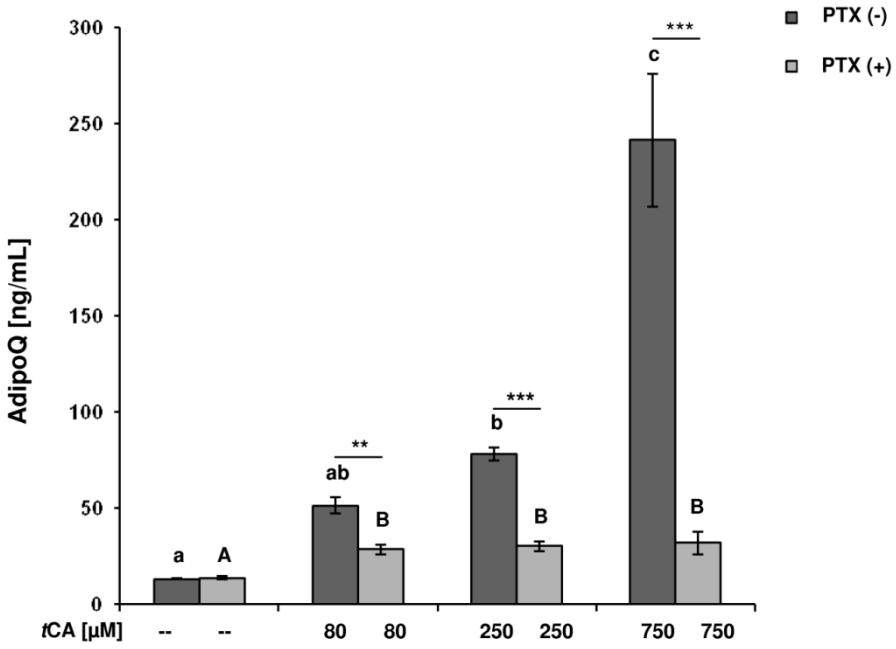
To test whether *t*CA has an effect on the phosphorylation of AMPK, the differentiated 3T3-L1 cells were stimulated with three different concentrations of *t*CA (80 µM, 250 µM, 750 µM) for 5 h. *Trans*-cinnamic acid increased ($p \leq 0.001$) the extent of phosphorylation of Thr 172 of AMPK in a dose dependent manner (Figure 1a). When compared with the controls, activation of the AMPK was 2 and 3 times higher in cells treated with 250 µM or 750 µM *t*CA ($p = 0.009$ and $p \leq 0.001$, respectively), whereas the pAMPK/AMPK ratio in 80 µM *t*CA treatment was similar to controls. To assess whether the effects of *t*CA were mediated by G_i/G_o-protein-coupled receptor signaling, the experiments were conducted following pre-incubation with PTX (100 ng/mL) for 16 h. Treatment with PTX dampened the increase of pAMPK/AMPK ratios after *t*CA treatment, no differences were observed neither among the treatment groups, nor in comparison to controls pre-incubated with PTX. Cells treated with 250 µM and 750 µM *t*CA, respectively, but without PTX pre-incubation showed two times higher pAMPK/AMPK ratios ($p = 0.011$ and $p = 0.026$) when compared to the same treatment groups with PTX incubation (Figure 1A). A representative picture of Western blot analyses is shown in Figure 1B.

Figure 1. *Trans*-cinnamic acid (*t*CA) affects the intracellular 5'adenosine monophosphate-activated protein kinase (AMPK) activation by phosphorylation (pAMPK) in differentiated 3T3-L1 cells. (A) *Trans*-cinnamic acid effects on pAMPK/AMPK ratios in differentiated 3T3-L1 cells. After 4h of starvation, the adipocytes were pre-incubated with (PTX (+)) or without pertussis toxin (PTX (−)) (100 ng/mL) for 16 h and then treated for 5 h with 80, 250 or 750 µM *t*CA, or with buffered saline (PBS) as controls respectively. Different lower case letters designate significant differences ($p \leq 0.01$) between *t*CA treatments and controls. Significant differences ($p \leq 0.05$) due to (+) or (−) PTX pre-incubation are designated with asterisks (*) for each *t*CA treatment group. Data are expressed as means ± SEM (n = 6); (B) Representative Western blot analyses. After gel electrophoreses, membranes were incubated with specific antibodies against AMPK, pAMPK or with β-actin as loading control.



Treatment with *t*CA increased ($p \leq 0.001$) AdipoQ concentrations in the cell culture supernatant dose dependently (Figure 2). When compared to controls, AdipoQ concentrations were increased five-fold (78 ± 3.4 ng/mL) after stimulation with 250 µM *t*CA ($p = 0.005$) and were about 18 times higher (241 ± 34.4 ng/mL) after treatment with 750 µM *t*CA ($p \leq 0.001$). For all *t*CA treatment groups, pre-incubation with PTX lowered the AdipoQ concentrations to values between 28.5 ± 2.6 and 32 ± 5.9 ng/mL, but consistently higher concentrations than in the related controls ($p \leq 0.002$) were retained.

Figure 2. *Trans*-cinnamic acid (*t*CA) effects on AdipoQ concentrations in cell culture supernatant of differentiated 3T3-L1 cells. After 4 h of starvation, the adipocytes were pre-incubated with (PTX (+)) or without pertussis toxin (PTX (−)) (100 ng/mL) for 16 h and then treated for 5 h with 80, 250 or 750 µM *t*CA, with PBS as controls, respectively. Different lower case letters designate significant differences ($p \leq 0.01$) between *t*CA treatments *vs*. controls for PTX (−) cells, different capital letters designate significant differences ($p \leq 0.01$) between *t*CA treatments *vs*. controls for PTX (+) cells. Significant differences (**: $p \leq 0.01$; ***: $p \leq 0.001$) due to PTX (+) or PTX (−) pre-incubation for each *t*CA treatment group are indicated with asterisks. Data are expressed as means ± SEM ($n = 6$).



Comparing PTX pre-incubation groups, AdipoQ concentrations decreased by 1.8 and 2.5 times after PTX pre-incubation in the 80 µM *t*CA ($p = 0.002$) and 250 *t*CA ($p \leq 0.001$) treatment groups, respectively, compared to the corresponding PTX (−) group. In addition, the AdipoQ

concentrations in the supernatant of the 750 µM treated cells decreased seven-fold ($p \leq 0.001$) with PTX pre-incubation.

Correlation analysis across all samples confirmed a linear relationship between the AdipoQ concentrations and the pAMPK/AMPK ratio ($p \leq 0.001$, $r = 0.534$).

3. Discussion

We investigated the changes in AdipoQ secretion and the prevalence of the phosphorylated form of AMPK in differentiated 3T3-L1 adipocytes stimulated with different concentrations of the recently identified GPR109A ligand *t*CA. In addition, it was to be characterized if these changes were mediated through G_i/G_o-protein-coupled receptor signaling. The major findings were as follows: 1. *t*CA increased secretion of AdipoQ and phosphorylation of AMPK; 2. Inhibition of GPR signaling by PTX abrogated the activating effect of *t*CA on secretion of AdipoQ and phosphorylation of AMPK but not completely. Several studies characterized CN to improve glucose and lipid profiles [1,2,16]. Various components and sources of CN were tested but we introduced *t*CA, another isolated compound, for the first time as the influencing variable on the AdipoQ system and, thereby, on glucose and fat metabolism. Corresponding to the study of Kim *et al*. [3] in which liquid Cinnamon bark extract was administered to mice, we showed that AdipoQ secretion increased in a dose dependent manner by *t*CA treatment. Adiponectin, one of the most important adipokines, improves insulin resistance and lipid metabolism [17]. The effects are mediated through its receptors AdipoR1/R2 and can at least partially be explained by their direct activation of AMPK in skeletal muscle, liver and adipose tissue [18]. Here, we showed a correlation between AdipoQ and the pAMPK/AMPK ratio, presuming an activation of AMPK subsequent to the increased secretion of AdipoQ after *t*CA treatment, supporting the study of Yamauchi *et al*. [19]. In our study, treatment with *t*CA-induced phosphorylation of AMPK up to three times more than in non-treated cells. That concurs with findings of Huang *et al*. [10] where activation of AMPK after treatment with cinnamaldehyde in 3T3-L1 adipocytes was observed and confirmed by dampened effects after adding compound C, a specific inhibitor of AMPK. In addition, Huang *et al*. [10] showed in consequence of cinnamaldehyde treatment an increase in phosphorylation and, thereby, inactivation of acetyl-CoA carboxylase (ACC), which is associated with a decreased lipogenic rate and reduced lipolysis [8]. Furthermore, phosphorylation of both proteins is said to be related to increased mitochondrial fatty acid oxidation in adipose tissue [8,10]. Although the beneficial effects of CN and its compounds were proven in several studies, little is known about its signaling pathway. Ren *et al*. [12] recently identified *t*CA as a ligand of GPR109A (PUMA-G in mice), mainly expressed in immune cells and adipocytes. Niacin as another ligand of GPR109A is known to increase AdipoQ secretion [20]. Plaisance *et al*. [15] demonstrated that the AdipoQ modulating effect of niacin is mediated through the GPR109A. Mice deficient in PUMA-G (GPR109A) showed no increase in serum AdipoQ concentration after treatment with niacin. To test if the modulating effect of *t*CA on AdipoQ and AMPK is mediated by G_i/G_o-protein-coupled receptors, we pre-incubated the adipocytes with PTX, an inhibitor of G-protein coupling. The *t*CA-induced activation of AMPK was abolished after blocking of G_i/G_o signaling, indicating this pathway is involved in the signal transmission of *t*CA. The AdipoQ secretion was, as expected, significantly decreased after receptor blocking irrespective of the *t*CA treatment group, but still significantly increased according to controls. This is in contrast to the findings of Plaisance *et al*. [15], who showed an abrogated increase

in AdipoQ secretion after stimulation with niacin and PTX in rat adipocytes. Due to diminished but still higher AdipoQ concentration after PTX incubation, another possible stimulator for AdipoQ after *t*CA treatment should be discussed. Kim and Choung [3] showed an up-regulated mRNA expression of peroxisome proliferation-activated receptor (PPARγ) in adipose tissue after treatment with an extract of cinnamon bark. The transcription factor PPARγ is a known stimulator of AdipoQ expression [21] and a regulator of several genes involved in controlling insulin sensitivity [22]. Besides increased PPARγ expression, an increase of AdipoQ secretion was observed after administration of cinnamon extract, supporting this regulation as possible stimulus of AdipoQ secretion after *t*CA treatment, besides the signaling through the GPR109A [3]. The data about the expression of GPR109A and its signaling capability in 3T3-L1 adipocytes is controversial. Zhang *et al*. [23] were unable to detect gene expression of GPR109A; Jeninga *et al*. [24] showed clearly an increasing mRNA as well as protein expression of GPR109A in 3T3-L1 cells throughout differentiation, which was increased by the PPARγ agonist rosiglitazone. Also, Digby *et al*. [25] observed the expression of GPR109A mRNA, which was upregulated by TNFα. Plaisance *et al*. [15] showed protein expression of GPR109A in 3T3-L1 cells but observed no effect on AdipoQ secretion after niacin treatment, whereas Ge *et al*. [26] detected increased glycerol release after stimulation with niacin. The findings of Plaisance *et al*. [15] were annihilated when the cells were transfected with the human GPR109A orthologon HM74A. In our study, the presence of GPR109A mRNA in the differentiated 3T3-L1 *in vitro* model was proven (data not shown). The GPR109A ligand *t*CA increased the AdipoQ secretion via GPR signaling in 3T3-L1 adipocytes. Due to our experimental design, it was not possible to specify the G_i/G_o-protein-coupled receptors mediating the effects of *t*CA, but we assume that further studies using e.g., a specific GPR109A agonist, or primary adipocytes from GPR109A knockout mice, will define the GPR109A being involved in *t*CA signaling pathways. Furthermore, our findings after PTX pre-incubation indicate a potential, but still unknown *t*CA mediated signaling pathway besides the one through GPR signaling that might be related to the activation of PPARγ, and which should be verified in the future.

