Institute of Animal Nutrition and Nutritional Physiology

(Director: Prof. Dr. K. Eder)

of the

Faculty of Agricultural Sciences, Nutritional Sciences and Environmental Management

of

Justus-Liebig-University Giessen

(Dean: Prof. Dr. Dr.-Ing. Peter Kämpfer)

"Investigations on the effect of nicotinic acid supplementation on muscle fiber distribution and muscle metabolic phenotype in pig and sheep"

Dissertation

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Muckta Khan (MS in Animal Science)

Born on 04.12.1979 in Tangail, Bangladesh

Supervisors: Prof. Dr. K. Eder

Prof. Dr. F. C. Mooren

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BHBA	β-hydroxybutyrate
CACT	Carnitine acylcarnitine translocase
cDNA	Complementary deoxyribonucleic acid
CETP	Cholesterol ester transfer protein
CoA	Coenzyme A
COX	Cytochrome c oxidase
CPT	Carnitine palmitoyltransferase
FATP	Fatty acid transport protein
G	Gastrocnemius
GPR	G protein-coupled receptor
G protein	Guanine nucleotide binding protein
HCA	Hydroxy-carboxilic acid
HDL	High-density lipoprotein
HM	Human
HSL	Hormone-sensitive lipase
LD	Longissimus dorsi
LDL	Low-density lipoprotein
Lp(a)	Lipoprotein (a)
M.	Muscle
MACP	Mitochondrial anion carrier proteins
MHC	Myosin heavy chain
MLC	Myosin light chains
mRNA	Messenger Ribonucleic acid
MYH	Myosin heavy chain encoded gene
NA	Nicotinic acid
NAD	Nicotinamide adenine dinucleotide

NADP	Nicotinamide adenine dinucleotide phosphate
NAM	Nicotinamide
NEB	Negative energy balance
OCTN	Novel organic cation transporter
PDE-3B	Phosphodiesterase-3B
PGC	Peroxisome proliferator-activated receptor-gamma coactivator
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptor
PUMA-G	Protein upregulated in macrophages by interferon-λ
QF	Quadriceps femoris
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SD	Standard deviations
SDHA	Succinate dehydrogenase subunit A
SLC	Solute carrier
SM	Semimembranosus
ST	Semitendinosus
TAG	Triacylglycerols
TCA	Tricarboxylic acid
UCP	Uncoupling proteins
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein

1. INTRODUCTION

1.1 Background

For more than five decades nicotinic acid (NA) has been used as a lipid-lowering drug (Karpe and Frayn, 2004; Bodor and Offermanns, 2008). At pharmacological doses, NA shows remarkable lipid-lowering activities, particularly on triacylglycerols (TAG), but also on total cholesterol, low-density lipoprotein (LDL) cholesterol, very low-density lipoprotein (VLDL) cholesterol and, interestingly, NA increases high-density lipoprotein (HDL) cholesterol (Carlson, 2005; Gille *et al.*, 2008; Kamanna and Kashyap, 2008) but to date, the underlying lipid-lowering mechanism of NA is only partially understood. However, the inhibition of lipolysis by NA in adipocytes through binding with the NA receptor G protein-coupled receptor 109A (GPR109A) causes reduction of plasma free fatty acids (FFA, also called non-esterified fatty acids, NEFA), which are substrates for hepatic TAG synthesis and VLDL formation and synthesis, is endorsed as the basic mechanism for the lipid-lowering effects of NA (Gille *et al.*, 2008; Kamanna and Kashyap, 2008). But this classic view of lipid-lowering mechanism of NA has been put into question because the circulating FFA level even become increased during long-term (at least 2 weeks) NA treatment due to a profound rebound on lipolysis even though its lipid-lowering effect keeps on (Choi *et al.*, 2011). Moreover, very recently Lauring *et al.* (2012) throw a challenge to this classic view by revealing that GPR109A is not the major molecular target responsible for NA lipid efficacy. In this circumstance, the explanation of the lipid-lowering effect of NA by the reduced FFAs delivery to the liver is obscure.

Skeletal muscle is the major tissue for whole body glucose and fatty acid metabolism (Wang *et al.*, 2004) and recently it has been found that NA has widespread effects on gene expression in major other tissues of lipid metabolism than adipose tissue, such as skeletal muscle (Choi *et al.*, 2011), raising the possibility of another underlying explanation of lipid-lowering effect of NA. Skeletal muscle fibers are generally classified as type I (oxidative/slow) and type II (glycolytic/fast) fibers. They display marked differences in respect to contraction, metabolism, and susceptibility to fatigue. Type I fibers are mitochondria rich and mainly use oxidative metabolism for energy production, on the other hand fast-twitch glycolytic type II fibers utilize glucose for energy production (Pette and Staron, 1990; Olson and Williams, 2000). Adult skeletal muscle shows marked plasticity and can undergo transformation between different fiber types in response to physical activity, such as exercise or modulation of motoneuron activity, mechanical loading/unloading or obesity (Pette, 1998; Olson and

Williams, 2000; Hood, 2001; Cassano *et al*., 2006; Fujita *et al.*, 2012). Notably, it has been shown in humans (Watt *et al.*, 2004) and recently in rats (Ringseis *et al.*, 2013) that high dose of NA induces the expression of two transcription factors, peroxisome proliferator-activated receptor (PPAR) δ and PPARγ coactivator-1α (PGC-1α) in skeletal muscle. Both of these transcription factors are key regulators of muscle fiber composition and the muscle's metabolic phenotype because they control genes involved in muscle fiber switching, fatty acid utilization, oxidative phosphorylation, mitochondrial biogenesis and function (Wang *et al*., 2004; Schuler *et al*., 2006) and angiogenesis (Chinsomboon *et al*., 2009). Based on the observation that NA up-regulates key regulators (PPARδ and PGC-1α) of fiber switching in skeletal muscle, it has been already investigated whether NA supplementation can inhibit the obesity-induced muscle fiber transition from oxidative type I to glycolytic type II and increases the number of type I fibers in skeletal muscle of obese Zucker rats (Ringseis *et al.*, 2013). Indeed, in this study, it was found that NA favored muscle fiber transition from type II to type I in obese Zucker rats (Ringseis *et al.*, 2013). Moreover, it was also found that the expression of genes involved in fatty acid transport, mitochondrial fatty acid import and oxidation, oxidative phosphorylation and angiogenesis and key regulators of muscle fiber switching PPARδ, PGC-1α and PGC-1β in skeletal muscle were elevated by NA treatment (Ringseis *et al.*, 2013). PGC-1β (another member of PGC-1 family) has been less extensively studied than PGC-1α, but recently it has been found that PGC-1β is a potential mediator of the development of oxidative sub-type IIX fibers in skeletal muscle of mice (Arany *et al*., 2007). PGC-1β also contributes to the regulation of contractile and metabolic phenotype of the skeletal muscle (Mortensen *et al*., 2006). It was also revealed that PGC-1β in skeletal muscle increases mitochondrial biogenesis, expression of genes encoding mitochondrial proteins and mitochondrial activity (Arany, 2008).

However, there is no investigation, to our knowledge, whether NA treatment also causes type II to type I muscle fiber switching and increases the type I fiber content of skeletal muscles in healthy non-ruminant and ruminant farm animals. Thus, on the base of these literature findings it has been hypothesized in the present thesis that NA treatment causes similar effects, namely transition of type II fiber to type I fiber in pig as a model of non-ruminant (**study 1**) and in sheep as a model of ruminant (**study 2**).

***Contextual talk of study 1*:** According to Liaubet *et al*. (2011), muscle fiber characteristics play a key role in meat quality of farm animals. Fiber type, fiber area, oxidative and glycolytic capacity, glycogen and lipid contents of muscle may strongly affect the energy metabolism of

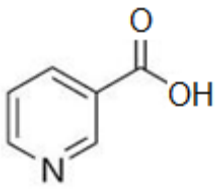
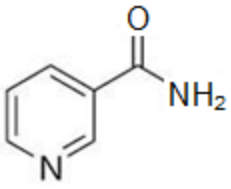
live animals as well as the postmortem conversion of muscle to meat, thus affecting ultimate quality of meat (Karlsson *et al*., 1999; Ryu and Kim, 2005; Choe *et al*., 2008; Choi and Kim, 2009). Muscles with a higher percentage of type I fibers and lower percentage of type IIb fibers showing lower lightness and drip loss of meat (Ryu and Kim, 2005; Choe *et al*., 2008; Ryu *et al*., 2008). Pre-slaughter metabolic response that takes place in different fiber types is an important factor for post-mortem changes and meat quality of meat producing animals (Karlsson *et al*., 1999), since oxidative muscles with a high percentage of type I fibers have a lower glycolytic potential (Monin *et al*., 1987; Fernandez *et al*., 1994) a darker color (Warner *et al*., 1993; Brewer *et al*., 2001) and a higher ultimate pH (Monin *et al*., 1987; Warner *et al*., 1993). Additionally, muscles with higher amount of oxidative fibers have a tendency to develop dark, firm and dry pork in response to intense physical activity and/or high psychological stress levels associated to preslaughter handling. Conversely, muscles with higher amount of glycolytic fibers may develop pale, soft, and exudative pork (Hambrecht *et al*., 2005). Therefore, a NA-induced change in the muscle's fiber type distribution (type II to type I muscle fiber switching) may influence meat quality of pigs.

Contextual talk of study 2: Ketosis (Hyperketonemia) and fatty liver (Hepatic lipidosis) are two common diseases, which are characterized by altered energy metabolism of high-yielding dairy cows (Block *et al*., 2001; Xu and Wang, 2008). During transition period (defined as 3 weeks prepartum until 3 weeks postpartum) of high-yielding dairy cows, a negative energy balance (NEB) is observed because of the increased requirement of energy for high milk production on the one side and a depression in the energy intake on the other side (Brockman, 1979; Xu *et al*., 2008; Pescara *et al*., 2010). In severe NEB animals start to mobilize body fat (adipose tissue) and release excessive amounts of FFAs which are quickly circulated to the liver, converted to acetyl-CoA by β-oxidation, overwhelming liver's ability to handle these excessive amount of acetyl-CoA either through tricarboxylic acid (TCA) cycle or through conversion to TAG and release in the form of lipoproteins. Then these huge volumes of acetyl-CoA are converted to ketone bodies, resulting in ketosis, or FFAs are deposited as TAG in hepatocytes resulting in fatty liver (Grummer, 1993; Vazquez-Añon *et al*., 1994). Since skeletal muscle, particularly type I fibers significantly contributes to whole body fatty acid utilization, an increased capacity of oxidative type I fiber for fatty acid utilization is expected to be useful during conditions where fatty acids are available at increased levels, such as in ketosis or fatty liver of high-yielding cows.

1.2 Niacin

Niacin or vitamin B3 is a water soluble vitamin of the vitamin B complex, which is a vital element of human and animal nutrition. It has two vitamers called NA (pyridine 3-carboxylic acid) and nicotinamide (NAM, pyridine 3-carboxylic acid amide), both are biologically active forms of niacin, which are very stable in light, heat and alkali, and therefore also stable in feed (Pond *et al.*, 2005). In addition to feed as a source of niacin, almost all species are capable to synthesise niacin from tryptophan (Pond *et al.*, 2005; McDowell, 2005) and quinolinate (Henderson, 1983). In many countries, especially in North America, the term 'niacin' means exclusively for NA (Harmeyer and Kollenkirchen, 1989; Bender, 2003). Thus causes some confusion between the generic and specific terms. NA acts in two aspects, one is as 'vitamin', effective in milligram doses and the other is as 'lipid drug', effective in gram doses; but interestingly although NA and NAM, chemically quite alike, and nutritionally equivalent, NAM has no plasma lipid-lowering property (Parsons and Flinn, 1959; DiPalma and Thayer, 1991; Carlson, 2005; Lukasova, *et al.*, 2011). The chemical name and structure of both forms of niacin are presented in **Table 1**.

Table 1: Chemical name and structure of vitamers of niacin

	Nicotinic acid	**Nicotinamide**
Chemical name	Pyridine 3-carboxylic acid	Pyridine 3-carboxylic acid amide
Chemical formula	$C_6H_5NO_2$	$C_6H_6N_2O$
Structure		

(Adapted from Bender, 2003)

1.2.1 Nutritional function of niacin

Both vitamers of niacin (NA and NAM) are the direct precursors of two important coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which have great importance in lipid, carbohydrate and protein metabolism (Bender, 2003; Pond *et al.*, 2005). NAD and NADP, synthesized from niacin are involved as proton

and electron carriers in a wide variety of energy-yielding oxidation and reduction reactions, which occur in animal and human tissues (Bender, 2003). Almost all cellular metabolisms are involved NAD/NADH or NADP/NADPH linked schemes, therefore, cellular functions as well as life itself would be impaired for the deficiency of niacin, and for that niacin is a critical nutrient for human and animal body (Henderson, 1983).