4. Experimental Section

4.1. Cell Culture

Murine 3T3-L1 fibroblast cells were seeded in 25 cm^2 flasks at a density of 4000 cells per cm^2 and cultured with Dulbecco's modified eagle's medium high glucose (DMEM) containing 10% fetal calf serum (FCS) and 10 mg/mL penicillin/streptomycin (pen/strep) (basic medium) (all from PAA, Pasching, Austria) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 24 h. To induce differentiation of the 3T3-L1 fibroblasts into adipocytes, 0.5 mM 3-isobutyl-methylxanthine (IBMX) (Applichem, Darmstadt, Germany), 0.25 µM dexamethasone and 5 µg/mL bovine insulin (both from Sigma-Aldrich, St. Louis, MO, USA) were added to the basic medium for 48 h. Cells were then maintained in basic medium supplemented with 5 µg/mL bovine insulin. Media were replaced every 2 days until 85%–

95% of the cells were differentiated (day 12 after initiation of differentiation), which was documented by the accumulation of lipid droplets (Oil Red O staining, 0.2%).

4.2. Treatment of Cells

Prior to the treatments, cells were cultured in basic medium for 24 h, then serum starved in DMEM supplemented only with 0.1% fatty acid-free bovine serum albumin (BSA) (Carl Roth, Karlsruhe, Germany) for 4 h. The adipocytes were subsequently incubated for 16 h with 100 ng/mL pertussis toxin (PTX) (Sigma-Aldrich, St. Louis, MO, USA), which selectively affects G_i/G_o signaling, to characterize possible effects of *t*CA by GPR signaling. Cells were then treated with 80 µM, 240 µM or 750 µM *t*CA (Sigma-Aldrich, St. Louis, MO, USA) for 5 h (n = 6). Equal volumes of the solvent (phosphate buffered saline (PBS)) were applied to controls instead of PTX and *t*CA, respectively. At the end of the incubation time, supernatant was collected and stored at −20 °C until analysis. The adherent adipocytes were washed twice with ice cold PBS and lysed with pre-chilled lysis buffer as described previously [27]. The cell lysates were harvested by scraping, transferred into pre-chilled 1.5 mL tubes and centrifuged at 16,000 *g* for 20 min at 4 °C. Protein concentrations were measured according to Bradford [28].

4.3. Western Blot

For the detection of AMPK and pAMPK, respectively, 18 µg total protein were treated with Laemmli buffer and reduced with 4% Dithiothreitol (DTT) (Applichem, Darmstadt, Germany), boiled for 5 min at 95 °C, centrifuged for 5 min at 10,000 *g* at 4 °C, and subsequently loaded in duplicates on a 10% Mini-PROTEAN TGX Precast Gel (Bio Rad Laboratories, Munich, Germany). After electrophoresis, the fractionated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK) by Trans Turbo Blot (Bio Rad Laboratories, Munich, Germany). To avoid unspecific antibody binding, the membranes were incubated in tris-buffered saline containing 0.05% Tween 20 (TBST) and 10% Rotiblock (Carl Roth, Karlsruhe, Germany) for 60 min at RT. The membranes were cut horizontally at 50–55 kDa. The upper parts of the membranes were exposed to the primary rabbit antibodies against AMPK in a dilution of 1:1000 or its phosphorylated form (pAMPK) (both 62 kDa), respectively (AMPKα, pAMPKα, Cell Signaling, Danvers, MA, USA) in a dilution of 1:500, each diluted in TBST with 5% BSA overnight at 4 °C. The lower parts of the membranes, with proteins ≤50 kDa, were incubated with a primary mouse antibody against β-actin (42 kDa) (Biovision, Milpitas, CA, USA) diluted 1:6000 in blocking solution under the same conditions. After rinsing, a horseradish peroxidase-labeled secondary anti-rabbit antibody (1:50,000; Cell Signaling, Danvers, MA, USA) or a horseradish peroxidase-labeled secondary anti-mouse antibody (1:20,000) (SouthernBiotech, Birmingham, AL, USA) were applied for 60 min at RT. Antigen-antibody immunocomplexes were revealed using enhanced chemiluminescence detection system (GE Healthcare) and densitometry analysis was performed using a Versa Doc 1000 and Image Lab software (both Bio Rad Laboratories Munich, Germany). Specific

band intensities were normalized to β-actin values as an internal standard. To be able to compare the band intensities from different membranes, a 3T3-L1 pool sample was electrophoresed and blotted in duplicates on each membrane and used as reference standard. The mean intensity of the duplicate bands of the samples in relation to the mean of the standard was estimated and the ratio of pAMPK to AMPK was calculated.

4.4. Measurement of AdipoQ Secreted from 3T3-L1 Adipocytes

The AdipoQ content in the cell supernatant was quantified by a recently developed in-house
ELISA [29] for which parallelism of mouse AdipoQ was approved. The intra- and interassay coefficients of variation were 7% and 11%, respectively.

4.5. Statistical Analyses

Data were analysed using IBM SPSS 20 (IBM, Ehningen, Germany) and are presented as means ± SEM. The results of the controls were not different and thus merged for further analyses, within the PTX (+) and PTX (−) treatment. For comparisons within treatment groups and between treatment and controls, ANOVA with either Bonferroni or Dunnett-T3 *post-hoc* analysis, depending on homogeneity of variances, was performed. To compare the PTX treated *versus* non PTX treated samples, data were examined using the Student's *t*-test. Spearman-Rho correlation coefficients were calculated between the results of AdipoQ and pAMPK/AMPK. Statistical significance was declared at $p \leq 0.05$.

5. Conclusions

In conclusion, treatment with *t*CA stimulated the secretion of AdipoQ and the phosphorylation of AMPK in 3T3-L1 adipocytes and therefore improves insulin sensitivity; the inhibitory effect of PTX points to a *t*CA stimulated G_i/G_o-protein-coupled receptor signaling pathway.

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER III: MANUSCRIPT 2

Nicotinic Acid Increases Adiponectin Secretion from Differentiated Bovine Preadipocytes through G-Protein-Coupled Receptor Signaling

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Abstract: The transition period in dairy cows (3 weeks prepartum until 3 weeks postpartum) is associated with substantial mobilization of energy stores, which is often associated with metabolic diseases. Nicotinic acid (NA) is an antilipolytic and lipid-lowering compound used to treat dyslipidaemia in humans, and it also reduces non-esterified fatty acids in cattle. In mice the G-protein coupled receptor 109A (GPR109A) ligand NA positively affects the secretion of adiponectin, an important modulator of glucose and fat metabolism. In cattle, the corresponding data linking NA to adiponectin are missing. Our objective was to examine the effects of NA on adiponectin and AMPK protein abundance and the expression of mRNAs of related genes such as chemerin, an adipokine that enhances adiponectin secretion *in vitro*. Differentiated bovine adipocytes were incubated with pertussis toxin (PTX) to verify the involvement of GPR signaling, and treated with 10 µM or 15 µM NA for 12 h or 24 h. NA increased adiponectin concentrations ($p \leq 0.001$) and the mRNA abundances of GPR109A ($p \leq 0.05$) and chemerin ($p \leq 0.01$). Pre-incubation with PTX reduced the adiponectin response to NA ($p \leq 0.001$). The NA-stimulated secretion of adiponectin and the mRNA expression of

chemerin in the bovine adipocytes were suggestive of GPR signaling-dependent improved insulin sensitivity and/or adipocyte metabolism in dairy cows.

Keywords: adiponectin; 5'-adenosine monophosphate-activated protein kinase; bovine adipocytes; G-protein coupled receptor 109A; nicotinic acid

1. Introduction

The peripartal period is associated with manifold endocrine and metabolic changes to adapt the cow for parturition and lactogenesis. In dairy cows the transition from late pregnancy to early lactation, defined as 3 weeks prepartum until 3 weeks postpartum, is attributed to increased energy demand due to fetal growth and lactogenesis; the energy requirement exceeds dietary energy intake [1]. Energy stores are mobilized, lipogenesis is reduced and lipolysis is increased substantially [2]. High non-esterified fatty acids (NEFA) concentrations are considered as one major risk factor for metabolic diseases, such as fatty liver and ketosis [3,4] in dairy cows during the transition period [5]. The understanding of the regulation of lipolysis and adipocyte metabolism is therefore fundamental to cope with production diseases around parturition.

Nicotinic acid (NA), also known as Niacin; has been recognized as a high-affinity ligand for the G_i/G_o-protein-coupled receptor 109A (GPR109A) in non-ruminants [6–8]. Nicotinic acid reduces triglycerides and low-density lipoprotein cholesterol and increases high-density lipoprotein cholesterol [9]. The seven transmembrane GPR109A, also known as hydroxycarboxylic acid receptor 2 (HCA_2), is activated by its endogenous ligand beta-hydroxybutyrate [10,11]. GPR109A is expressed in activated macrophages and in adipocytes [8,12] and has also been detected in bovine tissues [13]. Decreases in NEFA and beta-hydroxybutyric acid concentrations in dairy cows have been shown following treatment with NA [14–17]. The secretion of the adipokine adiponectin is stimulated by NA in rodents [18,19]. Several studies have demonstrated NA-induced increases in the expression and secretion of the adiponectin protein [20,21]. The secretion of adiponectin is inhibited by pertussis toxin (PTX), a G_i/G_o-protein-un-coupling compound, in 3T3-L1 adipocytes *in vitro,* and similar results have been obtained in GPR109A knockout mice; these findings support the involvement of GPR-signaling in this pathway [18]. Adiponectin is primarily expressed in adipocytes and modulates the glucose and lipid metabolism of insulin-sensitive tissues [22]. Adiponectin exerts its effects via binding to its receptors AdipoR1/R2 and the subsequent activations of the peroxisome proliferator-activated receptor α (PPARα) and 5'-adenosine monophosphate-activated protein kinase (AMPK) [23]. AMPK is a heterotrimeric kinase complex that consists of a catalytic α subunit and regulatory β and γ subunits [24]. This kinase is activated via the phosphorylation of threonine 172 in the α-subunit (pAMPK) [25]. Upon activation, AMPK acts as an intra-cellular energy sensor that turns on catabolic pathways (e.g., fatty-acid oxidation and glycolysis pathways) and inhibits anabolic processes, such as the syntheses of cholesterol, glycogen, and protein. Chemerin is synthesized as a proprotein and is a chemoattractant agent that is highly expressed in AT and the liver [26]. Chemerin modulates the innate immune system in both directions by acting as a pro- and anti-inflammatory protein [27,28]. Chemerin has also been identified as an adipokine that regulates adipogenesis and adipocyte metabolism [26]. In 3T3-L1 adipocytes, chemerin has been reported to enhance insulin-stimulated glucose uptake and adiponectin secretion [26,29] and thereby to positively regulate insulin sensitivity. Song *et al.* [30] recently cloned bovine chemerin and characterized its expression. These authors observed an increase in expression

throughout the differentiation of bovine adipocytes *in vitro*. Data regarding the effects of NA treatment on chemerin expression in bovine adipocytes is lacking.

In the present study, we hypothesized that NA affects adiponectin secretion and AMPK activation in bovine adipocytes. Furthermore, we analyzed the mRNA abundances of genes relating to NA and adiponectin signaling (e.g., GPR109A, AdipoR1/R2, and chemerin). For this purpose, a primary cell culture system consisting of differentiated bovine adipocytes was established. To investigate the importance of NA on GPR signaling in cows, the adipocytes were pre-incubated with PTX.

2. Results

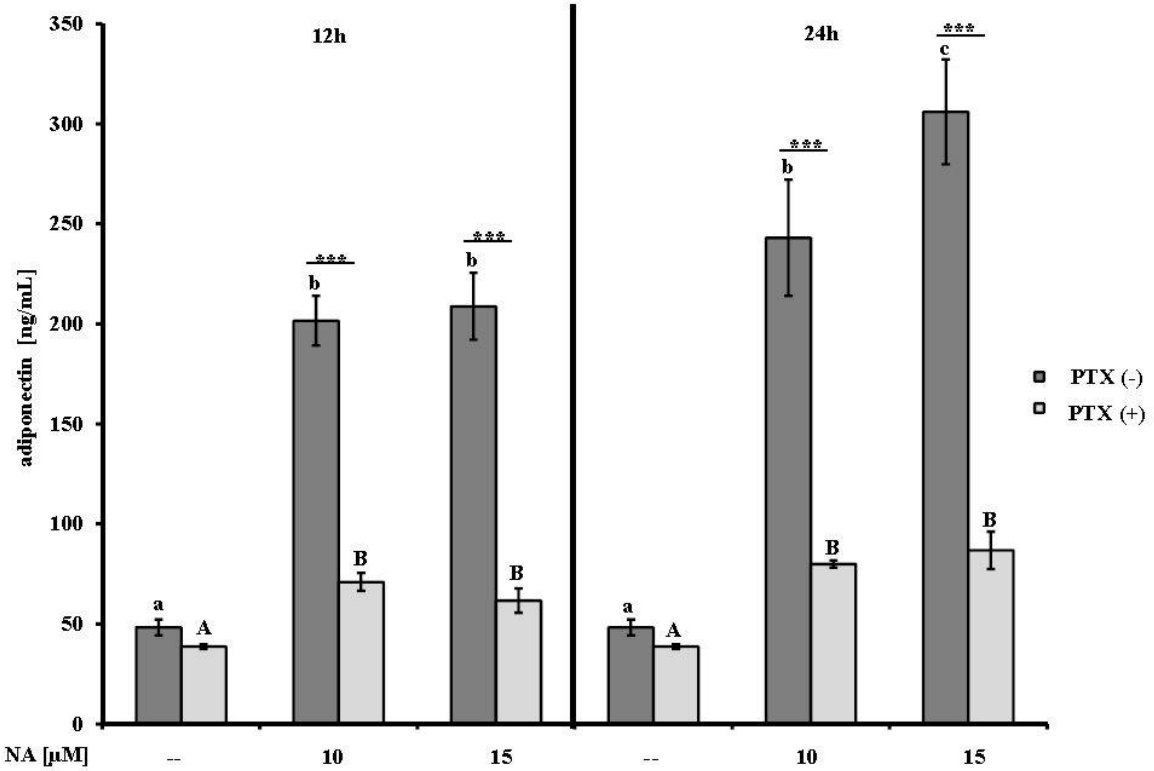
2.1. Effect of NA on Adiponectin Secretion

To test whether NA affects the secretion of adiponectin, the differentiated bovine adipocytes were stimulated with two different concentrations of NA (10 µM and 15 µM) for 12 h or 24 h. Compared to the NA-free controls, the adiponectin concentrations in the cell culture supernatants were 4-fold increased following stimulation with 10 µM or 15 µM NA for 12 h ($p \leq 0.001$) and approximately 5- and 6-fold increased following 24 h treatments with 10 µM and 15 µM NA, respectively ($p \leq 0.001$) (Figure 1). To assess whether the effects of NA were mediated by GPR signaling, the experiments were performed following pre-incubation with PTX (100 ng/mL) for 16 h. For both NA doses and both durations of treatment, the pre-incubation with PTX decreased the adiponectin concentrations to values between 62 and 87 ng/mL; however, these concentrations were consistently higher than those observed in the relevant NA-free control (39 ng/mL; $p \leq 0.001$). Comparisons of the PTX pre-incubation groups revealed that the adiponectin concentrations were approximately 3 times lower following PTX pre-incubation in the 10 µM NA group at both durations ($p \leq 0.001$). The adiponectin concentrations in the supernatants from the PTX (+) group were reduced by 3- and 3.5-fold ($p \leq 0.001$) following treatment with 15 µM NA regardless of time when compared with the corresponding PTX (−) group (Figure 1).

2.2. Effect of NA on AMPK

The phosphorylated form of AMPK remained undetected, therefore the obtained optical densities of AMPK were matched against the reference standard. Treatment with 15 µM NA for 24 h increased the AMPK/standard ratio 10-fold compared to the control ($p \leq 0.001$) (Figure 2a). Pre-incubation with PTX drastically limited this increase by 50%, but the values remained 5-fold higher than those of the respective controls ($p \leq 0.001$). A representative Western blot result is shown in Figure 2b.

Figure 1. The effects of nicotinic acid (NA) on adiponectin concentrations (means ± SEM) in cell culture supernatants of differentiated bovine adipocytes (n = 5). After 4 h of serum starvation, the adipocytes were pre-incubated with pertussis toxin (PTX (+)) or without (PTX (−)) (100 ng/mL) for 16 h and then treated for 12 or 24 h with 10 or 15 µM NA or PBS (vehicle control). The different lower case letters designate significant differences ($p \leq 0.005$) between the NA treatments and the controls for the PTX (−) cells; the different upper case letters designate significant differences ($p \leq 0.001$) between the NA treatments and the controls for the PTX (+) cells. Significant differences (******* $p \leq 0.001$) due to PTX (+) or PTX (−) pre-incubation for each NA treatment group are indicated with asterisks.



2.3. Effects of NA on the Abundances of AdipoR1/2, FABP4, and GPR109A mRNAs

Pre-incubation with PTX had no effect on the mRNA abundances of AdipoR1/2, FABP4, or GPR109A. Therefore, the PTX (+) and PTX (−) groups were merged for further analyses. Compared to the controls, incubation for 24 h with 15 µM NA increased the mRNA abundance of GPR109A ($p \leq 0.05$), and a trend ($p = 0.07$) toward an increase was observed following treatment with 10 µM NA after 24 h (Figure 3). In contrast, treatment with NA had no effect on the mRNA abundances of AdipoR1/R2 or FABP4 (Table 1).

Figure 2. Effects of nicotinic acid (NA) on 5' AMP-activated protein kinase (AMPK) protein abundance (means ± SEM) in differentiated bovine adipocytes (n = 5). After 4 h of starvation, the adipocytes were pre-incubated with (PTX (+)) or without pertussis toxin (PTX (−)) (100 ng/mL) for 16 h and then treated for 24 h with 15 µM NA or PBS (control). (**a**) The different lower case letters designate significant differences ($p \leq 0.001$) between the NA treatments and the controls for the PTX (−) cells. The different upper case letters designate significant differences ($p \leq 0.001$) between the NA treatments and the controls for the PTX (+) cells. Significant differences (****** $p \leq 0.01$) due to (+) or (−) PTX pre-incubation are designated with asterisks; (**b**) Representative Western blot results. After gel electrophoreses, the membranes were incubated with specific antibodies against AMPK or β-actin as a loading control. The obtained optical densities for AMPK were matched against a standard pool sample and are expressed as % relative to the standard.