1.2.2 Pharmacological use of nicotinic acid

NA, but not NAM, has been used for decades as a lipid-lowering drug (Hotz, 1983; Knopp, 1999; Karpe and Frayn, 2004; Bodor and Offermanns, 2008). It is the earliest lipid-regulating drug used in the treatment of lipid disorders and atherosclerotic coronary heart disease (Kamanna *et al*., 2009). In the landmark study, Altschul *et al*. (1955) reported, that NA in gram doses lowered plasma cholesterol in normal as well as hypercholesterolemic subjects. This milestone finding was consequently confirmed in numerous studies and NA has been gained the name **'the broad-spectrum lipid drug'** (Carlson, 1990). At pharmacological doses, NA decreases TAG, total cholesterol, VLDL, LDL, and lipoprotein(a) [Lp(a)] as well as increases HDL levels in blood plasma (Meyers *et al.*, 2004; Carlson, 2005; Gille *et al.*, 2008) (**Table 2**). The anti-lipolytic effect of NA was first found in rabbits (Altschul and Herman, 1954), later in mice (Carlson and Hanngren, 1964), goats (Schultz *et al.*, 1968), sheep (Nye and Buchanan, 1969) and cows (Schultz, 1971). NA is also known as an antiketogenic substance (Flachowsky, 1993), because the direct effect of NA on ketone bodies (β-hydoxybutyrate, BHBA) in dairy cows has been broadly observed (Waterman *et al*., 1972; Fronk and Schultz, 1979; Dufva *et al*., 1983; Jaster *et al*., 1983; Erickson *et al.*, 1992; Al-Abbasy, 2013). The antiketogenic effect of NA is based on the antilipolytic action of the substrates (Waterman and Schultz, 1973; Pires and Grummer, 2007; Al-Abbasy, 2013). In dairy cows, NA can decrease the plasma level of FFA and BHBA, resulting in reduced prevalence of metabolic disorders, such as ketosis and fatty liver by inhibiting TAG lipolysis (Schwab *et al*., 2005; Pires and Grummer, 2007). In humans, high doses of NA (3 g/day) decrease total plasma levels of cholesterol by almost 10% in healthy volunteers and by more than 20% in hypercholesterolemic patients (Altschul *et al*., 1955; Parsons and Flinn, 1959). NAM has no effect on the plasma lipid or lipoprotein concentration, undoubtedly indicating that the antilipolytic effect of NA is distinct to its role as a vitamin.

Table 2: Effects of nicotinic acid (>1.5 g / day) on plasma lipid profile at a glance

VLDL	(25–40%) ↓
LDL	(6–22%) ↓
HDL	(18–35%) ↑
Total cholesterol	(4–16%) ↓
TAG	(21–44%) ↓
Lp(a)	(16–36%) ↓

(Addapted from review Gille *et al.*, 2008)

1.2.3 Nicotinic acid receptor

About 50 years ago the rapid uptake, preferential distribution and accumulation of NA in adipose tissue was described by Carlson and Hanngren (1964). After that, specific binding sites for NA on plasma membranes of adipocytes and spleen cells were demonstrated (Lorenzen *et al*., 2001). In 2003, three independent research groups identified a G protein-coupled receptor as a specific and high affinity receptor for NA which mediates the antilipolytic effects of NA (Soga *et al*., 2003; Tunaru *et al*., 2003; Wise *et al*., 2003). The receptor was termed as GPR109A (HM74A in human and PUMA-G in mice, no specific term for sheep or pig has been found) and is expressed mainly in adipocytes and immune cells. In line with bioinformatics data NA receptor belongs to a subfamily of G protein-coupled receptors that includes GPR109A and GPR81, both of which are expressed in humans and in rodent species. A third member of this receptor family, GPR109B (HM74), has also been found (in humans, but not in rodent species) as a low-affinity receptor for NA (Soga *et al*., 2003; Wise *et al*., 2003). GPR81, GPR109A and GPR109B have recently been renamed hydroxy-carboxylic acid receptor 1, 2 and 3 (HCA1, HCA2 and HCA3), respectively (Offermanns *et al*., 2011). The endogenous ligand for GPR109A is BHBA (Gille *et al.*, 2008).

1.2.4 Mechanisms of antidyslipidemic effects of nicotinic acid-prevailing hypothesis

Although NA has the extraordinary capability to decrease TAG, VLDL cholesterol, LDL cholesterol and Lp(a), as well as to increase HDL cholesterol levels and thus improves the total plasma lipid profile, the underlying mechanisms by how NA exerts its antidyslipidemic effect have remained unclear (Gille *et al.*, 2008; Kamanna *et al*., 2009; Chapman *et al*., 2010; Lukasova *et al.*, 2011; Lauring *et al.*, 2012; Offermanns, 2012). One of the well-established classic views of action of NA is "FFA hypothesis" (Lauring *et al.*, 2012). According to this classic view, the antilipolytic effect in adipocytes causing reduction of plasma FFAs

concentrations, which modulates hepatic TAG synthesis and VLDL formation and synthesis, and subsequently results in decrease plasma levels of LDL cholesterol (Carlson and Hanngren, 1964; Wang *et al*., 2001; Gille *et al.*, 2008; Kamanna and Kashyap, 2008). NA binds to the GPR109A receptor in adipose tissue and activation of GPR109A by NA leads to inhibition of adenylyl cyclase activity and, consequently decreases cellular cyclic adenosine monophosphate (cAMP) levels. Decreased cAMP in adipocytes leads to an inactivation of protein kinase A (PKA), and accordingly decreases phosphorylation of hormone-sensitive lipase (HSL) and perilipin, which are obligatory for TAG hydrolysis. Reducing these enzyme activities leads to decrease TAG lipolysis, consequently reduce FFA production. The decreased FFA levels, induced by NA, resulted in a substrate shortage for hepatic TAG synthesis. Accordingly, less TAG and VLDL are produced in liver, and thus, plasma levels of TAG and VLDL as well as LDL are dropped (Gille *et al*., 2008; Kamanna and Kashyap, 2008). The mechanism by which NA increases the plasma HDL cholesterol is less clear, but has been shown to require the presence of cholesterol ester transfer protein (CETP) (Hernandez *et al*., 2007; van der Hoorn *et al*., 2008). However, this long-standing FFA hypothesis of NA efficacy has been put into question because the circulating FFA level even become increased during long-term NA treatment due to a profound rebound on lipolysis even though its lipid-lowering effect keeps on (Choi *et al.*, 2011). Some other previous studies have also questioned the FFA hypothesis because of the marked "rebound" or "baseline over-shoot" of both acute and more chronic dosing of NA (Jin *et al*., 1997; Wang *et al*., 2001; de Grooth *et al*., 2004; Ganji *et al*., 2004; Lamon-Fava *et al*., 2008; Hernandez *et al*., 2010), suggesting that the fundamental mechanisms other than anti-lipolysis may be responsible for lipid efficacy (Wang *et al*., 2001; Lukasova, *et al.*, 2011).Very recently, Lauring *et al*. (2012) provided additional evidence, that is very much contradictory to the prevailing FFA hypothesis. By using a humanized genetic mouse model and dyslipidemic patients, treated with NA and GPR109A agonists, Lauring *et al.* (2012) strongly suggest that GPR109A is not the major molecular target responsible for NA lipid efficacy and thus the long-standing FFA hypothesis has been challenged, but they did not investigate the mechanism further. The underlying mechanism is not discovered, so far.

1.3 Skeletal muscle

In mammals, skeletal muscle constitutes up to 50% of total body mass and thus making it the largest organ of the body (Ehrenborg and Krook, 2009) which is mainly involved in the

implementation of voluntary movement. It is also called voluntary muscle because its activities are usually under conscious regulation. It is also known as striated muscle because it contains fibers in an arrangement of altering dark and light bands, which create a striated appearance under the microscope. Skeletal muscles are the major site for carbohydrate and fatty acid metabolism, and heat generation (Kiens, 2006; Schuler *et al*., 2006; Houmard, 2008). As it constitutes a large part of the total body mass, by considering the whole volume, skeletal muscle metabolism affects the metabolic budget of the whole organism (Ehrenborg and Krook, 2009). The distinctive characteristics of skeletal muscle is its diversify composition with a large number of different types of muscle fibers (muscle cells), which differ according to their molecular, contractile and metabolic properties, and thus, can contribute to a diversity of functional capabilities (Pette and Staron, 2001) (structure of skeletal muscle **Figure 1**).

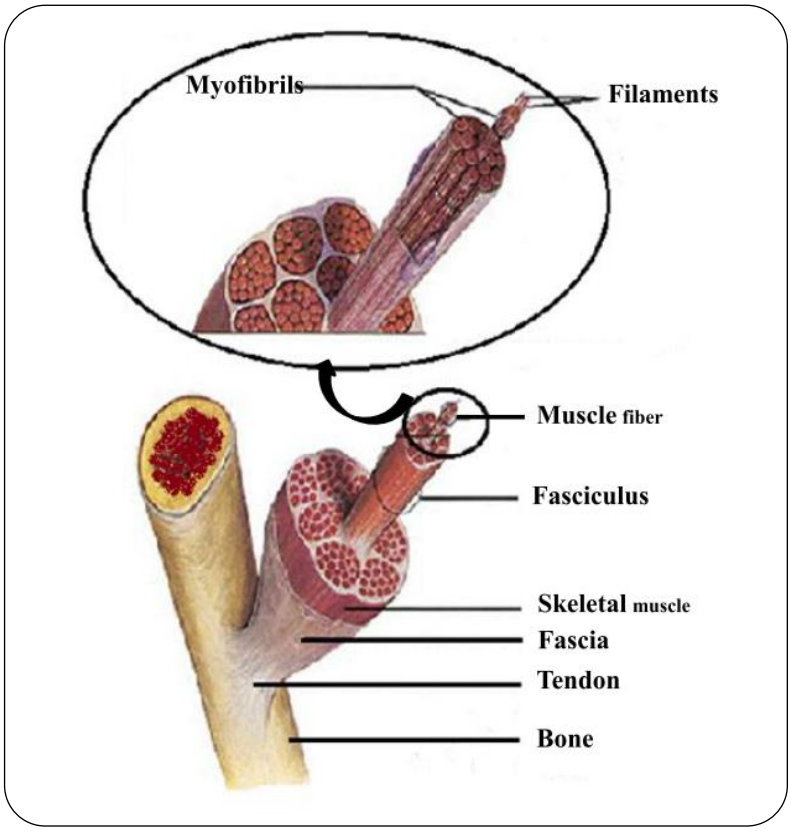


Figure 1 Structure of skeletal muscle (Adapted from URL, 2013)

1.3.1 Skeletal muscle fiber types

The functional unit of skeletal muscle tissue is the muscle fiber (cell), which may extend the entire length of the muscle (Karlsson *et al*., 1999). The muscle fibers are multinucleated, long, cylindrical cells, and composed of myofibrils, which constitute the contractile apparatus of the

muscle. Skeletal muscle fibers are generally classified into two broad categories: Type I and Type II fibers. They exhibit clear differences according to their contraction, metabolism and susceptibility to fatigue capabilities. Type I fibers are mitochondria rich, and also called oxidative, because these fibers use oxidative metabolism to generate adenosine triphosphate (ATP). They are also fatigue-resistant or slow twitch fibers, because they take more time for contraction, again also known as red fibers, because they contain oxygen binding protein myoglobin. Functionally, type I fibers are used for daily living aerobic activities requiring low level force production, such as walking, maintaining posture or holding head by neck muscle. Type II fibers have low level of mitochondria, and are also known as glycolytic, because these fibers use glycolytic metabolism to generate ATP and are susceptible to fatigue. They are also known as fast twitch fibers, because the contraction time is fast, again also called white fibers, because of absence or very low myoglobin content. Functionally, type II fibers are used for anaerobic activities such as locomotion, racing 400 meters or for short anaerobic high force producing activities such as sprinting, hurdling, weight lifting, jumping, kicking or biting. Type II fibers comprise two subtypes, IIa/IIx and IIb depending upon species. The oxidation and contraction ability of type IIa/IIx place between type I and IIb (Booth and Thomason, 1991; Berchtold *et al*., 2000; Olson and Williams, 2000; Wang *et al*., 2004; Schiaffino and Reggiani 2011). The major characteristics of different types of muscle fibers are summarized in **Table 3.**

Table 3: Properties of different types of skeletal muscle fibers

	Type I	**Type IIa/IIx**	**Type IIb**
Speed of contraction	Slow	Fast	Very fast
Resistance to fatigue	High	Intermediate	Low
Activity used for	Aerobic	Long term anaerobic	Short term anaerobic
Force production	Low	High	Very high
Metabolic type	Oxidative	Oxido-glycolytic	Glycolytic
Oxidative (aerobic) capacity	High	Intermediate	Low
Glycolytic (anaerobic) capacity	Low	High	High
Mitochondrial density	High	High/medium	Low
Capillary density	High	Medium/low	Low
Myoglobin content	High	High	Low

(Adapted from Lefaucheur and Gerrard, 2000)

1.3.2 Skeletal muscle plasticity and molecular mechanism of fiber type switching

Skeletal muscle tissue exhibits a unique capacity to undergo adaptive changes in response to functional demands by changing their phenotypic profiles, leading to a gradual switch from one fiber type to another, and this process is known as muscle plasticity (Jorquera, *et al*., 2013). It shows remarkable plasticity and has the ability to convert between different fibers types in response to exercise training or modulation of motoneuron activity (Pette and Staron, 1997; Booth and Thomason, 1991; Jarvis *et al*., 1996; Pette, 1998; Olson and Williams, 2000; Hood, 2001). Numerous factors, in particular altered neuromuscular activity, mechanical loading/unloading, circulating factors including different hormones (especially thyroid hormones) and aging are recognized to affect muscle fiber type composition (Schiaffino and Reggiani, 2011). The transformation of muscle fiber from fast-to-slow type is likely to be mediated by a calcium signalling pathway that involves calcineurin, calmodulin-dependent kinase, and the transcriptional cofactor PGC-1α (Naya *et al*., 2000; Olson and Williams, 2000; Lin *et al*., 2002a; Wu *et al*., 2001; Pette and Staron, 2001).