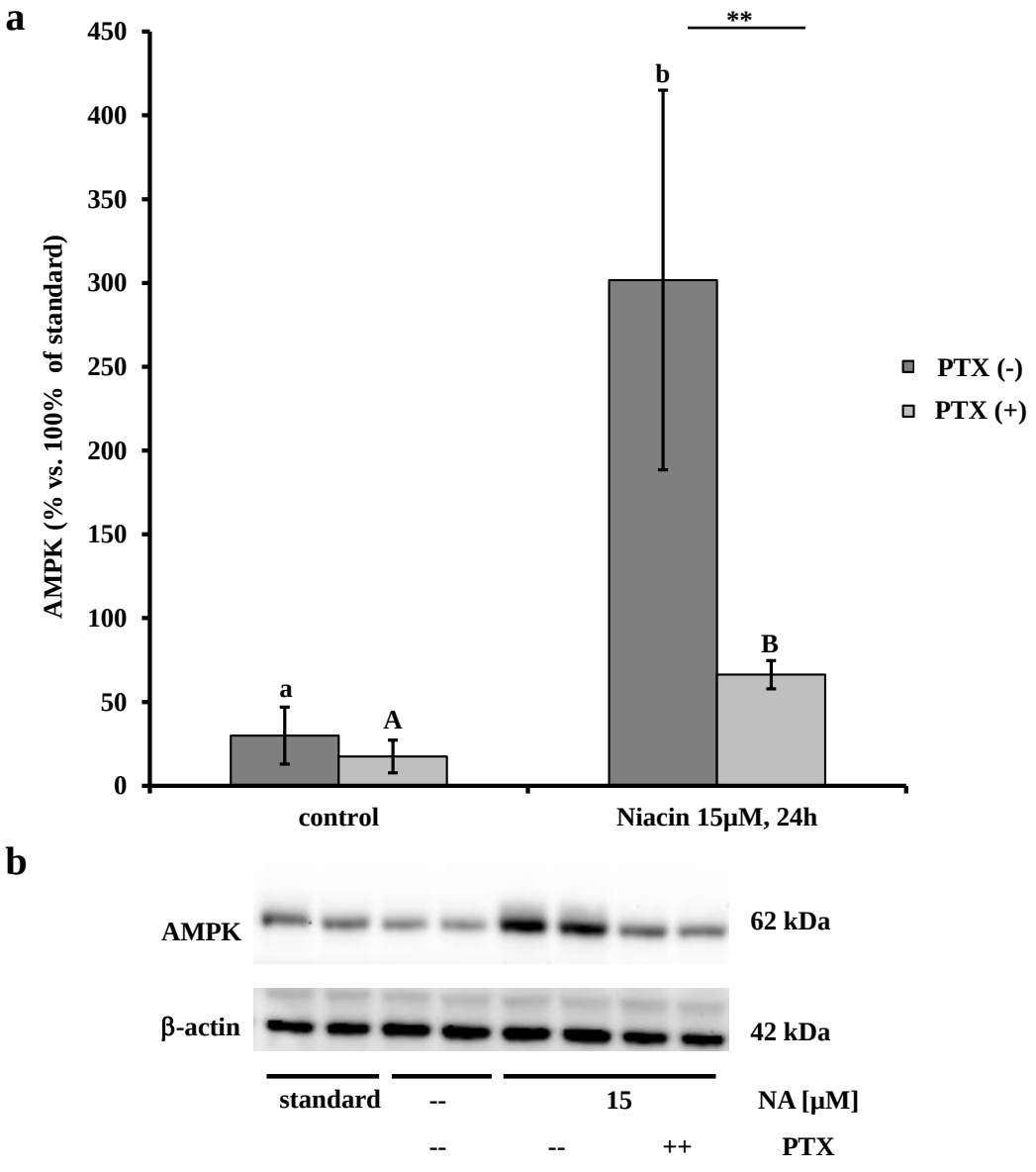


Figure 3. The effects of nicotinic acid (NA) on the mRNA abundance of G-protein-coupled receptor 109A (GPR109A) in differentiated bovine adipocytes cells (n = 10). After 4 h of starvation, the adipocytes were pre-incubated with (PTX (+)) or without pertussis toxin (PTX (−)) (100 ng/mL) for 16 h and then treated for 12 or 24 h with 10 or 15 µM NA or PBS (control). Due to the absence of differences, the PTX (+) and PTX (−) groups were merged for the analyses of the mRNA abundances. Significant differences ($p \leq 0.05$) and trends ($p \leq 0.1$) are designated with the corresponding p-values.

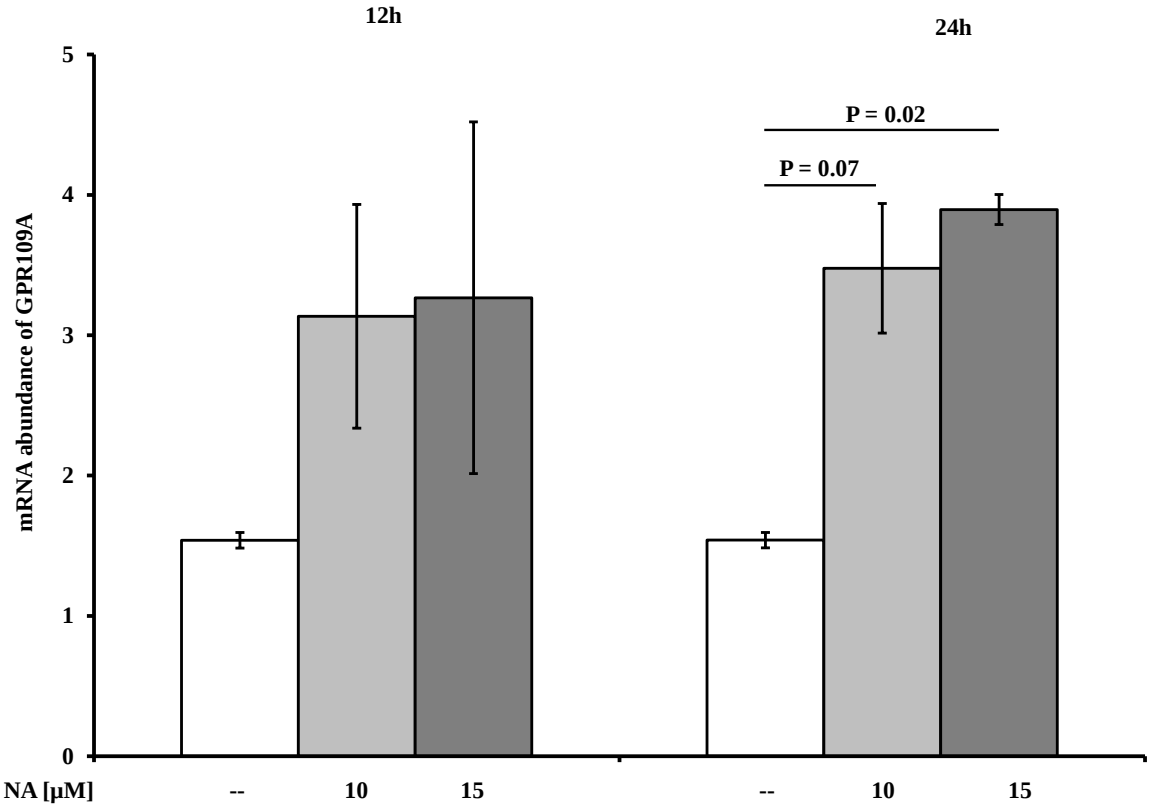


Table 1. Niacin effects on the mRNA abundance of selected genes.

Treatment/Genes	12 h					24 h				
NA [a][µM]	-	10	p	15	p	-	10	p	15	p
AdipoR1 [b]	1.1 ± 0.4	1.1 ± 0.5	n.s.	1.1 ± 0.4	n.s.	1 ± 0.3	1.1 ± 0.3	n.s.	1.4 ± 0,5	n.s.
AdipoR2 [c]	1 ± 0.1	1 ± 0.2	n.s.	1 ± 0.1	n.s.	1 ± 0.2	1 ± 0.2	n.s.	1 ± 0.1	n.s.
GPR109A [d]	1.2 ± 0.01	3.13 ± 0.7	n.s.	3.27 ± 1.25	n.s.	1.6 ± 0.05	3.48 ± 0.46	0.07	3.9 ± 0.1	0.02
FABP4 [e]	1.2 ± 0.3	1.2 ± 0.6	n.s.	1.1 ± 0.6	n.s.	1.1 ± 0.5	0.6 ± 0.5	n.s.	0.8 ± 0.5	n.s.
Chemerin	0.71 ± 0.19	0.76 ± 0.16	n.s.	0.67 ± 0.38	n.s.	0.76 ± 0.46	2.89 ± 1.42	0.006	1.59 ± 1.18	n.s.

The given values are means ± SEM. Significant differences ($p \leq 0.05$ and $p \leq 0.1$ as a trend) between control and NA treatment are depicted by bold values. Pre-incubation with pertussis toxin (PTX) had no effect of the analyzed mRNAs of AdipoR1/2, FABP4 or GPR109A. Therefore, the PTX (+) and PTX (−) groups were merged for further analyses. The mRNA abundance of chemerin is presenting only data of the PTX (−) group; [a] NA: Niacin; [b] AdipoR1: adiponectin receptor 1; [c] AdipoR2: adiponectin receptor 2; [d] GPR109A: G-protein coupled receptor 109A; [e] FABP4: fatty acid binding protein 4.

2.4. Effect of NA on the mRNA Abundance of Chemerin

The mRNA abundance of chemerin was increased by 3.3-fold ($p \leq 0.01$) compared to the controls following stimulation with 10 µM NA for 24 h. No effects were observed following the 12 h treatment or the treatments with 15 µM NA for either duration. Following pre-incubation with PTX, no differences between any treatment group and the NA-free controls were observed (Table 1).

3. Discussion

During the transition period the energy required exceeds the dietary energy intake, and this time period is linked with the health status [1] and thus with the profitability of the dairy cow. Due to this negative energy balance, lipogenesis is reduced and lipolysis is substantially increased [2]. Excess non-esterified fatty acids (NEFA) accumulate, alter liver function and increase the incidence of metabolic diseases, such as fatty liver and ketosis [3,4]. In dairy cows, the NEFA- and beta-hydroxybutyric acid-lowering effects of NA were first shown many years ago [14,15]. Recently, in dairy cows, NA and beta-hydroxybutyric acid were demonstrated to decrease isoproterenol-stimulated lipolysis *in vitro* by reducing the phosphorylation of hormone-sensitive lipase, which confirmed the presence of a GPR109A-mediated anti-lipolytic pathway in dairy cows [31]. In addition to the effects on lipolysis, NA has been linked to elevated mRNA expression and protein secretion of adiponectin in murine 3T3-L1 cells [20] and humans [21], respectively. Adiponectin is known to improve insulin sensitivity and lipid metabolism [32], but information about the effects of NA on adiponectin in cattle is lacking.

With our *in vitro* model, we were able to demonstrate effects of NA on differentiated primary bovine adipocytes *in vitro.* Our results might link the effects of NA to insulin sensitivity at the level of the adipocyte in bovines in a process that involves adiponectin secretion and AMPK protein expression. We observed an increase in the adiponectin secretion, in differentiated bovine adipocytes *in vitro*, following two different NA treatments after 12 h and another enhancement after 24 h of incubation. These observations are in line with those from a study by Plaisance *et al.* [18] in which the stimulation of isolated primary rat adipocytes with NA increased adiponectin secretion. Adiponectin exerts its effects via binding to its AdipoR1/R2 receptors and activating PPARα, and AMPK [23]. Yamauchi *et al.* [25] reported increased phosphorylation of AMPK following stimulation with adiponectin in myocytes and hepatocytes that resulted in the stimulation of glucose uptake and fatty-acid oxidation. Recently, adiponectin has been shown to activate AMPK in bovine hepatocytes, and this activation results in increased lipid oxidation and reduced lipid synthesis [33]. To our knowledge, no detailed information is available regarding this issue in bovine adipocytes, but it has been demonstrated that the overexpression of adiponectin in 3T3-L1 adipocytes *in vitro* increases lipogenesis and lipid accumulation [34]. Data regarding the activation of AMPK in AT are contradictory because both lipogenic and lipolytic effects have been described [35,36]. In dairy cows, the phosphorylation of AMPK increases during the transition period. As discussed by Locher *et al.* [37], this observation might be associated with the antilipolytic

function of AMPK in terms of the fine-tuning of NEFA release from triglycerides after parturition. However, the regulation of AMPK at the expression level was shown as response to physical activity or leptin administration [38–41]. In addition Martinez-Agustin *et al.* [42] demonstrated a direct correlation between AMPK protein abundance and adiponectin expression in human AT, supporting adiponectin as discrete activator of AMPK [43,44]. Similarly, treatment with 15 µM NA for 24 h increased the AMPK amount by up to 10-fold, which presumably resulted from an elevation in AMPK protein expression subsequent to the increased secretion of adiponectin following NA treatment. In addition to AMPK phosphorylation, the increase in AMPK abundance might be in line with the antilipolytic effects of NA that have been shown in dairy cows [14,15] and its effects on the fine-tuning of lipolysis [37]. The concentrations of NA used by Kenéz *et al.* [31] were discussed as reflective of physiological concentrations following NA feeding, and the concentrations we used were also within this range. In humans and rodents, NA exerts its lipid-lowering effects by binding to GPR109A [7]. Furthermore, mice that are deficient in PUMA-G (the mouse ortholog of GPR109A) exhibit no increase in serum adiponectin concentration following treatment with NA [7]. Titgemeyer *et al.* [13] identified GPR109A mRNA and protein in various bovine tissues. To test whether the modulating effects of NA on adiponectin and AMPK are mediated by GPR109A in cattle, we pre-incubated bovine adipocytes with PTX, a standard inhibitor of G_i/G_o-protein-coupling. Adiponectin secretion was significantly decreased after blocking G_i/G_o-protein signaling irrespective of the amount or duration of NA treatment. Adiponectin increases the abundance of AMPK [42] and might be therefore responsible for the decrease in the abundance of the AMPK amount in consequence of the treatment with PTX. However, adiponectin secretion was still significantly increased compared to the controls. This finding contrasts those of Plaisance *et al.* [18] in rat adipocytes; these authors observed no increase on adiponectin secretion following stimulation with NA after PTX pre-treatment. Therefore, we suggest that, in addition to GPR signaling-dependent pathways, GPR-signaling independent pathways are involved in the regulation of adiponectin secretion following stimulation with NA in bovines. In 3T3-L1 adipocytes, NA has been shown to increase PPARγ mRNA abundance [45,46]. An increase of PPARγ mRNA by NA was also observed in GPR109A knockout mice [19]. Kim and Choung [47] reported an up-regulation of the mRNA expression of PPARγ in AT following treatment with an extract of cinnamon bark, the main compounds of which are known to be ligands of GPR109A. The transcription factor PPARγ is a known stimulator of adiponectin expression [48] and a regulator of several genes that are involved in the control of insulin sensitivity [49]. In addition to an increase in PPARγ expression, an increase in adiponectin secretion has been observed following the administration of cinnamon extract [47]. These findings support the notion that this regulatory pathway is a possible NA-induced stimulator of adiponectin secretion independent of GPR109A signaling, and suggest PPARγ as an interesting target gene for further studies in the bovine. Corresponding to the results observed for adiponectin, the protein abundance of AMPK decreased following PTX incubation but remained 4-fold higher than the levels observed in the controls. These findings might be due to a subsequent effect of the PTX-independent increase in adiponectin concentration and its direct activation

of AMPK [25]. The NAD/NADH redox potential as another metabolic sensor might be involved [50]. Nicotinic acid is a substrate for the synthesis of NAD^+ after conversion to nicotinamide [51]. However, increasing NADH concentrations may down regulate AMPK activity [50]. The importance of this link on the abundance and activity of AMPK should be analysed in future experiments in the bovine.

In addition to the analysis of adiponectin and AMPK, the mRNA expression of related genes were quantified by PCR. FABP4 is widely known as a marker for mature adipocytes; it regulates the transport of NEFA and PPARγ agonists and also interacts with proteins linked to lipid metabolism and insulin sensitivity [52]. However, the mRNA abundance of FABP4 unexpectedly remained unchanged following treatment with NA. The effects of adiponectin are mediated through its receptors, although the mRNA abundance of AdipoR1/R2 did not change in our study. Therefore, neither NA nor adiponectin affected the receptor mRNA abundance in our *in vitro* model. Similarly, we have observed that adiponectin mRNA increased during the 13 d of adipocyte differentiation *in vitro* by 2500-fold in parallel with the relatively constant levels of the mRNAs of both adiponectin receptors [53]. Increased mRNA abundances of GPR109A were observed following 24 h of treatment with both NA concentrations. This effect was not blocked by PTX and was therefore not G_i/G_o-protein-coupling dependent. Another possibility could be signaling by PPARγ or stimulation of the NAD/NADH redox system by NA as discussed above. Correlation between PPARγ and GPR109A mRNA in epididymal white adipose tissue of mice has been shown *in vivo* [54], and the direct regulation of GPR109A by PPARγ in 3T3-L1 adipocytes was demonstrated by Jeninga *et al.* [55]. We recently have shown in dairy cows a high correlation between the mRNA abundance of PPARγ and GPR109A in subcutaneous adipose tissue ($p = 0.782$) but not in liver *in vivo* [56] which may support our speculation. All of the suggested mechanisms need to be checked in future experiments. The regulation of GPR109A mRNA by NA is in contrast with the results of the *in vivo* study by Titgemeyer *et al.* [13] who used Holstein steers and showed no alterations in GPR109A mRNA or protein expression following abomasal infusions of 16 g/d of NA in the AT or other tissues. The discrepancy between the results of our study that utilized differentiated adipocytes and those of Titgemeyer *et al.* [13] might be linked to the use of *in vitro vs. in vivo* models; this possibility should be clarified in further experiments. Chemerin is known to be involved in the control of immune responses via its action as a chemoattractant for antigen-presenting cells. Chemerin has anti-inflammatory and pro-inflammatory functions depending on the model studied [57]. The protein is highly expressed as prochemerin in the liver, AT and placenta [57] and was recently identified as an adipokine that regulates adipogenesis and adipocyte metabolism [58]. Furthermore, chemerin enhances insulin-stimulated glucose uptake, insulin signaling and adiponectin secretion and therefore improves insulin sensitivity in murine adipocytes [26,29]. Our study showed for the first time that treatment with 10 µM NA for 24 h increased the chemerin mRNA abundance 3-fold compared to the controls. We speculate that the increased chemerin mRNA might be indicative of enhanced adipocyte insulin sensitivity and/or improved adipocyte metabolism due to NA. Following pre-incubation with PTX, the chemerin mRNA abundance in the

treatment group was similar to that of the controls, which confirms the hypothesized signaling pathway of NA through GPR signaling.

4. Experimental Section

4.1. Isolation of Bovine Preadipocytes

Subcutaneous (sternum) AT was collected from five Holstein-Friesian cows at a local abattoir. The tissue was rinsed in isopropanol for 60 s to minimize contamination and was then transported in sterile 50-mL tubes to the laboratory. All of the following steps were performed under sterile conditions and are based on the modified method of Grant *et al.* [59]. The outer layer of the AT was cut off, a block of approximately 3 g was cut into 1 mm^3 pieces in cutting medium that contained Dulbecco's modified Eagle's medium low glucose (DMEM-LG; PAA, Pasching, Austria), 10 mg/mL penicillin/streptomycin (pen/strep) and 0.25 µg/mL amphotericin (all from PAA, Pasching, Austria). The cutting medium was drained off, and the AT pieces were transferred to 50-mL tubes and digested in DMEM-LG containing 2 mg/mL collagenase (244 U/mg) (Biochrom, Berlin, Germany) and 2% fatty acid-free BSA (Carl Roth, Karlsruhe, Germany). The samples were incubated at 37 °C for 15 min during which time the vials were mixed every 5 min. Each sample was then transferred to an incubator and further digested with shaking for 90 min at 37 °C, 370 rpm and at a 45° angle. The digested material was then sequentially filtered through 100-µm, 70-µm and 40-µm sterilized cell strainers into sterile 50-mL tubes and centrifuged at 800× *g* for 10 min at room temperature (RT). To eliminate erythrocyte contamination, 4 mL ultra pure sterile H_2O was added, and the pellets were resuspended for 20 s. To adjust the osmotic pressure, the same amount of 2× PBS was added. After centrifugation at 800× *g* for 10 min at RT, the pellet was resuspended in growth medium (DMEM-LG, 10 mg/mL pen/strep, 0.25 µg/mL amphotericin, 33 µM biotin, 17 µM pantothenate, and 100 µM ascorbate (all substances were from AppliChem GmbH, Darmstadt, Germany, unless otherwise stated), supplemented with 10% fetal calf serum (FCS) (PAA) and seeded on 10 cm^2 petri dishes. The medium was replaced after 24 h and on every 2nd day thereafter. After reaching confluence (90%–95%), the cells were washed twice with PBS, harvested with 0.5 g trypsin/EDTA and collected by centrifugation at 800 × *g* for 10 min at RT. The pellets were resuspended in freezing medium that contained Dulbecco's modified Eagle's medium high glucose (DMEM-HG) (PAA), 20% FCS and 10% dimethyl sulfoxide (DMSO, Carl Roth), frozen consecutively at −20 °C and −80 °C for 24 h each and then stored in liquid nitrogen until further use.

4.2. Differentiation of Bovine Preadipocytes

A pool of equal proportions of preadipocytes from five different animals was seeded in 25 cm^2 flasks at a density of 2500 cells per cm^2 and cultured with growth medium in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 24 h. To induce differentiation of the preadipocytes, 0.5 mM 3-isobutyl-methylxanthine (IBMX) (Applichem), 0.25 µM dexamethasone, 5 µg/mL bovine insulin (both from Sigma-Aldrich, St. Louis, MO, USA) and

5 µM troglitazone (Cayman Chemical, Ann Arbor, MI, USA) were added to DMEM-HG containing 5% FCS and 10 mg/mL pen/strep for 48 h. The cells were then maintained in post-differentiation medium (DMEM-HG, 5% FCS, 10 mg/mL pen/strep, 5 µg/mL bovine insulin and 5 µM troglitazone). The media were replaced every 2nd day, and the cells were used for the experiment at d 12 after the initiation of differentiation. Only adipocytes from those animals were used for the experiment that showed a differentiation rate of 60% at this time period, which was documented by the accumulation of lipid droplets (Oil Red O staining, 0.2%).

4.3. Treatment of the Cells with NA

Prior to the treatments at d 12, the cells were cultured in insulin-free DMEM-HG containing 5% FCS and 10 mg/mL pen/strep for 24 h and then serum starved in DMEM-LG supplemented with 0.1% BSA for 4 h. According to Tunaru *et al.* using CHO-K1 cells and Soliman *et al.* using differentiated bovine preadipocytes [7,60], the adipocytes were incubated for 16 h, with or without 100 ng/mL PTX (Sigma-Aldrich), to inhibit G_i/G_o-protein coupled signaling. The cells were then treated with 10 or 15 µM NA (Sigma-Aldrich) for 12 h or 24 h, respectively. Equal volumes of PBS were applied in place of the PTX and NA for the controls. At the end of the incubation time, the supernatant was collected and stored at −20 °C until analysis. The adherent adipocytes were washed twice with ice-cold PBS, lysed with 1 mL Qiazol (Qiagen, Hilden, Germany) and subsequently frozen at −80 °C for total RNA and protein extractions. The differentiation and treatment procedure was independently repeated five times (n = 5).