1.4 Genes involved in fatty acid catabolism pathways, thermogenesis and angiogenesis

The first step of the oxidative pathway is the transport of fatty acids from the cytoplasm into the mitochondrial matrix. This step is controlled by the carnitine palmitoyltransferase (CPT) system (McGarry and Brown, 1997). The protein encoded by CPT1B gene is carnitine palmitoyl- transferase 1B [also known as muscle-type CPT1 (M)] (Price *et al*., 2003), a member of the carnitine/choline acetyltransferase family, is the integral rate-controlling enzyme of the long-chain fatty acid β-oxidation pathway in the mitochondria of muscle fiber. This enzyme is located on the outer mitochondrial membrane and required for the net transport of long-chain fatty acetyl-CoAs from the cytoplasm into the mitochondria (McGarry *et al*., 1978; Ramsay *et al*., 2001). Fatty acid transport protein 1 (FATP1), also named solute carrier family 27 member 1 (SLC27A1), gene encodes a member of a family of fatty acid transport proteins, which are involved in transport of long-chain fatty acids across the cell membrane (Martin *et al*., 2000). Moreover, FATP1 also has a role in mitochondrial fatty acid oxidation in collaboration with CPT1 (Sebastián *et al*., 2009). Carnitine-acylcarnitine translocase (CACT), also known as SLC25A20, embedded in the inner mitochondrial membrane, is essential for mitochondrial oxidation of long-chain fatty acids (Indiveri *et al*., 1997), because this protein catalyzes a mole-to-mole exchange of carnitines and acylcarnitines, so that the fatty acid moieties can be translocated into the mitochondrial matrix

(Peluso *et al*., 2005). Novel organic cation transporter 2 (OCTN2), also named SLC22A5, is the second member of the OCTN family, positioned within the cell membrane (Wu *et al.*, 1998; Ohashi *et al*., 2001). It is a physiologically important Na^+ dependent transporter for carnitine, which is a hydrophilic nutrient essential to carry long-chain fatty acids into mitochondria for β-oxidation (Tamai *et al*., 1998; Ohashi *et al*., 2001). Cytochrome c oxidase (COX), a terminal enzyme of the respiratory chain of mitochondria, is a key enzyme of aerobic respiration, meaning a decrease of COX activity decreases ATP production (Villani and Attardi, 1997; Hosler *et al*., 2006; Pacelli *et al*., 2011; Yoshikawa *et al*., 2011; Hüttemann *et al*., 2012). COX4/1, COX5A and COX6A1 are subunits of COX, which are embedded in the inner mitochondrial membrane and catalyze the transfer of electrons from cytochrome c to molecular oxygen and contribute to a proton electrochemical gradient across the inner mitochondrial membrane (Villani *et al*., 1998; Shoubridge, 2001; Cui *et al*., 2006). Succinate dehydrogenase (SDH) is a multimeric enzyme that is bound to the inner membrane of mitochondria (Oyedotun and Lemire 2004). The SDHA protein is the active subunit of SDH that performs the conversion of succinate, and it also helps to transfer electrons to the oxidative phosphorylation pathway. In oxidative phosphorylation, the electrons help to create an electrical charge that provides energy for the production of the cell's main energy source ATP (Kantorovich *et al*., 2010). The uncoupling protein (UCP) is a subfamily of the larger family of mitochondrial anion carrier proteins (MACP), located in the inner mitochondrial membrane (Krauss *et al*., 2005). These anion-carrier proteins transport protons (H^+) to the mitochondrial matrix and in turn dissipate the proton motive force as heat and uncouple the substrate oxidation from the production of ATP, also referred to as the mitochondrial proton leak. UCP2 and UCP3 are the member of UCP expressed in several tissues and thought to participates in thermogenesis (Dulloo and Samec, 2001; Henry *et al*., 2011). They play a role in uncoupling oxidative phosphorylation, as a result energy is dissipated in the form of heat, and maintains energy homeostasis (Krauss *et al*., 2005; Liu *et al*., 2013). Type I fibers exhibit a higher expression of angiogenic factors, such as vascular endothelial growth factor (VEGF). VEGF subunit A (VEGFA) is one of the most potent inducers of angiogenesis and vasculogenesis, and is a key regulator of both physiological and pathological angiogenesis. It causes proliferation, sprouting, migration and tube formation of endothelial cells (Ferrara *et al*., 2003). This also contributes to the preferential use of fatty acids by type I fibers, because angiogenic factors increase capillary density and thereby blood perfusion (Hagberg *et al*., 2010).

2. AIMS AND HYPOTHESES

The overall aim for the present PhD studies was, to investigate the effect of NA administration at a pharmacological dose on fiber type distribution and metabolic phenotype of different skeletal muscles of pig as a model for non-ruminants and sheep as a model for ruminants.

Study 1: *Khan M, Ringseis R, Mooren FC, Krüger K, Most E, Eder K (2013) Niacin supplementation increases the number of oxidative type I fibers in skeletal muscle of growing pigs. BMC Veterinary Research 9:177.*

General aim of study 1: *In* obese Zucker rats, it was observed that NA supplementation switches muscle fiber from glycolytic type II to oxidative type I in skeletal muscle and these effects were likely mediated by the induction of key regulators of fiber transition, PGC-1α and PGC-1β, leading to muscle fiber switching and up-regulation of genes involved in mitochondrial fatty acid import and oxidation, citrate cycle, oxidative phosphorylation, mitochondrial biogenesis (Ringseis *et al.*, 2013). In contrast, it has not been known whether high levels of NA also causes type II to type I muscle fiber switching in metabolically healthy animals. This question may be of particular interest in farm animals used for meat production, such as growing pigs because a change in the muscle's fiber type distribution is expected to influence meat quality. To address this issue, the aim of the present study was to investigate whether NA administration also influences fiber distribution and the metabolic phenotype of different skeletal muscles in pig as a model for non-ruminant farm animals.

Specific hypotheses of study 1: The following three hypotheses were tested in the present study-

(i) NA supplementation switches muscle fiber from type II (fast-glycolytic) to type I (slow- oxidative), and thereby induces an oxidative metabolic phenotype of skeletal muscle in pigs.

(ii) The relative mRNA levels of key regulators of fiber transition, PGC-1α and PGC-1β are upregulated in NA treated pigs compared to control pigs.

(iii) The relative mRNA levels of genes involved in mitochondrial fatty acid catabolism (CACT, FATP1, OCTN2), citrate cycle (SDHA), oxidative phosphorylation (COX4/1,

COX6A1), and thermogenesis (UCP2, UCP3) in skeletal muscle are higher in the NA group compared to control group pigs.

Study 2: *Khan M, Couturier A, Kubens JF, Most E, Mooren FC, Krüger K, Ringseis R, Eder K (2013) Niacin supplementation induces type II to type I muscle fiber transition in skeletal muscle of sheep. Acta Veterinaria Scandinavica, 55:85.*

General aim of study 2: It has been observed that NA supplementation increases the number of type I fibers in skeletal muscle of obese Zucker rats (Ringseis *et al*., 2013) and pigs (**Study 1),** and these effects were likely mediated by the induction of key regulators of fiber transition, PPARδ, PGC-1α and PGC-1β, leading to type II to type I fiber transition and upregulation of genes involved in fatty acid oxidation, mitochondrial oxidative phosphorylation, and angiogenesis (Ringseis *et al*., 2013). The aim of the present study was to investigate whether NA administration also influences fiber type distribution and the metabolic phenotype of different skeletal muscles in sheep as a model for ruminant farm animals.

Specific hypotheses of study 2: The following three hypotheses were tested in the present study-

(i) NA supplementation induces muscle fiber transition from type II (fast-glycolytic) to type I (slow-oxidative), and thereby creates an oxidative metabolic phenotype of skeletal muscle in sheep.

(ii) The relative mRNA and protein levels of key regulators of fiber transition (PGC-1α, PGC-1β and PPARδ) are upregulated in NA treated sheep than in control sheep.

(iii) The relative mRNA levels of genes involved in mitochondrial fatty acid uptake (CPT1B, SLC25A20), TCA cycle (SDHA), mitochondrial respiratory chain (COX5A, COX6A1), and angiogenesis (VEGFA) in skeletal muscle are higher in the NA treated sheep compared to control sheep.

3. Original works

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Niacin supplementation increases the number of oxidative type I fibers in skeletal muscle of growing pigs

Muckta Khan[1], Robert Ringseis[1], Frank-Christoph Mooren[2], Karsten Krüger[2], Erika Most[1] and Klaus Eder[1*]

Abstract

Background: A recent study showed that niacin supplementation counteracts the obesity-induced muscle fiber switching from oxidative type I to glycolytic type II and increases the number of type I fibers in skeletal muscle of obese Zucker rats. These effects were likely mediated by the induction of key regulators of fiber transition, PGC-1α and PGC-1β, leading to muscle fiber switching and up-regulation of genes involved in mitochondrial fatty acid import and oxidation, citrate cycle, oxidative phosphorylation, mitochondrial biogenesis. The aim of the present study was to investigate whether niacin supplementation causes type II to type I muscle and changes the metabolic phenotype of skeletal muscles in growing pigs.

Results: 25 male, 11 wk old crossbred pigs (Danzucht x Pietrain) with an average body weight of 32.8 ± 1.3 (mean ± SD) kg were randomly allocated to two groups of 12 (control group) and 13 pigs (niacin group) which were fed either a control diet or a diet supplemented with 750 mg niacin/kg diet. After 3 wk, the percentage number of type I fibers in three different muscles (*M. longissimus dorsi*, *M. quadriceps femoris*, *M. gastrocnemius*) was greater in the niacin group and the percentage number of type II fibers was lower in the niacin group than in the control group ($P < 0.05$). The mRNA levels of PGC-1β and genes involved in mitochondrial fatty acid catabolism (CACT, FATP1, OCTN2), citrate cycle (SDHA), oxidative phosphorylation (COX4/1, COX6A1), and thermogenesis (UCP3) in *M. longissimus dorsi* were greater in the niacin group than in the control group ($P < 0.05$).

Conclusions: The study demonstrates that niacin supplementation induces type II to type I muscle fiber switching, and thereby an oxidative metabolic phenotype of skeletal muscle in pigs. Given that oxidative muscle types tend to develop dark, firm and dry pork in response to intense physical activity and/or high psychological stress levels preslaughter, a niacin-induced change in the muscle´s fiber type distribution may influence meat quality of pigs.

Keywords: Niacin, Pig, Muscle fiber transition, Oxidative type I fiber

Background

Niacin, also called nicotinic acid, is a water-soluble vitamin which belongs to the vitamin B complex and is essential for the metabolism of carbohydrates, fats and many other substances. At pharmacological doses, niacin exerts pronounced lipid-lowering activities, particularly on triacylglycerols (TAG), but also on total cholesterol and LDL cholesterol [1], and, interestingly, niacin increases HDL cholesterol [2]. Besides these well-documented effects on blood lipid profile, high doses of niacin were also shown to cause profound, but less recognized changes in gene expression in several tissues [3]. In this regard it is worth mentioning, that studies in both, humans [4] and rats [5] revealed that high levels of niacin cause an up-regulation of transcription factors in skeletal muscles, like PPARγ coactivator-1α (PGC-1α) and PGC-1β, which are key regulators of fiber distribution in skeletal muscle [6,7]. In principle, two major fiber types of skeletal muscle can be distinguished: type II fibers, also called glycolytic fibers, which have few mitochondria and largely generate ATP through glycolytic metabolism, and type I fibers, also called oxidative fibers, which are mitochondria-rich and utilize mainly oxidative phosphorylation for

* Correspondence: klaus.eder@ernaehrung.uni-giessen.de
[1]Institute of Animal Nutrition and Nutrition Physiology, Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26-32, Giessen 35390, Germany
Full list of author information is available at the end of the article

energy production [8,9]. Remarkably, the type II to type I fiber distribution in a given muscles displays high plasticity and can be induced to switch depending on various factors, like exercise, mechanical unloading or obesity [10-13]. Since the muscle´s fiber distribution determines its metabolic phenotype, fiber switching induced by exercise, mechanical unloading or obesity results in a change of the functional and metabolic phenotype of skeletal muscle [10-13]. Based on the observation that niacin upregulates key regulators of fiber switching in skeletal muscle, it has been investigated whether niacin supplementation can prevent the obesity-induced muscle fiber switching from type I to type II and causes an elevation in the number of type I fibers in skeletal muscle of obese Zucker rats [5]. This study indeed showed that niacin prevents the obesity-induced muscle fiber switching from type I to type II and elevates the number of type I fibers in skeletal muscle of obese Zucker rats [5]. Corresponding to this niacin-induced increase in the muscle´s type I fiber content niacin supplementation to the obese Zucker rats caused the development of a more oxidative metabolic phenotype of skeletal muscle as evidenced by an increased expression of genes involved in mitochondrial fatty acid import and oxidation, citrate cycle, oxidative phosphorylation, mitochondrial biogenesis and angiogenesis [5]. This obvious improvement in the muscle´s capacity for oxidative utilization of fatty acids has likely contributed, at least partially, to the strong lowering effect of niacin on blood levels of TAG and non-esterified fatty acids (NEFA) in the obese Zucker rats [5], which are characterized by markedly elevated blood levels of TAG and NEFA.

It is currently unknown whether high levels of niacin also causes type II to type I muscle fiber switching in metabolically healthy animals. This question may be of particular interest in farm animals used for meat production like growing pigs because a change in the muscle´s fiber type distribution is expected to influence meat quality considering that several studies have reported that oxidative muscles with a high percentage of type I fibers have a lower glycolytic potential [14,15], a darker color [16,17] and a higher ultimate pH [14,16]. In addition, it was shown that oxidative muscle types tend to develop dark, firm and dry pork in response to intense physical activity and/or high psychological stress levels preslaughter [18]. Therefore, the present study aimed to investigate whether niacin supplementation causes type II to type I muscle fiber switching, thereby, resulting in an increased type I fiber percentage in skeletal muscle of growing pigs.

Methods

Animals, housing, and experimental design

The experiment was performed at the Institute of Animal Nutrition and Nutrition Physiology, University of Giessen, Germany. A total of 25 male, 11 wk old crossbred pigs (Danzucht × Pietrain) with an average body weight of 32.8 ± 1.3 (mean ± SD) kg were randomly allocated to two groups of 12 (control group) and 13 pigs (niacin group), respectively. The pigs were kept individually in pens in a room under controlled temperature at 23 ± 2°C and relative humidity at 55 to 60% with light from 06.00 to 18.00 hrs. Both groups of pigs received a nutritionally adequate commercial diet (RWZ-UNIVERSAL-START HE Press, RWZ, Köln, Germany) for growing pigs containing (in g/kg) wheat (226), barley (200), soybean meal (149), triticale (100), corn (100), wheat gluten (100), dried distiller´s grains (31), rapeseed meal (20), wheat bran (20), calcium carbonate (16.1), vegetable oil (10), sodium chloride (3.9), monocalcium phosphate (2), and vitamin-mineral premix (22). The vitamin-mineral premix provided 34 mg of niacin per kg diet, a dose which is sufficient to meet the niacin requirement of growing pigs [19]. In the niacin group, the commercial diet was supplemented with additional 750 mg of niacin (obtained from Lonza, Basel, Switzerland) per kg as a pharmacological dose. The diets and water were given ad libitum. The feeding experiment lasted 21 days. All experimental procedures were in strict accordance with the recommendations in the guidelines for the care and use of laboratory animals [20] and the Appendix A of European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. In accordance with article 4 par. 3 of the German Animal Welfare Law all animals were humanely killed for scientific purpose approved by the Animal Welfare Officer of the Justus-Liebig-University.

Sample collection

After 21 days the animals were slaughtered after a 12 h fasting period at a commercial slaughterhouse near by the Institute. Blood samples were taken into EDTA polyethylene tubes (Sarstedt, Nürnbrecht, Germany) and plasma was collected by centrifugation (1,100 × g; 10 min, 4°C). Samples from three different skeletal muscles [*M. longissimus dorsi* (LD), *M. quadriceps femoris* (QF), *M. gastrocnemius* (G)] were excised and samples were shock frozen with liquid nitrogen and stored at −80°C pending analysis.

Determination of type I and type II fiber percentages in skeletal muscle

Determination of type I and type II fiber percentages in skeletal muscle was carried out as recently described in detail [5]. In brief, 30 μm thick, serial cross sections were prepared using a cryostat microtome, mounted on cover slips and stained for myosin ATPase (mATPase) using a modified method of Hämäläinen and Pette [21].

Subsequently, the sections were analyzed by light microscopy (Leica DMI 6000B) for calculating the type I and type II fiber percentages.

Determination of TAG and NEFA concentrations in plasma

Concentrations of TAG and NEFA in plasma were determined by enzymatic reagent kits from Merck Eurolab (ref. 113009990314) and from Wako Chemicals (ref. RD291001200R), respectively.

Determination of nicotinic acid and nicotineamide concentrations in plasma

Concentrations of nicotinic acid and nicotineamide in plasma were determined by LC-MS/MS according to the method from Liu et al. [22].

RNA isolation, cDNA synthesis and qPCR analysis

RNA isolation, cDNA synthesis and qPCR analysis were performed as described recently in detail [23]. In brief, total RNA was extracted from 50–60 mg skeletal muscle aliquots using peqGOLD TriFast™ RNA Extraction reagent (Peqlab, Erlangen, Germany) according to the manufacturer´s protocol, and RNA concentration and purity were estimated from the optical density at 260 and 280 nm (Infinite 200 M microplate reader, Tecan, Männedorf, Switzerland). cDNA synthesis was carried out within one week after RNA isolation using dT18 primer and M-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany). qPCR analysis was performed using KAPA SYBR FAST qPCR Universal Mastermix (Peqlab, Erlangen, Germany) and gene-specific primer pairs which are listed in Table 1. Calculation of gene expression data and normalization by GeNorm normalization factor were carried out as described recently [23]. The normalization factor was calculated as the geometric mean of expression data of the three most stable out of five tested potential reference genes. Means and SD were calculated from normalized expression data for samples of the same treatment group. The mean of the group control group was set to 1 and mean and SD of the niacin group were scaled proportionally. Data on qPCR performance for genes measured in skeletal muscle are shown in Table 1.

Statistical analysis

Data were statistically analysed by one-way ANOVA using the Minitab Statistical Software (Rel. 13.0, State College, PA, USA). Means of the two groups were compared by Fisher's multiple range test. Means were considered significantly different for $P < 0.05$. Data presented are shown as means ± SD.

Results

Feed intake, body weight development, carcass weights and feed conversion ratios

Feed intake, initial and final body weights, total and daily body weight gain, carcass weights and feed conversion ratio did not differ between the control group and the niacin group (Table 2).

Concentrations of nicotinic acid and its metabolite nicotineamide in plasma

The plasma concentrations of nicotinic acid (NA) and its metabolite nicotineamide (NAM) were greater in the niacin group than in the control group (NA: < 5 ng/mL (limit of detection) vs. 32.0 ± 13.0 ng/mL; NAM: 0.34 ± 0.07 vs. 3.88 ± 2.02 μg/mL; control group vs. niacin group; $P < 0.05$).

Concentrations of TAG and NEFA in plasma

In order to assess whether lipid concentrations in plasma are influenced by niacin supplementation, we measured the concentrations of TAG and NEFA in plasma of the pigs. The plasma concentrations of both, TAG and NEFA were not different between the two groups of pigs (TAG: 0.51 ± 0.12 vs. 0.51 ± 0.10 mmol/L; NEFA: 0.47 ± 0.27 vs. 0.65 ± 0.21 mmol/L; control group vs. niacin group).

Fiber type distribution of different skeletal muscles

To study whether niacin supplementation causes type II to type I fiber switching, we determined the fiber type distribution in different skeletal muscles (LD, QF and G). The percentage number of type I fibers in all three muscles considered was greater in the niacin group than in the control group, whereas the percentage number of type II fibers was less in niacin group than in the control group ($P < 0.05$, Figure 1).

Transcript levels of genes encoding fiber-specific MHC isoforms and regulators of muscle fiber distribution in LD muscle

In order to explore whether the niacin-induced fiber switching is reflected by changes in the expression of fiber-specific MHC isoforms, we determined the transcript levels of different MHC isoforms, from which three isoforms exist in pigs, namely one type I isoform (MHCI encoded by MYH7) and two type II isoforms (MHCIIA encoded by MYH2, and MHCIIB encoded by MYH4), in LD muscle. In line with the decreased type II fiber percentage the transcript levels of MYH2 and MYH4, which are expressed in type II fibers, were significantly reduced ($P < 0.05$) or tended to be reduced ($P < 0.15$), respectively, in LD muscle in the niacin group compared to the control group (Table 3). The transcript level of the MHC isoform MYH7, which is expressed in

Table 1 Characteristics and performance data of primers used for qPCR

Gene	Forward primer (3′-5′) Reverse primer (5′-3′)	Product length (bp)	NCBI Genbank	Slope	$R^{2\#}$	Efficiency*
Reference genes						
ATP5G1	CAGTCACCTTGAGCCGGGCGA TAGCGCCCCGGTGGTTTGC	94	NM_001025218	−3.42	0.999	1.96
ACTB	GACATCCGCAAGGACCTCTA ACATCTGCTGGAAGGTGGAC	205	XM_003124280	−3.60	0.998	1.89
RPS9	GTCGCAAGACTTATGTGACC AGCTTAAAGACCTGGGTCTG	325	XM_003356050	−3.64	0.999	1.88
Target genes						
COX4/1	GTGGAACTGTACCGCCTGAA TTGTCGTAGTCCCACTTGGC	257	XM_003355730	−3.44	1.000	1.95
COX6A1	CTCAGCTCGCATGTGGAAGA GATGCGAAGATGGGGGTAGG	139	NM_001190221	−3.34	0.996	1.99
CACT/SLC25A20	GCAAAGCCCATTAGCCCTCT GAGCACATCCTCTGGGTGTT	235	XM_003483178	−3.21	0.988	2.05
PPARGC1A	TAAAGATGCCGCCTCTGACT TGACCGAAGTGCTTGTTCAG	168	NM_213963	−3.94	0.993	1.79
PPARGC1B	AAGTGCGGCTTCGTCACCTA GCTGTCGAAATCCATGGCTT	216	XM_003124093	−3.28	0.998	2.02
SLC22A5	TGCATTTGGCTACATGCTGC ATGATCACCTCAGCTTCCTG	174	XM_003123912	−3.76	0.995	1.85
SDHA	CTACGCCCCCGTCGCAAAGG AGTTTGCCCCCAGGCGGTTG	380	DQ402993	−3.24	1.000	2.03
MYH2	GGCCCTTTGATGCCAAGACA GGCCATGTCCTCGATCTTGT	188	NM_214136	−3.45	1.000	1.95
MYH4	GTGCCCTGCTGCCATCAATA TGCGTAACGCTCTTTGAGGT	363	NM_001123141	−3.53	1.000	1.92
MYH7	TGCCAGCTTGAGCCTCTTTC GTAGCGCTCCTTGAGGTTGT	380	NM_213855	−3.33	0.999	2.00
FATP1	GGTTCCAGCCTGTTGAATGT AACAAAACCTTGGTGCTTGG	275	NM_001083931	−3.44	0.990	1.95
UCP2	AGTGTGAGACCTGACGAAGC GCTTGACGGAGTCGTAGAGG	435	NM_214289	−3.64	0.996	1.88
UCP3	GCCACTTTGTCTCTGCCTTC CAAACATCACCACGTTCCAG	219	NM_214049	−3.49	0.998	1.93

#Coefficient of determination of the standard curve.
*The efficiency is determined by $[10^{(-1/\text{slope})}]$.

type I fibers, in LD muscle tended to be increased in the niacin group compared to the control group ($P < 0.15$, Table 3).

To elucidate the mechanisms underlying type II to type I fiber transition in skeletal muscle of pigs in response to niacin supplementation, we determined the transcript levels of two key regulators of muscle fiber transition, PGC-1α and PGC-1β, in LD muscle. The transcript level of PGC-1β in LD muscle was greater in the niacin group than in the control group ($P < 0.05$; Table 3). In addition, the transcript level of PGC-1α in LD muscle was numerically greater in the niacin group than in the control group but this effect was not significant ($P > 0.05$; Table 3).

Table 2 Feed intake, body weight gain, feed conversion ratio and carcass weight of pigs fed either a control diet or a diet supplemented with 750 mg niacin/kg diet for 3 wk

	Control	Niacin	*P* value
	n = 12	n = 13	(ANOVA)
Feed intake (kg/d)	2.14 ± 0.27	2.13 ± 0.26	0.838
Initial body weight (kg)	32.7 ± 1.3	32.9 ± 1.5	0.829
Final body weight (kg)	53.5 ± 2.4	53.7 ± 3.9	0.864
Total body weight gain (kg)	20.7 ± 2.2	20.9 ± 2.6	0.915
Daily body weight gain (kg)	0.99 ± 0.10	0.99 ± 0.13	0.915
Carcass weight (kg)	40.5 ± 1.9	41.0 ± 2.9	0.567
Feed conversion ratio (kg feed/kg weight gain)	2.21 ± 0.42	2.22 ± 0.42	0.899

Values are means ± SD.

Transcript levels of genes involved in fatty acid utilization, citrate cycle, oxidative phosphorylation and thermogenesis in LD muscle

Given that induction of PGC-1α and PGC-1β results in the induction of genes involved in mitochondrial fatty acid catabolism (CACT, FATP1, OCTN2), citrate cycle (SDHA), oxidative phosphorylation (COX4/1, COX6A1), and thermogenesis (UCP2, UCP3), we determined transcript levels of genes representing these pathways in LD muscle. The transcript levels of CACT, FATP1, OCTN2, SDHA, COX4/1, COX6A1 and UCP3 in LD muscle were greater in the niacin group than in the control group ($P < 0.05$; Table 4). The transcript level of UCP2 in LD muscle tended to be elevated in the niacin group compared to the control group ($P < 0.15$; Table 4).

Discussion

The main finding of the present study is that supplementation of a pharmacological niacin dose, similar with that recently used in Zucker rats (Pigs: 30–49 mg/kg body weight; Zucker rats: 40–54 mg/kg body weight [5]) in pigs causes type II to type I muscle fiber switching, thereby, resulting in an increased type I fiber percentage in skeletal muscle in comparison to pigs receiving a diet with a nutritionally adequate niacin concentration. In contrast to our study in Zucker rats, in which fiber distribution of only one muscle (M. *rectus femoris*) was studied, we analyzed fiber distribution of three different skeletal muscles (LD, QF and G) in the pigs in the present study. These muscles contained predominantly type II fibers but varied in their type II to type I fiber type ratios (control group: 4.6, 7.6 and 2.8 for LD, QF and G, respectively). We observed that niacin supplementation decreased this ratio in all three muscles considered (niacin group: 2.8, 2.6 and 1.6 for LD, QF and G, respectively) indicating that niacin exerts its effect on muscle fiber distribution independently of the muscle type, which extends our knowledge with regard to the effect of niacin supplementation on muscle fiber distribution. This effect was also reflected by a reduced expression of the type II fiber-specific transcript levels of MYH2 ($P < 0.05$) and MYH4 isoform ($P < 0.15$) and an increased expression of the type I fiber-specific isoform MYH7 ($P < 0.15$) in LD muscle of the niacin group compared to the control group.