4.4. RNA Extraction, cDNA Synthesis and mRNA Quantification

Total RNA from the Qiazol cell lysate (Qiagen, Hilden, Germany) was isolated with the Invitrap Universal RNA mini kit (Stratec Molecular, Berlin). To do this, the aqueous Qiazol phase was transferred and mixed with an equal volume of lysis solution TR containing DNA-binding particles. After binding of the residual DNA to the particles, the samples were centrifuged, and the supernatants containing the total RNA were subsequently purified with spin columns (Invitrap Universal RNA mini kit, Stratec Molecular). Total RNA concentrations and purities were analyzed by absorbance readings at 260 nm and 280 nm (Nanodrop 1000, peQLab Biotechnology, Erlangen, Germany). The total RNA integrity was verified using denaturing RNA gel electrophoresis. Additionally, the quality of the total RNA was rechecked in random samples by microcapillary electrophoresis using the Bioanalyzer 2100 and the RNA 6000 Nano Kit system (Agilent, Waldbronn, Germany) to determine the RNA integrity numbers (RIN = 9.05 ± 0.73). For cDNA synthesis, reverse transcription of 350 ng of total RNA per 20 µL reaction volume was performed with RevertAid™ reverse transcriptase (Thermo Fisher, Schwerte, Germany) according to the manufacturer's instructions with the exception that only 1 µL of dNTP mix was used (10 mM of each dNTP, Thermo Fisher). Reverse transcription was performed in a Multicycler PTC 200 (MJ Research, Watertown, MA, USA) using a negative template control and one control per run in

which no reverse transcriptase was included. For inter-run normalization of the PCR runs, the pooled RNA was additionally reverse transcribed. Reverse transcription was performed in duplicate for each sample, and the duplicate products were then combined for quantitative PCR (qPCR).

Characteristics of the primers and the quantitative real-time PCR conditions are displayed in Table 2. The selection of the reference genes and the data normalization were based on the methods of Saremi et al. [61] using qbase+ (Biogazelle, Gent, Belgium). Triplicates with 2 µL cDNA (diluted 1:4) as the templates and 5 µL SYBR Green Jump Start Taq Readymix (Sigma–Aldrich) or DyNAmo ColorFlash SYBR Green qPCR kit (Thermo Fisher) were run in total volumes of 10 µL in an Mx3000P cycler (Stratagene, Amsterdam, Netherlands). A negative template control, a control lacking reverse transcriptase and an additional two inter-run calibrators were run in each run. The efficiencies were estimated with PCR amplicon standard curves. The PCR products were verified by sequencing.

Table 2. Characteristics of the primers and the quantitative real-time PCR conditions.

Gene [a]	Forward Primer Sequence (5'-3') Reverse Primer Sequence (5'-3')	Acc. No.[d]	Base Pairs	Con. (nM) [e]	Mean Cq [f]	Annealing (second/°C) [g]	Efficiency
AdipoR1 [b]	GCTGAAGTGAGAGGAAGAGTC GAGGGAATGGAGTTTATTGCC	NM_001034055	118	800	23.9	35/61	99.9
AdipoR2 [b]	GGCAACATCTGGACACATC CTGGAGACCCCTTCTGAG	NM_001040499	200	400	24.2	45/60	90.7
GPR109A [c]	GGACAGCGGGCATCATCTC CCAGCGGAAGGCATCACAG	XR_028237	140	200	31.9	30/61	86.5
FABP4 [b]	CATCTTGCTGAAAGCTGCAC AGCCACTTTCCTGGTAGCAA	X89244	160	800	22.9	30/60	120.4
Chemerin [b]	GAAGAAAGACTGGAGGAAAG TTGAACCTGAGTCTGTATGG	FJ594406	139	200/100	23.2	60/60	89.1
MARVELDI [c]	GGCCAGCTGTAAGATCATCACA TCTGATCACAGACAGAGCACCAT	NM_001101262	100	400	23.4	45/59	101.2
EMD [b]	GCCCTCAGCTTCACTCTCAGA GAGGCGTTCCCGATCCTT	NM_203361	100	400	23.4	45/59	101.7
LRP10 [b]	CCAGAGGATGAGGACGATGT ATAGGGTTGCTGTCCCTGTG	Bc149232	139	400	22.7	30/61	101.1
EIF3K [b]	CCAGGCCCACCAAGAAGAA TTATACCTTCCAGGAGGTCCATGT	NM_001034489	125	400	23.4	45/59	97.5
POLII [b]	GAAGGGGGAGAGACAAACTG GGGAGGAAGAAGAAAAAGGG	X63564	86	800	23.1	60/60	97.4

[a] AdipoR1: adiponectin receptor 1 [62], AdipoR2: adiponectin receptor 2 [62], GPR109A: G-protein coupled receptor 109A [62], FABP4: fatty acid binding protein 4 [63], MARVELD1: marvel domain containing 1 [64], EMD: emerin [64], LRP10: lipoprotein receptor-related protein 10 [63], EIF3K: eukaryotic translation initiation factor 3, subunit K [64], POLII: RNA polymerase II [63]; [b] DyNAmo ColorFlash SYBR Green qPCR kit (Thermo Fisher); [c] SYBR Green Jump Start Taq Readymix (Sigma–Aldrich); [d] NCBI Accession Number; [e] Concentrations for each primer (forward/reverse); [f] median cycle threshold; [g] Initial denaturation for 10 min at 90 °C; denaturation for 30 s at 95 °C, extension at 72 °C, 60 seconds.

4.5. Protein Extraction from the Cell Lysate

For the analysis of the AMPK activation due to the NA treatment (15 µM NA for 24 h and the corresponding controls), DNA was precipitated from the Qiazol interphase and phenol phase. The resulting phenol-ethanol supernatant was used, after centrifugation, for protein precipitation according to the methods of Chey *et al.* with minor modifications [65]. Briefly, 1.75 mL of 100% ethanol was added to 700 µL of the phenol-ethanol supernatant, followed by 470 µL bromochloropropane (Applichem) and 1.4 mL H_2O. After centrifugation (3900× *g*, 30 min, RT), the upper aqueous phase was discarded, and 1 mL 100% ethanol was added for protein precipitation. Pelleting of the protein was performed by centrifugation (3900× *g*, 10 min, RT); to purify the pellet, the addition of ethanol and centrifugation were repeated once. Next, the pellet was dried for 10 min and subsequently dissolved in 300 µL 4% SDS (Carl Roth) by shaking for 30 min at 55 °C. The protein content was quantified according to the

method of Bradford [66] using the Nanodrop 1000 (Peqlab Biotechnology). The samples were frozen at −20 °C until the Western blot analyses.

4.6. Western Blot

To detect α-AMPK and pAMPK, 9 µg of total cell protein was treated with Laemmli buffer and reduced with 4% dithiothreitol (DTT) (Applichem), heated for 5 min at 95 °C, centrifuged for 5 min at 10,000× *g* at 4 °C, and subsequently loaded in duplicate on a 10% Mini-PROTEAN TGX Precast Gel (Bio Rad Laboratories, Munich, Germany). After electrophoresis, the fractionated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK) using the Trans Turbo Blot (Bio Rad Laboratories). To minimize nonspecific binding, the membranes were incubated in tris-buffered saline containing 0.05% Tween 20 (TBST) and 10% Rotiblock (Carl Roth) for 60 min at RT. The membranes were cut horizontally at 50–55 kDa. The upper parts of the membranes were either incubated with the primary rabbit antiserum against α-AMPK or its phosphorylated form (pAMPK) (both from Cell Signaling, Danvers, MA, and both 62 kDa) in dilutions of 1:500 or 1:200, respectively, in TBST with 5% BSA overnight at 4 °C. The bottom parts of the membranes with proteins ≤50 kDa were incubated with a primary mouse antibody against β-actin (42 kDa) (Biovision, Milpitas, CA, USA) diluted to 1:6000 in blocking solution under the same conditions. After rinsing, a horseradish peroxidase-labeled secondary goat anti-rabbit antibody (1:50,000; Cell signaling) or a horseradish peroxidase-labeled secondary goat anti-mouse antibody (1:20,000, SouthernBiotech, Birmingham, ALA) was applied for 60 min at RT. The immunocomplexes were revealed using the enhanced chemiluminescence detection system (GE Healthcare), and densitometric analyses were performed using the Versa Doc 1000 and the Image Lab software (both from Bio Rad Laboratories). The intensities of the specific bands were normalized to the β-actin values for the internal standards. To compare the band intensities from different membranes, a pooled sample of lysed 3T3-L1 differentiated adipocytes was electrophoresed and blotted in duplicate on each membrane for use as a reference standard. The mean intensities of the duplicate bands of the samples in relation to the means of the standards (100%) were calculated. Due to the missing values for pAMPK, the obtained optical densities of AMPK were matched against the reference standard.

4.7. Measurement of Adiponectin Secreted from Bovine Adipocytes

The adiponectin concentrations in the cell culture supernatants were quantified with a bovine adiponectin-specific ELISA that was developed in-house [67]. The intra- and interassay coefficients of variation were 7% and 11%, respectively.

4.8. Statistical Analyses

The data were analyzed using IBM SPSS 20 (IBM, Ehningen, Germany) and are presented as the means ± the SEMs. The results of the NA-free controls did not vary across time; thus,

these data were merged across the PTX (+) and PTX (−) treatments for further analyses. For comparisons within the treatment groups and between the treatments and controls, ANOVAs with Bonferroni post-hoc analyses, depending on the homogeneities of the variances, was performed. Student's *t*-tests were used to compare the PTX (+)- and PTX (−)-treated samples. Statistical significance was declared at $p \leq 0.05$.

5. Conclusions

In conclusion, treatment with NA stimulated the secretion of adiponectin, the expression of AMPK protein, and the expression of chemerin mRNA in bovine adipocytes and therefore might improve insulin sensitivity and/or adipocyte metabolism in dairy cows. The inhibitory effect of PTX and the increase in the abundance of GPR109A mRNA suggest a G_i/G_o-protein-coupled receptor signaling pathway in cows and we speculate that GPR109A is at least partially involved in the NA-stimulated adiponectin and AMPK signaling pathways in bovine adipocytes.