Muscle fiber switching was reported to be initiated through the up-regulation of key regulators of muscle fiber distribution and muscle metabolic phenotype [6,24-26],

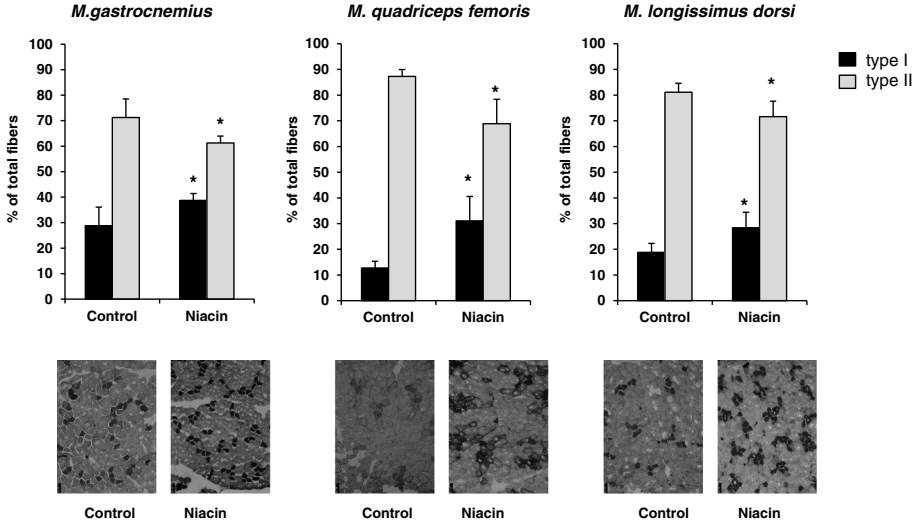

Figure 1 Muscle fiber type distribution of *M. gastrocnemius*, *M. quadriceps femoris* and *M. longissimus dorsi* of pigs fed either a control diet or a diet supplemented with 750 mg niacin/kg diet for 3 wk. Bars represent means ± SD, n = 12 (control) and 13 (niacin) pigs/group. Images from cross sections representing one animal per group are shown at the bottom. Asterisk denotes difference from control group, $P < 0.05$.

Table 3 Transcript levels of genes encoding fiber-specific MHC isoforms and regulators of muscle fiber distribution in LD muscle of pigs fed either a control diet or a diet supplemented with 750 mg niacin/kg diet for 3 wk

	Control	Niacin	*P* value
	n = 12	n = 13	(ANOVA)
	Relative mRNA level (fold of control)		
Fiber-specific MHC isoforms			
MYH7 (type I-specific)	1.00 ± 0.29	1.26 ± 0.42	0.139
MYH2 (type IIA-specific)	1.00 ± 0.40	0.65 ± 0.21	0.023
MYH4 (type IIB-specific)	1.00 ± 0.51	0.67 ± 0.27	0.086
Regulators of fiber distribution			
PGC-1α	1.00 ± 0.91	1.49 ± 0.98	0.309
PGC-1β	1.00 ± 0.24	1.33 ± 0.31	0.021

Values are means ± SD.

and we have recently shown that niacin supplementation causes an up-regulation of two of these key regulators, namely PGC-1α and PGC-1β, in rectus femoris muscle of rats [5]. Like in rats, we observed in the present study that the transcript level of PGC-1β was elevated in LD muscle of pigs of the niacin group. In addition, the transcript level of PGC-1α in LD muscle was also increased in pigs of the niacin group, even though this effect was not significant, which is attributed to the relatively high standard deviation of this parameter in both groups of pigs. PGCs regulate the muscle metabolic phenotype by binding to and activating a variety of nuclear receptors and additional transcription factors. For example, PGC-1α dramatically co-activates PPARα and/or PPARδ in various cell types and tissues and thereby induces the expression of genes involved in fatty acid catabolism and thermogenesis. Similarly, co-activation by PGC-1α and PGC-1β has also been shown for the myocyte enhancer factor 2 family of transcription factors, which stimulate specifically the expression of MHC genes from oxidative fibers [25,27], and for nuclear respiratory factor-1 and estrogen-related receptor α, which are required for oxidative phosphorylation and mitochondrial biogenesis [28]. In line with the up-regulation of key regulators of type II to type I fiber switching in LD muscle, we observed that pigs of the niacin group had elevated transcript levels of genes involved in mitochondrial fatty acid catabolism (CACT, FATP1, OCTN2), citrate cycle (SDHA), oxidative phosphorylation (COX4/1, COX6A1), and thermogenesis (UCP3) in LD muscle. All these genes are abundantly expressed in type I fibers, which is responsible for the oxidative metabolic phenotype and the preferred utilization of oxidative phosphorylation for energy production of type I fibers [8,29]. Thus, the abovementioned changes in gene expression in LD muscle of pigs of the niacin group are consistent with the niacin-induced increase of type I fiber content in LD muscle. It is currently unknown how niacin mediates the observed up-regulation of key regulators of skeletal muscle phenotype because the skeletal muscle does not express the niacin receptor. This suggests that the effect of niacin involves niacin receptor-independent mechanisms. In this context it noteworthy that niacin has been reported recently to induce several humoral changes, like increases in the plasma levels of epinephrine, corticosterone and glucagon [30]. In addition, niacin supplementation also causes an elevation in the plasma levels of growth hormone, adiponectin and leptin [31,32], all of which are well-documented to influence gene expression and cellular signaling in different tissues. Thus, future studies have to clarify whether these niacin-induced humoral changes are responsible for the observed muscle fiber switching.

Table 4 Transcript levels of genes involved in fatty acid utilization, citrate cycle, oxidative phosphorylation and thermogenesis in LD muscle of pigs fed either a control diet or a diet supplemented with 750 mg niacin/kg diet for 3 wk

	Control	Niacin	*P* value
	n = 12	n = 13	(ANOVA)
	Relative mRNA level (fold of control)		
Fatty acid utilization			
CACT	1.00 ± 0.43	1.55 ± 0.62	0.038
FATP1	1.00 ± 0.31	1.33 ± 0.27	0.020
OCTN2	1.00 ± 0.31	1.44 ± 0.44	0.018
Citrate cycle			
SDHA	1.00 ± 0.34	1.53 ± 0.58	0.037
Oxidative phosphorylation			
COX4/1	1.00 ± 0.28	1.40 ± 0.45	0.033
COX6A1	1.00 ± 0.36	1.57 ± 0.62	0.022
Thermogenesis			
UCP2	1.00 ± 0.63	1.53 ± 0.84	0.130
UCP3	1.00 ± 0.45	1.46 ± 0.49	0.036

Values are means ± SD.

In contrast to our recent study in obese Zucker rats [5] niacin supplementation did not induce the well-documented plasma TAG-lowering effect in pigs. The lack of effect, however, is probably not due to an insufficient niacin dose because the dose was similar as in our rat study [5] and the administered niacin dose caused a significant increase in plasma nicotinic acid and particularly nicotineamide levels indicating sufficient bioavailability. It is more likely that plasma TAG concentration of pigs was not lowered because it was yet within the normal range making a further reduction unlikely. In addition, in opposite to the well-documented antilipolytic effect of niacin [1] the plasma NEFA

concentration in pigs of the niacin group was also not reduced but even increased, at least numerically. This result, however, is in agreement with recent observations that chronic niacin administration for at least 2 weeks results in elevated plasma NEFA levels [33]. The basis for this rebound phenomenon on lipolysis during long-term niacin treatment is only incompletely understood, but recent findings indicate that niacin favors an increase in the net rate of lipolysis through reducing TAG synthesis and expression of perilipin in adipocytes [34].

Conclusions

The present study demonstrates that niacin supplementation induces type II to type I muscle fiber switching, and thereby an oxidative metabolic phenotype of skeletal muscle in pigs as a farm animal model. The observed up-regulation of key regulators of fiber distribution in skeletal muscle in response to niacin supplementation is likely causative for the induction of muscle fiber switching in pigs. Given that oxidative muscle types tend to develop dark, firm and dry pork in response to intense physical activity and/or high psychological stress levels preslaughter [18], a niacin-induced change in the muscle´s fiber type distribution may influence meat quality of pigs which would be worth of being investigated in future studies.

Abbreviations

CACT: Carnitine-acylcarnitine translocase; COX: Cytochrome c oxidase; FATP: Fatty acid transport protein; G: Gastrocnemius; HDL: High-density lipoprotein; LD: Longissimus dorsi; LDL: Low-density lipoprotein; MHC: Myosin heavy chain; MYH: Myosin heavy chain encoded gene; NA: Nicotinic acid; NAM: Nicotineamide; NEFA: Non-esterified fatty acids; OCTN: Novel organic cation transporter; PGC-1: Peroxisome proliferator-activated receptor-gamma coactivator-1; PPAR: Peroxisome proliferator-activated receptor; QF: Quadriceps femoris; SDHA: Succinate dehydrogenase subunit A; TAG: Triacylglycerol; UCP: Uncoupling protein.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MK conducted the animal experiment, performed fiber typing, PCR analyses, blood lipid analyses, statistical analyses, and wrote the manuscript, RR participated in the design and coordination of the study, supervised PCR analyses, and statistical analysis and helped to draft the manuscript, FCM and KK analysed data from muscle fiber typing, EM performed nicotinic acid and nicotineamide analyses in blood, KE conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Author details

[1]Institute of Animal Nutrition and Nutrition Physiology, Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26-32, Giessen 35390, Germany. [2]Department of Sports Medicine, Justus-Liebig-University Giessen, Kugelberg 62, Giessen 35394, Germany.

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Niacin supplementation induces type II to type I muscle fiber transition in skeletal muscle of sheep

Muckta Khan[1], Aline Couturier[1], Johanna F Kubens[1], Erika Most[1], Frank-Christoph Mooren[2], Karsten Krüger[2], Robert Ringseis[1] and Klaus Eder[1*]

Abstract

Background: It was recently shown that niacin supplementation counteracts the obesity-induced muscle fiber transition from oxidative type I to glycolytic type II and increases the number of type I fibers in skeletal muscle of obese Zucker rats. These effects were likely mediated by the induction of key regulators of fiber transition, PPARδ (encoded by PPARD), PGC-1α (encoded by PPARGC1A) and PGC-1β (encoded by PPARGC1B), leading to type II to type I fiber transition and upregulation of genes involved in oxidative metabolism. The aim of the present study was to investigate whether niacin administration also influences fiber distribution and the metabolic phenotype of different muscles [*M. longissimus dorsi* (LD), *M. semimembranosus* (SM), *M. semitendinosus* (ST)] in sheep as a model for ruminants. For this purpose, 16 male, 11 wk old Rhoen sheep were randomly allocated to two groups of 8 sheep each administered either no (control group) or 1 g niacin per day (niacin group) for 4 wk.

Results: After 4 wk, the percentage number of type I fibers in LD, SM and ST muscles was greater in the niacin group, whereas the percentage number of type II fibers was less in niacin group than in the control group ($P < 0.05$). The mRNA levels of PPARGC1A, PPARGC1B, and PPARD and the relative mRNA levels of genes involved in mitochondrial fatty acid uptake (CPT1B, SLC25A20), tricarboxylic acid cycle (SDHA), mitochondrial respiratory chain (COX5A, COX6A1), and angiogenesis (VEGFA) in LD, SM and ST muscles were greater ($P < 0.05$) or tended to be greater ($P < 0.15$) in the niacin group than in the control group.

Conclusions: The study shows that niacin supplementation induces muscle fiber transition from type II to type I, and thereby an oxidative metabolic phenotype of skeletal muscle in sheep as a model for ruminants. The enhanced capacity of skeletal muscle to utilize fatty acids in ruminants might be particularly useful during metabolic states in which fatty acids are excessively mobilized from adipose tissue, such as during the early lactating period in high producing cows.

Keywords: Niacin, Sheep, Muscle fiber transition, Oxidative type I fiber

Background

Pharmacological doses of niacin have long been known to lower the levels of blood lipids, especially triacylglycerols (TAG), but the mechanism underlying this effect is only incompletely understood. Even though it has been established that niacin inhibits lipolysis in adipocytes through binding to the niacin-receptor HCA_2 and thereby reduces the supply of non-esterified fatty acids (NEFA) for hepatic TAG synthesis [1], this effect can only insufficiently explain the lipid-lowering effect because blood NEFA levels even become elevated during long-term niacin treatment due to a strong rebound phenomenon on lipolysis while the TAG lowering effect remains [2]. However, less well-documented niacin treatment also causes significant changes in gene expression in other tissues than adipose tissue, like skeletal muscle [2], a tissue which due to its great mass is particularly important for whole body fatty acid utilization. Noteworthy, it has been recently shown in humans that niacin administration induces the expression of two transcription factors, peroxisome proliferator-activated receptor δ (PPARδ, encoded by PPARD) and PPARγ coactivator-1α (PGC-1α, encoded by PPARGC1A) in skeletal muscle [3]. Both transcription factors are key regulators of muscle fiber composition and the muscle's metabolic phenotype because they control

* Correspondence: klaus.eder@ernaehrung.uni-giessen.de
[1]Institute of Animal Nutrition and Nutrition Physiology, Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26-32, 35390 Giessen, Germany
Full list of author information is available at the end of the article

genes involved in muscle fiber switching, fatty acid utilization, oxidative phosphorylation, mitochondrial biogenesis and function [4,5], and angiogenesis [6]. Skeletal muscle contains two major types of muscle fibers which differ in their contractile proteins and their metabolic capacity [7]. The type II fibers ("glycolytic fibers") have a little number of mitochondria and largely generate ATP through glycolytic metabolism, whereas type I fibers ("oxidative fibers") are mitochondria-rich and thus utilize mainly oxidative phosphorylation [8,9]. Interestingly, the distribution of type I and type II fibers of skeletal muscles shows high plasticity and can be altered by diverse factors, such as exercise, mechanical unloading, obesity or diabetes, resulting in a change of the muscle's functional and metabolic phenotype [10-13]. In an attempt to study whether the induction of PPARδ and PGC-1α in skeletal muscle by pharmacological niacin doses leads to a change of muscle fiber distribution and the muscle's metabolic phenotype, we have previously tested the effect of niacin supplementation at a dose used for reduction of serum lipids in obese Zucker rats [14] and pigs [15]. Both studies revealed that niacin supplementation induces muscle fiber transition from type II to type I and increases the number of type I fibers in skeletal muscle [14,15]. Moreover, we found that the expression of genes involved in fatty acid transport, mitochondrial fatty acid import and oxidation, oxidative phosphorylation and angiogenesis and genes encoding PPARδ, PGC-1α and PGC-1β (encoded by PPARGC1B), which, like PGC-1α, is a key regulator of skeletal muscle's oxidative and contractile phenotype [16], in skeletal muscle is elevated by niacin treatment [14,15]. Thus, these findings suggest that niacin induces a change in the muscle metabolic phenotype which is indicative of an increased capacity of muscle for oxidative utilization of fatty acids and which might be useful during metabolic states where TAG and NEFA are strongly elevated, such as during early lactation in high producing dairy cows [17]. However, whether niacin treatment also causes type II to type I muscle fiber switching and increases the type I fiber content of skeletal muscles in ruminants has not been investigated yet. Thus, the present study aimed to investigate whether niacin administration at a pharmacological dose influences fiber distribution and the metabolic phenotype of different skeletal muscles in sheep as a model for ruminants. Niacin was administrated by drenching ensuring that the main part of the administrated niacin bypasses the rumen and reaches the small intestine.

Methods

Animals, housing, and experimental design

The experiment was located at the Research Station of the Institute of Animal Breeding and Genetics at the University of Giessen, Germany. A total of 16 male, 11 wk old Rhoen sheep with an average body weight of 29.6 ± 3.0 (mean ± SD) kg were randomly allocated to two groups of 8 sheep each (control group and niacin group). All sheep within one group were kept together in a barn on straw. All sheep received hay *ad libitum* and 1.5 kg concentrate per day and sheep. The hay contained (% of dry matter) 47.5% nitrogen-free extractable substances, 30.3% crude fiber, 7.0% crude protein, 6.1% crude ash and 1.1% crude fat. The concentrate (RWZ-Schaf 18 Uni Press, RWZ, Köln) consisted of (g/kg): Root pulp (250), wheat (200), dried distillers grains with solubles (120), wheat bran (104), wheat gluten feed (100), rapeseed extraction meal (100), soybean extraction meal (37), calcium carbonate (22), soy hulls (20), molasses (20), vinasse (10), monocalcium phosphate (8), sodium chloride (1.9), magnesium oxide (1.6) and a premix supplying vitamins and minerals (5.5; amounts of vitamins and minerals supplied per kg: vitamin A, 8,000 IE; vitamin D3, 1,000 IE; vitamin E, 65 mg; zinc, 40 mg as zinc sulfate monohydrate; manganese, 20 mg as manganese (II) sulfate monohydrate; selenium, 0.2 mg as sodium selenite; cobalt, 0.2 mg as cobalt (II) sulfate monohydrate; iodine, 0.1 mg as calcium iodate). According to the manufacturer's declaration the concentrate contained 10.6 MJ ME/kg and 18% crude protein. Additionally, sheep of the niacin group received 1 g niacin (obtained from Lonza, Basel, Switzerland) dissolved in 100 ml drinking water by drenching daily at eleven a.m. Sheep of the control group were given the same amount of drinking water by drenching without addition of niacin. Since the concentrate did not contain any supplemental niacin, the sheep of the control group received only the niacin contained in the hay and the feed components of the concentrate, from which no actual concentrations of niacin are available. Based on literature data, the niacin concentration in hay and concentrate is below 100 mg/kg dry matter [18]. The experimental period during which sheep were administered either no (control group) or 1 g niacin per day (niacin group) lasted for 4 wk. Water was given *ad libitum*. All experimental procedures were in strict accordance with the recommendations in the guidelines for the care and use of laboratory animals [19] and the Appendix A of European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. In accordance with article 4 par. 3 of the German Animal Welfare Law all animals were humanely killed for scientific purpose approved by the Animal Welfare Officer of the Justus-Liebig-University.

Sample collection

After 4 wk the animals were slaughtered at a commercial slaughterhouse located in the near of the Research Station. Blood samples were taken into EDTA polyethylene tubes (Sarstedt, Nürnbrecht, Germany) and plasma was

collected by centrifugation (1,100 × g; 10 min, 4°C). Samples from three different skeletal muscles [*M. longissimus dorsi* (LD), *M. semimembranosus* (SM), *M. semitendinosus* (ST)] were excised nearly at the same location and samples were shock frozen with liquid nitrogen and stored at −80°C pending analysis.

Muscle fiber typing

Fiber typing was performed as recently described in detail [14]. In brief, 30 μm thick, serial cross sections were taken using a cryostat microtome, mounted on cover slips and stained for myosin ATPase (mATPase) using a modified method of Hämäläinen and Pette [20]. In brief, sections were pre-incubated for 5 min in sodium acetate (54.3 mM) – sodium barbital (32.6 mM) solution adjusted with hydrogen chloride to pH 4.6. After washing, the sections were incubated for 30 min at 37°C in substrate solution (2.7 mM ATP, 100 mM glycin, 54 mM calcium (II) chloride, 100 mM sodium chloride, pH adjusted to 9.6). Following incubation in 1% calcium (II) chloride and 2% cobalt (II) chloride, a black insoluble compound was developed in 1% ammonium sulfide for 50 s leading to a black staining of type I fibers and grey staining of type II fibers. Subsequently, the sections were analyzed by light microscopy (Leica DMI 6000B) for calculating the type I and type II fiber percentages. Fiber typing was carried out in the best five images out of ten stained sections per muscle and animal, and all fibers within a 100 cm^2 area were calculated. This area corresponded to about 60 fibers. Thus, a total of 300 fibers were calculated per animal and muscle.

Determination of nicotinic acid and nicotinamide concentrations in plasma

Concentrations of nicotinic acid and nicotineamide in plasma were determined by LC-MS/MS according to the method from Liu et al. [21].

Determination of plasma lipids

The plasma concentrations of TAG and NEFA were measured using enzymatic reagent kits from Merck Eurolab (ref. 113009990314) and from Wako Chemicals (ref. RD291001200R), respectively.

RNA isolation and qPCR analysis

RNA isolation, cDNA synthesis qPCR analysis were performed as described recently in detail [22]. In brief, total RNA was isolated from 25–30 mg skeletal muscle aliquots using Trizol™ reagent (Invitrogen, Karlsruhe, Germany), and RNA concentration and purity were estimated from the optical density at 260 and 280 nm (Infinite 200 M microplate reader, Tecan, Männedorf, Switzerland). RNA integrity was assessed by confirming intact bands corresponding to the 18S and 28S ribosomal RNA subunits using 1% agarose gel electrophoresis. Following cDNA synthesis within one week after RNA isolation using dT18 primer and M-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany), qPCR analysis was performed as described recently in detail [22]. Features of gene-specific primer pairs are listed in Table 1. Calculation of gene expression data and normalization by GeNorm normalization factor were carried out as described recently [22]. The normalization factor was calculated as the geometric mean of expression data of the three most stable out of six tested potential reference genes (RPL19, YWHAZ, RPS26, MDH1, B2M, and GAPDH). In each muscle the three most stable reference genes were the same (the stability score M as calculated by GeNorm is shown in brackets): LD muscle: RPL19 (0.025), YWHAZ (0.026), and RPS26 (0.029); SM muscle: RPL19 (0.026), YWHAZ (0.028), and RPS26 (0.028); ST muscle: RPL19 (0.033), YWHAZ (0.037), and RPS26 (0.040). Means and SD were calculated from normalized expression data for samples of the same treatment group. The mean of the group control was set to 1 and mean and SD of the niacin group were scaled proportionally. Data on qPCR performance for target and reference genes measured in skeletal muscle are shown in Table 2.

Immunoblotting

Preparation of homogenates, determination of protein concentration and immunoblotting were performed as described recently in detail [23]. In brief, proteins were separated by 12,5% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with primary antibodies against PGC-1α (dilution 1:1000; polyclonal anti-PGC-1α antibody; Millipore, Temecula, CA), PPARδ (dilution 1:1000; polyclonal anti-PGC-1α antibody; Abcam, Cambridge, UK), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (dilution 1:5000; monoclonal anti-GAPDH antibody, Abcam, Cambridge, UK) as a reference protein. Nitrocellulose membranes were washed, and subsequently incubated with a horseradish peroxidase conjugated secondary monoclonal anti-mouse-IgG antibody (Abcam, Cambridge, UK) for GAPDH and polyclonal anti-rabbit-IgG antibody (Sigma-Aldrich, St. Louis, Germany) for PGC-1α, and PPARδ at room temperature. Finally, blots were developed by either ECL Select or ECL Prime (both GE Healthcare, Munich, Germany), respectively, and the intensities of the specific bands detected with a Bio-Imaging system (Syngene, Cambridge, UK) and quantified by Syngene GeneTools software (nonlinear dynamics).

Statistics

Data were statistically analysed by Student's t-test using the Minitab Statistical Software (Rel. 13.0, State College, PA, USA). Means were considered significantly different for $P < 0.05$. Data presented are shown as means ± SD.

Table 1 Characteristics of primers used for qPCR

Gene	Forward primer (3′-5′)	Reverse primer (5′-3′)	Product length (bp)	T_m (°C)	NCBI Genbank
Reference genes					
B2M	GCGTATTCCAGAGGTCCAGG	CGGCAGCTGTACTGATCCTT	234	60	NM_001009284
GAPDH	GGCGTGAACCACGAGAAGTA	GCAGGGATGATGTTTTGGGC	227	60	AF022183
MDH1	TACGTGTTCCCTGGAGTTGC	TGCTTCCTTGTTTGGGGGTT	249	57	NM_001135220
RPL19	AGCCTGTGACTGTCCATTCC	TTCTCGGGCATTCGAGCATT	118	57	JN811679
RPS26	ACAACGGTCGTGCCAAAAAG	AAATCGGGGTGGAGGTGTTC	284	57	NM_001009435
YWHAZ	AGACGGAAGGTGCTGAGAAA	TGGGGATCAAGAACTTTTCCAA	120	57	JN811681
Target genes					
COX5A	GCTCGCTGGGTGACATACTT	ACCTCTAGGATGCGAACTGC	173	60	AF233074
COX6A1	TGCAGCTGAGTCGGTGTATG	GAACTCGGGTCTCTCCTCCT	161	60	GU585577
CPT1B	GACGTTTCCATGGGACTGGT	GCCAGCGTCTCCATTCGATA	389	60	NM_001009259
MHCI	TCGTCAAGGCCACAATTTTG	CTGTCGCAACACCTGGTCCT	100	60	AB058898
MHCIIA	AAGCCTTTTGATGCCAAGACAT	TTCACCGTCACTTTCCCACC	100	60	AB058896
MHCIIX	CTTCGTGGCGGACCCTAAG	CAGTTACTGTCGCCCCAGCT	100	60	AB058897
PPARD	TCAGCGTGCACGTCTTCTAC	CAGGAATTCCCGGGTGACAA	230	59	XM_004018769
PPARGC1A	GGTGACCATGACTATTGTCAG	CTCGGATTTCCTGGTCTTGAA	216	58	XM_004009738
PPARGC1B	CTGGACCGAGTTCTCCATCC	CACGTGCCCTTTCACCTGCA	244	61	XM_004008965
SDHA	GTTTGAGCAGCACTGGAGGA	AGTCGGTCTCGTTCAAAGTCC	110	60	DQ386895
SLC25A20	CCGAGGGATCTACAAGGGGA	CCTTCATCCCGGATCAGCTC	288	61	NM_001127277
VEGFA	GGACATCTTCCAGGAGTACC	GCATGGTGATGTTGAACTCCT	137	58	EU857623

Table 2 qPCR performance data

Gene	Slope	$R^{2\#}$	Efficiency*
B2M	−3.20	0.999	2.05
COX5A	−3.22	0.999	2.04
COX6A1	−2.99	0.997	2.16
CPT1B	−3.79	0.996	1.84
GAPDH	−2.97	0.999	2.17
MDH1	−3.21	0.999	2.05
MHCI	−3.37	1.000	1.98
MHCIIA	−3.21	0.998	2.05
MHCIIX	−3.29	0.999	2.01
PPARD	−3.05	0.967	2.13
PPARGC1A	−3.34	0.999	1.99
PPARGC1B	−3.29	0.956	2.01
RPL19	−3.31	0.997	2.00
RPS26	−3.72	0.998	1.86
SDHA	−3.12	0.999	2.09
SLC25A20	−3.81	0.980	1.83
VEGFA	−3.31	0.997	2.00
YWHAZ	−3.34	0.993	1.99

#Coefficient of determination of the standard curve.
*The efficiency is determined by $[10^{(-1/\text{-slope})}]$.

Results

Final body weight, body weight gain and carcass weight

Final body weights, daily body weight gain and carcass weights did not differ between the control group and the niacin group (Final body weight: 37.4 ± 2.3 vs. 37.8 ± 3.7 kg; daily body weight gain: 308 ± 50 vs. 308 ± 41 g; carcass weight: 17.2 ± 1.4 vs. 17.3 ± 2.4 kg; control group vs. niacin group; n = 8/group).

Concentrations of nicotinic acid and its metabolite nicotinamide in plasma

The plasma concentrations of nicotinic acid and its metabolite nicotinamide were greater in the niacin group than in the control group (nicotinic acid: 0.41 ± 0.31 vs. 0.75 ± 0.42 μg/mL; nicotinamide: 0.46 ± 0.25 vs. 3.42 ± 0.90 μg/mL; control group vs. niacin group; $P < 0.05$).

Lipid concentrations in plasma

In order to assess the lipid-lowering properties of niacin in sheep, we determined the plasma concentrations of NEFA and TAG. The plasma TAG concentration tended to be lower in the niacin group than in the control group (0.20 ± 0.02 vs. 0.17 ± 0.03 mmol/L; control group vs. niacin group; $P < 0.1$). The plasma NEFA concentration did not differ between the niacin and the control group (0.32 ± 0.11 vs. 0.29 ± 0.14 mmol/L; control group vs. niacin group).