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Author Contributions

Christina Kopp, Afshin Hosseini, Petra Regenhard and Manfred Mielenz conceived and designed the experiments; Christina Kopp, Shiva P. Singh and Hamed Khalilvandi-Behroozyar performed the experiments; Christina Kopp and Manfred Mielenz analyzed the data; Christina Kopp, Helga Sauerwein and Manfred Mielenz wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER IV: GENERAL DISCUSSION AND CONCLUSION

The adipokine AdipoQ is known to improve insulin sensitivity and lipid metabolism and takes part in the protection against the development of obesity and type 2 diabetes [Tishinsky et al., 2012]. Due to its insulin-sensitizing effect, AdipoQ is not only of great interest in humans, but also for dairy cows during the transition period. During this period the energy required exceeds the voluntary energy intake. This time period is linked with the potential development of many diseases [Grummer 1995] and thus with the profitability of the dairy cow. The negative energy balance during this period is characterized by increased lipolysis and reduced insulin sensitivity in peripheral tissues, such as AT and muscle. Hence, strategies to improve the synthesis and secretion of AdipoQ are of major interest in various species. In this thesis we examined the effect of AdipoQ expression after the stimulation with *t*CA in murine or NIA, i. e., its compound NA, in bovine adipocytes. Furthermore, an involvement of the receptor GPR109A in signal transduction of both compounds was researched.

The herein used 3T3-L1 is a cell line, derived from murine 3T3 cells, that is commonly used in biological research on AT. This population consists only of preadipocytes, with a fibroblastic phenotype, which can be differentiated to adipocytes in the presence of serum supplemented medium, insulin and glucocorticiods [Zebisch et al., 2012]. Besides gaining first experiences in cell culture of differentiated adipocytes, this cell line was used to introduce *t*CA, a compound of cinnamon, not only as an influencing variable on AdipoQ secretion, but also its GPR specific signaling could be shown for the first time. In case of NA supplementation, a bovine primary cell culture model was established. Briefly, sc AT was dissociated by enzymatic digestion, the stromal vascular fraction was isolated by sieving and centrifugation steps and subsequently cultivated. We considered this model as advantageous compared to an explant model [Hosseini et al., 2012; Kenez et al., 2014] since animal specific differences, such as genetic or lifetime imprinting and differences in adipocyte number could be largely avoided. To minimize possible differences in the proliferation and differentiation abilities in the primary cell culture model, the cells were tested before the study and only cells with similar characteristics were used. A great benefit in explant models is the preserved AT composition (including mature cells, preadipocytes, macrophages etc.), including the paracrine signals of this tissue by maintaining the cross-talk among the different cell types [Thalmann et al., 2008]. Several cell co-culture studies showed the importance of this cross talk [Sell et al., 2006; Wang et al., 2006]. However, this cross talk may also provoke possible interferences due to the trauma of the tissue by biopsy (e.g., activation of inflammatory adipokines) [Fain et al., 2005] and may falsify the results. The model used in this study

consists not only of preadipocytes, like the 3T3-L1 cells, but, as shown in human and rodents [Hausman et al., 2008] also of stromal vascular cells, which include besides preadipocytes, fibroblasts, pericytes, blood and endothelial cells, diverse precursor cells and immune cells, such as macrophages and T-lymphocytes. This heterogeneity ensures a cell-to-cell communication, similar to explant culture, which is combined with positive capacities of a cell culture model (e.g., generating larger cultures with alike imprinting and characteristics).

In this thesis increased AdipoQ secretion was demonstrated after *t*CA and NA treatment. In addition, not only the effect of *t*CA and NA on AdipoQ expression should have been analyzed in this thesis, but also the signaling pathway was subject of research. Since both compounds were identified as ligands of the GPR109A, we hypothesized that this pathway is involved in a *t*CA/NA-induced effect on AdipoQ, supported by the findings of Plaisance et al., [2009] who reported inhibited secretion of AdipoQ after GPR blocking and NA treatment. Both, murine and bovine adipocytes, were pre-incubated with pertussis toxin (PTX), a commonly used but nonspecific uncoupling agent of G_i/G_0-proteins. A decrease in AdipoQ concentrations was shown after pre-incubation with PTX in both culture systems, indicating a distinct involvement of a G_i/G_0-protein-coupled receptor. Due to the non-specific receptor blocking, a direct signal transduction by GPR109A could not be proven absolutely. To give evidence of a specific signal transduction via the GPR109A, further studies should use specific GPR109A antagonist or RNA interference. However, at least to our knowledge no antagonist is available so far [Offermanns et al., 2015].

Interestingly, in both studies (murine and bovine) PTX pre-incubation (followed by *t*CA or NA treatment) decreased the secretion of AdipoQ significantly, but the concentrations remained elevated when compared with non-treated adipocytes. To our knowledge we are the first who reported this implication and contrast those findings of Plaisance et al. [2009] who observed an abrogated increase in AdipoQ secretion after stimulation with NA and PTX in rat adipocytes. Therefore, we suggest, in addition to GPR signaling-dependent pathways, a GPR independent pathway in the regulation of AdipoQ secretion after stimulation with *t*CA and NA. Interestingly, this GPR-independent pathway occured in an immortalized, pure adipocyte cell culture (3T3-L1) as well as in a primary cell population, consisting of a mixture of stromal vascular fraction cells, indicating an adipocyte specific pathway. The forecited transcription factor PPARγ is highly expressed in AT and important for adipocyte differentiation. Furthermore, PPARγ is a known stimulator of AdipoQ expression [Iwaki et al., 2003], and as regulator of several genes it is also involved in the control of insulin sensitivity [Chiarelli & Di, 2008]. Treatment with an extract of cinnamon bark (containing

*t*CA) up-regulated the mRNA expression of PPARγ, as well as the AdipoQ secretion in mice [Kim & Choung, 2010]. Wanders et al. [2013] reported an increase of PPARγ mRNA by NIA in GPR109A knockout mice. In addition to our analysis of AdipoQ secretion, the mRNA abundance of GPR109A (and other related genes) was quantified after NA stimulation in the bovine cells. Interestingly, the observed increase in GPR109A gene expression was not blocked by PTX, verifying a G_i/G_o-protein-coupling independent effect. A direct regulation of GPR109A mRNA by PPARγ in 3T3-L1 cells was demonstrated [Jeninga et al., 2009], as well as a high correlation between mRNA abundance of PPARγ and GPR 109A in sc AT of dairy cows [Friedrichs et al., 2014]. These findings support PPARγ as a possible *t*CA and NA-induced stimulator of AdipoQ secretion independent of GPR109A signaling, and suggest PPARγ as an interesting target gene for further studies especially in the bovine to complete the knowledge on NIA dietary effects and its impact on the AdipoQ system of dairy cows.

In addition, the expression of PPARγ mRNA was reported to be higher in retroperitoneal (rp) AT than in sc AT depots of rats [Palou et al., 2009]. The physiological significance of functional differences between AT localisations were discussed in a broad manner in monogastrics suggesting a higher impact of visceral (vc) depots (e.g. rp) on metabolism. This may result from a higher release of adipokines [Wajchenberg et al., 2002] in these depots. In addition, vc AT mass is stronger correlated with insulin sensitivity [Cefalu et al., 1995] than sc AT. As aforementioned, sc AT from the sternum depot was used in this study, since information about the functional differences between AT depots in the bovine was missing. Recently, Locher et al. [2011, 2012] showed higher protein expression and extent of phosphorylation of AMPK and HSL in rp versus sc AT in dairy cows during early lactation, indicating a greater lipolytic potential for this AT depot. In addition, our group [Singh et al., 2014] assessed the AdipoQ concentrations in different AT depots of primiparious dairy cows during early lactation. In rp AT, the lowest AdipoQ concentration as well as total amount of AdipoQ were found compared to sc and other vc AT. Since AdipoQ is known to have an inhibitory effect on lipolysis in murine adipocytes [Qiao et al., 2001], the decreased AdipoQ secretion may contribute to the abovementioned higher lipolytic rate in this AT depot. On the ground of the newly revealed functional differences in bovine AT and the promising results seen in sc AT cell culture, a bovine primary cell culture model based on rp AT should be considered for further studies, since a comparison of different AT depots concerning expression of AdipoQ as well as related genes like PPARγ, after stimulation with determining factors might be of great interest.

In conclusion, this thesis studied two factors potentially improving AdipoQ secretion. In the first instance, *t*CA, as isolated cinnamon compound, was introduced as stimulator of AdipoQ in murine adipocytes. With our established cell culture model, we were able to demonstrate enhancing effects of NA on AdipoQ secretion in differentiated primary bovine adipocytes *in vitro*. Therefore, treatment with NIA might improve insulin sensitivity and/or adipocyte metabolism in dairy cows. Furthermore, participation of GPR signaling as well as another, GPR-independent, *t*CA/NA-induced pathway to stimulate AdipoQ was detected. These results may clear the way for further studies to understand and improve the metabolic changes during the transition period in bovine AT *in vitro* and may establish a basis for upcoming *in vivo* studies.

SUMMARY

White adipose tissue (AT) was believed to be just an energy-storage organ, but it is now recognized to be an active participant in energy homoeostasis and physiological functions such as immunity and inflammation. Adipose tissue secretes multiple metabolically important proteins known as adipokines. One of the most abundant adipokine in circulation is adiponectin (AdipoQ), which is known as an important modulator of glucose and fat metabolism and a key regulator of insulin sensitivity and thereby takes part in protection against the development of obesity and type 2 diabetes. Adiponectin exerts its effects via binding to its receptors AdipoR1/R2 and activation of peroxisome proliferator-activated receptor α (PPARα) and 5'adenosine monophosphate-activated protein kinase (AMPK).

Dairy cows undergo various metabolic changes during the transition period. This time from late pregnancy to early lactation is crucial for the potential development of many diseases and metabolic disorders, such as mastitis, metritis or ketosis. Energy requirements increase due to the nutrient demand of the fetus and the initiation of lactation and cannot be covered by feed intake only. The resulting negative energy balance is characterized by increased lipolysis and reduced insulin sensitivity in peripheral tissues, such as AT and muscle. Due to its insulin-sensitizing effect, AdipoQ is not only of great interest in humans, but also for dairy cows during the transition period. Hence, strategies to improve synthesis and secretion of AdipoQ are of major interest in various species.