Muscle fiber type composition and expression of fiber-specific myosin heavy chain (MHC) isoforms in skeletal muscles

To evaluate an effect of niacin on fiber type distribution, muscle fiber typing and transcript level measurement of fiber-specific MHC isoforms was carried out. As shown in Figure 1A, the percentage number of type I fibers in LD muscle, SM muscle and ST muscle was greater in the niacin group than in the control group, whereas the percentage number of type II fibers was less in niacin group than in the control group ($P < 0.05$). The PCR primers used to detect transcript levels of sheep MHC isoforms corresponding to MHCI, MHCIIA and MHCIIX have been designed based on available sheep partial-length cDNA sequences [24]. Specific PCR primers for sheep MHCIIB transcripts could not be designed because no sheep MHCIIB cDNA sequence is available in nucleic acid databases. As shown in Figure 1B, the mRNA level of type I-specific MHCI in SM muscle and ST muscle was increased in the niacin group compared to the control group ($P < 0.05$). In LD muscle, the mRNA level of MHCI was numerically greater in the niacin group relative to the control group ($P = 0.24$). The mRNA level of MHCIIA was decreased in LD muscle and SM muscle of the niacin group compared to the control group ($P < 0.05$), but did not differ between groups in ST muscle. The mRNA level of MHCIIX in LD muscle and SM muscle was significantly less ($P < 0.05$) and tended to be less ($P < 0.15$),

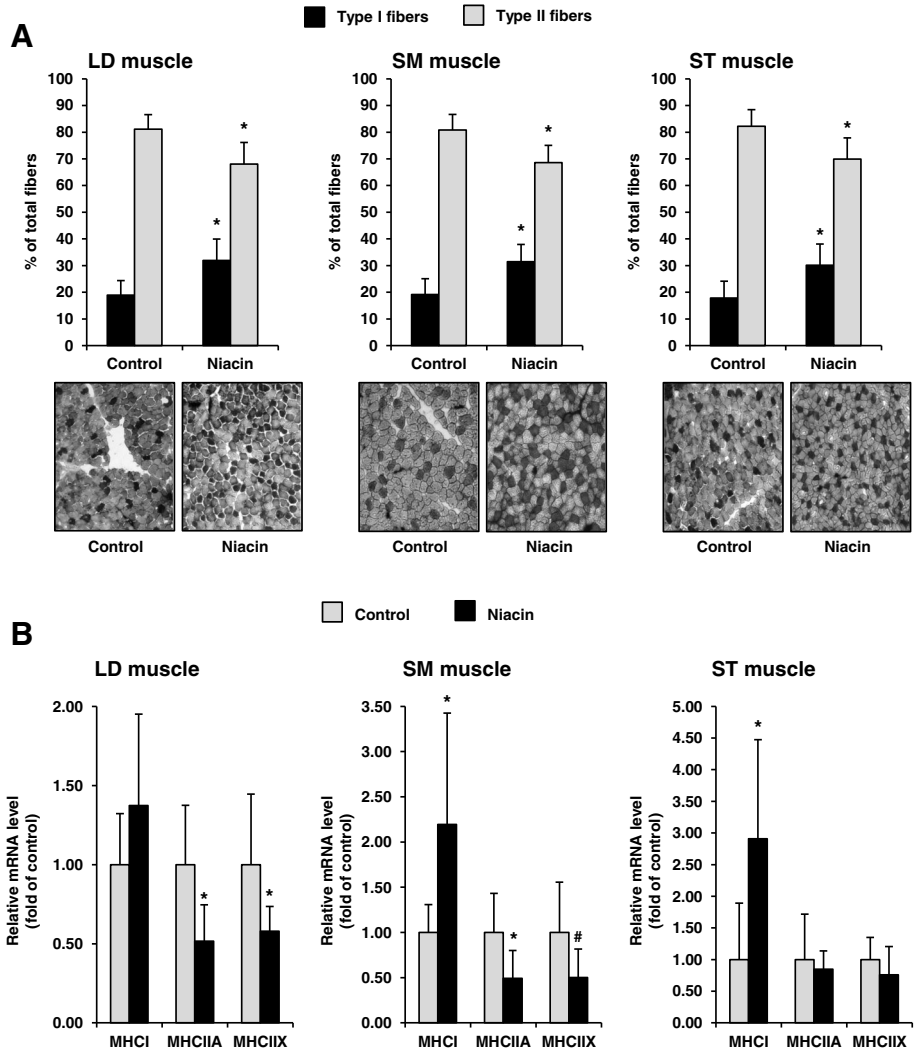

Figure 1 Muscle fiber type distribution (A), and relative mRNA levels of fiber-specific MHC isoforms (B) of LD muscle, SM muscle, and ST muscle of sheep administered either no (control group) or 1 g niacin per day (niacin group) for 4 wk. Bars represent means ± SD, n = 8 sheep/group. Representative images from fiber typing for each group are shown below the graph showing fiber type distribution, with the "black" areas being the type I fibers and the "grey" ones being the type II fibers. *different from control group, $P < 0.05$, #different from control group, $P < 0.15$.

respectively, in the niacin group than in the control group. In ST muscle, the mRNA level of MHCIIX was not different between groups.

Expression of key regulators of muscle fiber transition in skeletal muscles

To explore the mechanisms underlying the niacin-induced muscle fiber transition we determined mRNA and/or protein levels of the key regulators of muscle fiber transition, PGC-1α, PGC-1β and PPARδ, in the three muscles. The mRNA level of PPARGC1A in all three muscles was greater in the niacin group than in the control group ($P < 0.05$; Figure 2). The mRNA level of PPARGC1B was greater in LD muscle ($P < 0.05$) and tended to be greater in SM muscle and ST muscle ($P < 0.15$) of the niacin group than in the control group (Figure 2). The mRNA level of PPARD was increased in LD muscle and ST muscle ($P < 0.05$) and tended to be increased in SM muscle ($P < 0.15$; Figure 2). The protein level of PGC-1α was elevated in LD muscle and SM muscle of the niacin group compared to the control group ($P < 0.05$), but did not differ in ST muscle between groups (Figure 2). The protein level of PPARδ in all three muscles did not differ between groups.

Expression of genes involved in fatty acid oxidation, mitochondrial respiratory chain and angiogenesis in skeletal muscles

Since PGC-1α and PPARδ are important regulators of genes involved in fatty acid oxidation, mitochondrial respiratory chain and angiogenesis, we determined mRNA levels of CPT1B and SLC25A20, which encode two enzymes of the carnitine shuttle system, SDHA, which encodes the tricarboxylic acid cycle (TCA) enzyme succinate dehydrogenase, COX6A1 and COX5A, which encode two subunits of the respiratory chain complex IV (cytochrome c oxidase), and VEGFA encoding the angiogenic factor VEGF-a. Relative mRNA levels of COX5A, COX6A1, VEGFA, CPT1B, and SLC25A20 in all three muscles were greater in the niacin group than in the control group ($P < 0.05$; Figure 3). In addition, the relative mRNA level of SDHA in SM muscle and ST muscle was greater in the niacin group than in the control group ($P < 0.05$; Figure 3). In LD muscle, the mRNA level of SDHA tended to be greater in the niacin group than in the control group ($P < 0.15$; Figure 3).

Discussion

In the present study we tested the hypothesis that, like in rats and pigs [14,15], niacin supplementation induces muscle fiber transition from type II (glycolytic) to type I (oxidative), and thereby an oxidative metabolic phenotype of skeletal muscle in sheep as a ruminant model. The dietary niacin dosage (1 g niacin per day) given to the sheep related to 27–35 mg/kg body weight which is only slightly below that given to the rats (40–54 mg/kg body weight [14]) and pigs (30–49 mg/kg body weight [15]) in our recent studies and which was shown to induce a muscle fiber switch from type II to type I in skeletal muscle. The niacin dosage administered by drenching to the sheep of the niacin group was markedly higher than that taken up from the feed ration (hay and concentrate) by the sheep of the control group, because according to literature data the native concentration of niacin in hay and the main components of the concentrate is below 100 mg/kg dry matter [18]. In line with this, the niacin administration to the sheep caused a significant increase in the plasma concentration of the nicotinic acid metabolite nicotinamide. In addition, it has to be considered that the sheep used in this study had already fully developed rumen. This means that the niacin requirement for the sheep was covered from niacin synthesized by the rumen microbes and that the niacin from the ingested hay and concentrate was largely degraded by rumen microbes [25]. In contrast, the drenching procedure, which was used to administer the daily niacin bolus, is a suitable approach to ensure that the main part of the administered niacin bypasses the rumen and reaches the small intestine. In the present study, we considered three different skeletal muscles, LD, SM and ST, containing predominantly type II fibers (the type II fiber percentage in all three muscles in the control group was approximately 81%), because we expected an effect of niacin only in skeletal muscles with a high percentage of type II fibers. The main finding of the present study is that supplementation of niacin induces muscle fiber switching also in skeletal muscles of sheep. Muscle fiber typing revealed that the type I fiber percentage in the three muscles investigated increased from approximately 18–20% in the control group to 30–31% in the niacin group, whereas the type II fiber percentage decreased from 81% to 69%. In line with this, we observed that the mRNA level of the type I-specific MHCI was significantly greater in SM muscle and ST muscle and tended to be greater in LD muscle, but the mRNA levels of type II-specific MHC isoforms in LD and SM muscle were less in the niacin group than in the control group.

Regarding that muscle fiber transition is induced on the molecular level by an increased activity of PGC-1α, PGC-1β and PPARδ [4,5,26,27], we determined the mRNA and/or protein levels of these key regulators in the three muscles. We found that the mRNA level of PPARGGC1A in all three muscles was markedly elevated, and the mRNA levels of PPARGC1B and PPARD in all three muscles were either significantly increased or tended to be increased in the niacin group compared to the control group. In addition, the protein level of PGC-1α in two of three muscles was greater in the niacin than

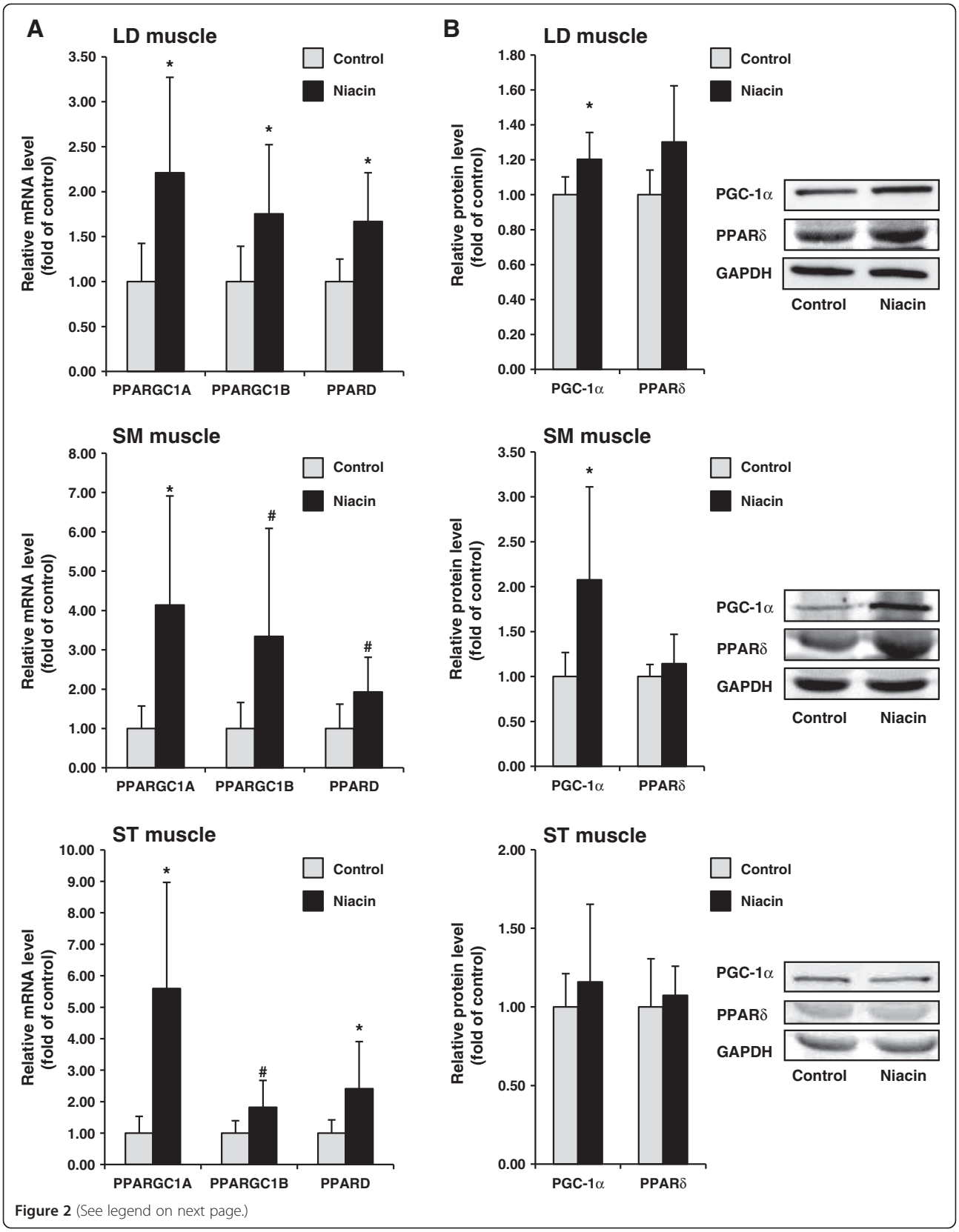

Figure 2 (See legend on next page.)