Cinnamon (CN) is known to exert several beneficial effects by improving insulin sensitivity and lipid profiles. In mice treated with an extract of cinnamon bark, the concentration of AdipoQ was increased. In this thesis we focused on the barely explored cinnamon compound *trans*-cinnamic acid (*t*CA) as potential AdipoQ stimulus. In addition, Niacin (NIA) is known to decrease lipolysis and the circulating NEFA concentrations in lactating cows and to increase AdipoQ blood concentrations in rodents. It may therefore have the potential to emend the metabolic situation of dairy cows during the transition period via improving AdipoQ. Since nicotinic acid (NA), a compound of NIA, as well as *t*CA are both ligands of the G-protein-coupled receptor (GPR)109A, not only the effects on AdipoQ were of interest but also the signaling pathway. Therefore, this thesis aimed (1) to investigate the effects of *t*CA on the secretion of AdipoQ and the activation of AMPK (represented as pAMPK/AMPK ratio) in murine adipoyctes, (2) to evaluate the changes of AdipoQ secretion and mRNA abundance of selected genes after supplementation with NA in bovine adipocytes and (3) to characterize the involvement of the GPR109A in the signaling pathway towards AdipoQ. In

Manuscript 1, differentiated 3T3-L1 cells were incubated with varying *t*CA concentrations. Treatment with *t*CA increased the secreted AdipoQ and the pAMPK/AMPK ratio ($p \leq 0.001$). To prove signaling by GPR, the adipocytes were additionally pre-incubated with pertussis toxin (PTX), an inhibitor of G_i/G_o protein coupling. Treatment with PTX abolished the increased pAMPK/AMPK ratio and AdipoQ secretion. By this study, we introduced *t*CA for the first time as an influencing variable on the AdipoQ system, identifying that *t*CA is an effective compound of cinnamon, with the capability to improve glucose and fat metabolism. Furthermore, an involvement of the GPR signaling, induced by *t*CA, was shown. After establishing a bovine primary cell culture model, the potential effect of NA on AdipoQ was tested in an analogous study **(Manuscript 2)**. The differentiated bovine adipocytes were incubated with PTX to verify the involvement of GPR signaling, and treated with 10 or 15 µM NA for 12 or 24 h. Nicotinic acid increased the concentrations of AdipoQ ($p \leq 0.001$) and the mRNA abundance of GPR109A ($p \leq 0.05$). Pre-incubation with PTX reduced the AdipoQ response to NA ($p \leq 0.001$). The NA-stimulated secretion of AdipoQ and the mRNA expression of GPR109A in bovine adipocytes were suggestive for GPR signaling-dependent improved insulin sensitivity and adipocyte metabolism in dairy cows. In addition, in both studies (murine and bovine) PTX pre-incubation (followed by *t*CA or NA treatment) decreased the AdipoQ concentrations significantly, but remained increased according to non-treated adipocytes. These observations contrast other findings that observed an completely abrogated increase in AdipoQ secretion after stimulation with NIA and PTX in rat adipocytes. Therefore, we suggest, in addition to GPR signaling-dependent pathways, a GPR-independent pathway in the regulation of AdipoQ secretion after stimulation with *t*CA and NA. The transcription factor PPARγ is a known stimulator of AdipoQ expression and a regulator of several genes involved in the control of insulin sensitivity, supporting PPARγ as a possible *t*CA and NA-induced stimulator of AdipoQ secretion, independent of GPR109A signaling.

This thesis presents two compounds improving AdipoQ secretion in murine and bovine adipocytes, respectively. Furthermore, participation of GPR signaling as well as another, GPR-independent *t*CA/NA-induced pathway to stimulate AdipoQ was detected. These results may pave the way for further studies to understand and improve the metabolic changes during transition period in bovine AT *in vitro* and may establish a basis for upcoming *in vivo* studies.

ZUSAMMENFASSUNG

Das Fettgewebe wurde lange Zeit lediglich als Energiespeicher des Körpers angesehen. Untersuchungen der letzten Jahre stellten das Fettgewebe jedoch als endokrin aktives Gewebe dar, das mit verschiedenen physiologischen Funktionen wie Insulinsensitivität, Immunität und Entzündungsreaktionen in Verbindung steht. Das Fettgewebe sezerniert eine große Anzahl an metabolisch bedeutenden Proteinen, bekannt als Adipokine. Adiponektin (AdipoQ) ist eines der am häufigsten in der Zirkulation vorkommenden Adipokine und ein wichtiger Modulator des Fett- und Glucosestoffwechsels. Des Weiteren gilt AdipoQ als wichtige Einflußgröße in der Verbesserung der Insulinsensitivität und somit als Schutz vor der Entstehung von Übergewicht und Typ 2 Diabetes. Adiponektin vermittelt seine Wirkung über die Bindung an seine beiden Rezeptoren AdipoR1/2 und anschließender Aktivierung des Peroxisomen-Proliferator-aktivierten Rezeptors α (PPARα) und der 5'Adenosinmonophosphat-aktivierten Proteinkinase (AMPK).

Milchkühe sind in der sogenannten Transitionsperiode vielen metabolischen Veränderungen ausgesetzt. Der Zeitraum von der späten Trächtigkeit bis zur frühen Laktation ist kritisch für die mögliche Entstehung verschiedener Erkrankungen wie z.B. Mastits, Metritis oder Ketose. Aufgrund des Nährstoffbedarfs des Fötus und der einsetzenden Laktation steigt der Energiebedarf der Kuh so stark an, dass er über eine erhöhte Futteraufnahme nicht mehr gänzlich gedeckt werden kann. Folge dieser negativen Energiebilanz sind eine gesteigerte Lipolyse und reduzierte Insulinsensitivität in peripheren Geweben wie dem Fettgewebe und der Muskulatur. Aufgrund seiner insulin-sensitivierenden Wirkung ist AdipoQ nicht nur für die humane Forschung interessant, sondern auch für die Gesunderhaltung der Milchkühe während der Transitionsperiode. Daher sind Strategien zur Verbesserung der Synthese und Sekretion von AdipoQ bei verschiedensten Spezies von besonderem Interesse. Zimt ist seit vielen Jahren dafür bekannt, einen positiven Einfluss auf die Insulinsensitivität und den Lipidstoffwechsel zu nehmen. Eine erhöhte AdipoQ-Konzentration konnte nach Verabreichung eines Zimtrindenextraktes bei Mäusen beobachtet werden. In dieser Arbeit wurde der Fokus auf *trans*-Zimtsäure (*t*CA) als AdipoQ-Stimulator gelegt, ein bis jetzt wenig beachteter Inhaltsstoff von Zimt. Darüber hinaus soll das Vitamin Niacin (NIA) in supranutritiver Dosierung die Lipolyseaktivität bei laktierenden Kühen verringern und die AdipoQ-Konzentration bei Nagern erhöhen. Es könnte daher die kritische Situation der Milchkühe während der Transitionsperiode durch eine Erhöhung des AdipoQ-Status verbessern. Da sowohl Nikotinsäure (NA), ein Bestandteil des NIA, als auch *t*CA Liganden

des G-Protein-gekoppelten Rezeptors (GPR) 109A sind, war nicht nur der Effekt dieser beiden Substanzen auf AdipoQ von Interesse, sondern auch der Signalweg. Ziel dieser Arbeit war daher, (1) den Einfluss von *t*CA auf die AdipoQ-Sekretion und AMPK-Aktivierung in murinen Adipozyten (3T3-L1) zu untersuchen, (2) die Veränderungen auf die AdipoQ-Konzentrationen und auf die mRNA-Expression zugehöriger Gene nach Stimulation mit NA in bovinen Adipozyten zu zeigen und (3) die Beteiligung des GPR109A als Signalweg zu charakterisieren. In der ersten Studie (**Manuskript 1**) wurden differenzierte 3T3-L1-Zellen mit verschiedenen Mengen von *t*CA inkubiert. Sowohl die AdipoQ-Konzentrationen als auch die Aktivierung von AMPK wurden signifikant gesteigert (p=0,001). Um die Beteiligung des GPR zu zeigen, wurden die Zellen zusätzlich mit Pertussis-Toxin (PTX) inkubiert. Dieses katalysiert die Bindung des inaktiven G-Proteins im Rezeptor und führt somit zu einer Unterbrechung in der Signalweiterleitung. Die Behandlung mit PTX führte zu einer verringerten AdipoQ-Konzentration und AMPK-Aktivierung trotz anschließender *t*CA-Supplementation. Mit dieser Studie konnte *t*CA als Einflussgröße auf AdipoQ etabliert werden. Somit ist *t*CA nicht nur aktiver Bestandteil von Zimt, sondern hat auch die Fähigkeit zur Verbesserung des Glucose- und Fettstoffwechsels. Des Weiteren konnte eine *t*CA-induzierte Signalweiterleitung durch GPRs gezeigt werden. Nach der Etablierung einer bovinen Adipozytenzellkultur wurde in einer analogen Studie der potentielle Effekt von NA auf AdipoQ getestet (**Manuskript 2**). Die differenzierten Adipozyten wurden mit 10 oder 15 µM NA für 12 oder 24 h inkubiert. Sowohl die Sekretion von AdipoQ als auch die mRNA-Expression von GPR109A wurden gesteigert ($p \leq 0.001$ und $p \leq 0.05$). Eine vorhergehende Inkubation mit PTX verringerte die AdipoQ-Antwort auf NA ($p \leq 0.001$). Die NA-stimulierte Sekretion von AdipoQ und GPR109A-mRNA-Expression in bovinen Adipozyten deuten auf eine GPR-abhängige Verbesserung der Insulinsensitivität über AdipoQ hin. Interessanterweise führte die Inkubation mit PTX (gefolgt von der Supplementation mit NA oder *t*CA) in beiden Studien zu signifikant verringerten AdipoQ-Konzentrationen, die allerdings immer noch erhöht waren im Vergleich zu nicht behandelten Adipozyten. Diese Ergebnisse stehen im Widerspruch zu anderen Studien, in denen ein vorher gezeigter NIA-stimulierter Anstieg von AdipoQ nach PTX Behandlung nicht mehr nachweisbar war. Daher unterstellen wir neben einem GPR-abhängigen Signalweg zusätzlich eine GPR-unabhängige Signalweiterleitung bei der Regulation von AdipoQ nach Stimulation mit *t*CA und NA. Der Transkriptsionsfaktor PPARγ ist ein bekannter Stimulator der Expression von AdipoQ und Regulator verschiedener Gene, die in die Kontrolle der Insulinsensitivität involviert sind und

stellt daher einen möglichen *t*CA/NA-induzierten Stimulator von AdipoQ, unabhängig vom GPR109A dar.

Diese Arbeit stellt zwei Faktoren vor, welche die Sekretion von AdipoQ in murinen und bovinen Adipozyten verbessern. Des Weiteren wurde sowohl die Beteiligung des GPR Signalweges als auch eines GPR-unabhängigen, *t*CA/NA-induzierten Pfades zur Stimulation von AdipoQ gezeigt. Diese Ergebnisse könnten den Weg für weitere Studien zum Verständnis und zur Verbesserung der metabolischen Veränderungen während der Transitionsperiode im bovinen Fettgewebe *in vitro* ebnen und somit eine Grundlage für künftige *in vivo* Studien schaffen.

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PUBLICATIONS DERIVED FROM THIS DOCTORATE THESIS

Published manuscripts:

Kopp C, Singh S, Khalilvandi-Behroozyar H, Regenhard P, Müller U, Sauerwein H, Mielenz M. Nicotinic acid increases adiponectin secretion from differentiated bovine preadipocytes through G-protein-coupled receptor signaling. Int J Mol Sci. 2014; 15:21401-18.

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