(See figure on previous page.)
Figure 2 Relative mRNA levels of PPARGC1A, PPARGC1B and PPARD (A), and relative protein levels of PGC-1α and PPARδ (B) in LD muscle, SM muscle, and ST muscle of sheep administered either no (control group) or 1 g niacin per day (niacin group) for 4 wk. Bars represent means ± SD, n = 8 (mRNA) and 6 (protein) sheep/group. Representative immunoblots specific to PGC-1α, PPARδ and GAPDH as internal control are shown for one animal per group; immunoblots for the other animals revealed similar results. *different from control group, $P < 0.05$, #different from control group, $P < 0.15$.

in the control group, whereas the protein level of PPARδ in all muscles was not different between groups. The PGC-1β protein level could not be determined, because no appropriate antibody to reliably detect PGC-1β was available. We cannot definitely explain the lack of effect of niacin on PPARδ protein levels, but this may be due to the comparatively small sensitivity of the western blotting technique making it difficult to detect slight differences between groups. However, the unaltered protein level of PPARδ does not exclude that its DNA-binding

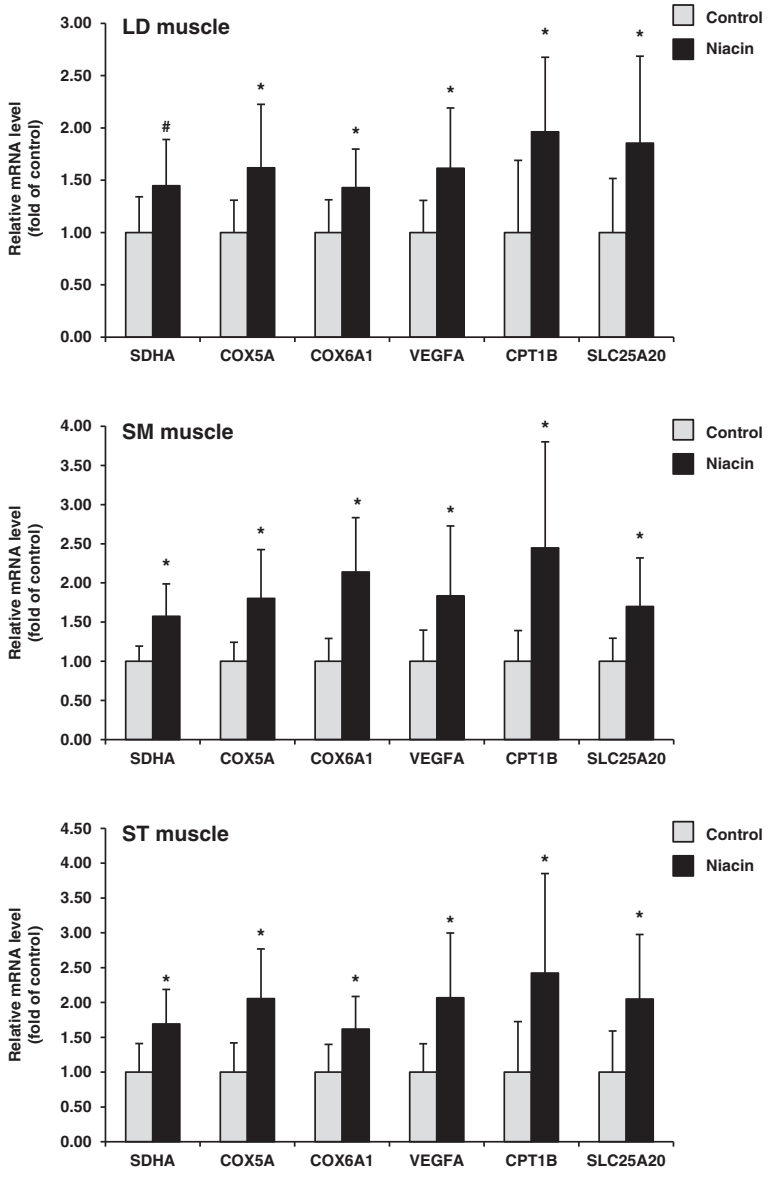

Figure 3 Relative mRNA levels of SDHA, COX5A, COX6A1, VEGFA, CPT1B, and SLC25A20 in LD muscle, SM muscle and ST muscle of sheep administered either no (control group) or 1 g niacin per day (niacin group) for 4 wk. Bars represent means ± SD for n = 8 sheep/group. *different from control group, $P < 0.05$; #different from control group, $P < 0.15$.

activity was increased because it is known that PGC-1α and PGC-1β, whose genes expression was clearly increased, act as coactivators of PPARδ and enhance the transactivation activity of PPARδ [28]. Therefore, our finding suggests that niacin supplementation increases the transcriptional activity of these critical regulators of muscle fiber transition, and thus provides an explanation for the increased type I fiber content in skeletal muscles of niacin-treated sheep.

Type I fibers, also called slow-twitch oxidative fibers, contain a high number of mitochondria, have a high oxidative capacity, and preferentially use fatty acids for energy production [8,9]. This oxidative metabolic phenotype of type I fibers is the consequence of a markedly higher expression of genes involved in fatty acid transport and uptake, β-oxidation, carnitine shuttle, TCA cycle and respiratory chain compared to glycolytic type II fibers [26,27]. In addition, type I fibers exhibit a higher expression of angiogenic factors, like VEGFA, which favors the preferential use of fatty acids by type I fibers because angiogenic factors increase capillary density and thereby blood perfusion but also the expression of fatty acid transport proteins [29]. In the present study we could demonstrate that several genes encoding proteins involved in oxidative metabolism (SDHA, COX5A, COX6A1, VEGFA, CPT1B, SLC25A20) were up-regulated in the muscles of the niacin group compared to the control group which is in line with the niacin-induced changes in fiber type distribution and expression of MHC isoforms. Although we did not provide data showing that the increased expression of oxidative genes is also accompanied by an enhanced activity of the encoded enzymes and an elevated capillary density, we suggest that the niacin-induced changes in skeletal muscle mRNA levels are indicative of an improved oxidative capacity because it is well known that the changes in the muscle's metabolic and contractile phenotype are induced at the transcriptional level through an enhanced activity of PGC-1α and PPARδ [26,27].

Conclusions

The results of this study show that niacin supplementation in sheep as a model for ruminants induces muscle fiber transition from type II (glycolytic) to type I (oxidative) being indicative of a change of the muscle's metabolic phenotype towards a more oxidative one. An enhanced capacity of skeletal muscle to utilize fatty acids in ruminants might be particularly useful during metabolic states in which fatty acids are extensively mobilized from adipose tissue, such as during the early lactating period in high producing cows. In addition, considering that several studies have reported that oxidative muscles with a high percentage of type I fibers have a lower glycolytic potential, a darker color and a higher ultimate pH [30-32], the niacin-induced change in the muscle's fiber type distribution may influence meat quality. At least in pigs it was demonstrated that oxidative muscle types tend to develop dark, firm and dry pork in response to intense physical activity and/or high psychological stress levels preslaughter [33]. Thus future studies have to investigate whether niacin administration influences meat quality from sheep.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MK conducted the animal experiment, performed fiber typing, PCR analyses, blood lipid analyses and statistical analyses, and wrote the manuscript. AC and JFK performed immunoblotting. EM performed nicotinic acid and nicotinamide determination in blood. FCM and KK analysed data from muscle fiber typing. RR supervised PCR analyses, immunoblotting and statistical analysis and helped to draft the manuscript. KE conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Author details

[1]Institute of Animal Nutrition and Nutrition Physiology, Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26-32, 35390 Giessen, Germany. [2]Department of Sports Medicine, Justus-Liebig-University Giessen, Kugelberg 62, 35394 Giessen, Germany.

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4. DISCUSSION

The overall aim of the present thesis was to test the hypothesis that, like in obese Zucker rats (Ringseis *et al*., 2013) NA supplementation induces muscle fiber transition from type II (glycolytic) to type I (oxidative), and thereby increases an oxidative metabolic phenotype of skeletal muscle in pig as a non-ruminant model and sheep as a ruminant model of farm animals. Although NA is only necessary in minor quantities to meet metabolic requirements (NRC, 2001) for the normal functioning of the body, pharmacological doses of NA have the capability to inhibit lipolysis (Pires *et al*., 2007). NA has been shown to reduce total cholesterol and LDL cholesterol levels by an average of 20 to 30%, TAG levels by 35 to 55%, and increase HDL cholesterol levels by 20 to 35% (Berge et *al*., 1961; Rivin, 1962; Knopp *et al*., 1985; Alderman *et al*., 1989). These findings have led to the use of high doses of NA, for the treatment of hypertriglyceridemia/hypercholesterolemia for more than five decades (Bodor and Offermanns, 2008). The lipid-lowering effects of NA have been extensively investigated, and traditionally attributed to its antilipolytic effect in adipocytes (Carlson and Hanngren, 1964; Lukasova *et al*., 2011), the prevailing mechanisms underlying the antidyslipidemic effects of NA are shown in **Figure 2**. NA can decrease the concentration of FFA and BHBA in blood thus reducing the incidence of ketosis and fatty liver in lactating dairy cows by inhibiting TAG lipolysis (Schwab *et al*., 2005; Pires and Grummer, 2007; Pires *et al*., 2007). However, the circulating FFA level rebound due to long-term NA treatment even though its lipid-lowering effects persists (Jin *et al*., 1997; Wang *et al*., 2001; de Grooth *et al*., 2004; Ganji *et al*., 2004; Lamon-Fava *et al*., 2008; Hernandez *et al*., 2010; Choi *et al*., 2011). Thus, in spite of a long history of clinical use, the precise mechanism by which NA lowers circulating lipids, remains unclear. Nevertheless, it does not appear to be related to vitamin coenzyme actions because NAM does not have a similar effect (Altschul *et al*., 1955; Carlson, 2005). In search for a novel mechanism underlying lipid-lowering effect of NA, in the present thesis studies, it has been demonstrated for the first time that NA induces muscle fiber transition from type II (glycolytic) to type I (oxidative) in metabolically healthy growing pig and sheep. The diagrammatical picture of potential mechanisms underlying the lipid-lowering effect of NA is presented in **Figure 3**.

In the present studies, the dietary NA doses given to pigs, related to 30-49 mg/kg body weight and to sheep, related to 27-35 mg/kg body weight, which is only slightly below that given to Zucker rats in the recent study (40-54 mg/kg body weight) (Ringseis *et al*., 2013), and which was shown to induce a muscle fiber switch from type II to type I in *rectus femoris* muscle of Zucker rats. Doses of NA of the Zucker rats study chosen were based on amounts

of NA used for the reduction of serum lipids in humans, typically in the range of 2-6 g/d (Rosen *et al*., 1987), which relates to ~30–90 mg/kg body weight for an individual weighing 70 kg. Similar doses of NA were also used in epidemiological studies with diabetic humans, in which up to 2.5 g of NA/d were administered for a 70 kg body weight person (Meyers and Kashyap, 2004).

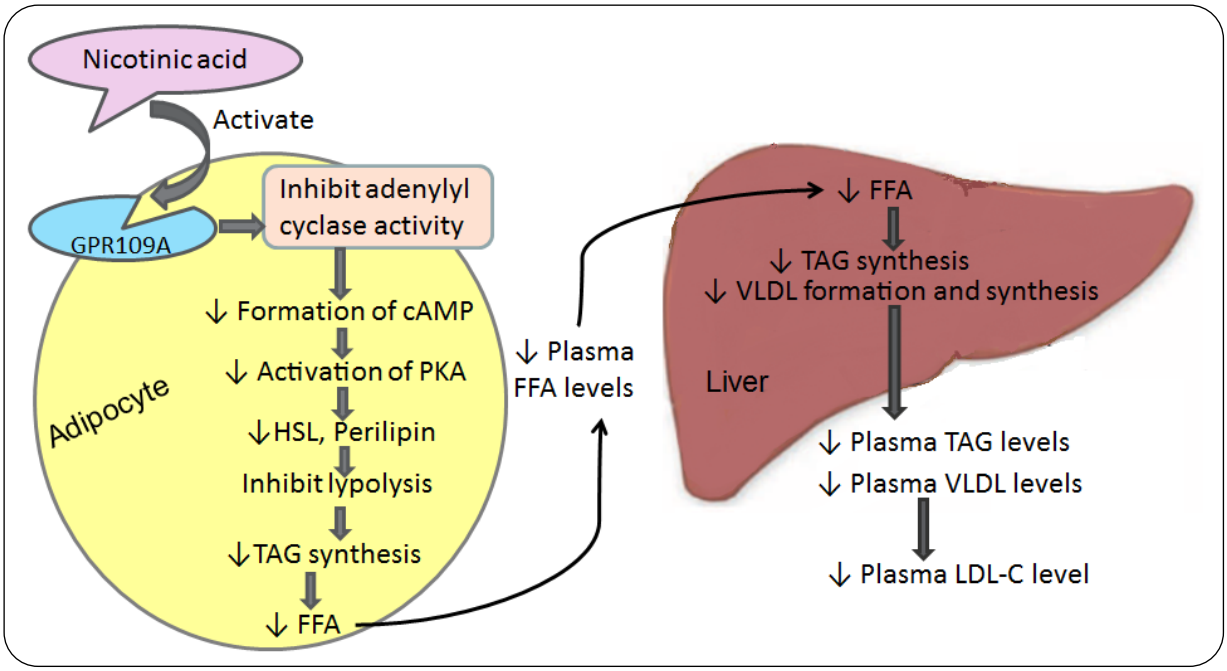

Figure 2: Prevailing mechanisms underlying the antidyslipidemic effects of nicotinic acid. Nicotinic acid binds to the nicotinic acid receptor GPR109A in adipose tissue. Activation of GPR109A by nicotinic acid leads to inhibition of adenylyl cyclase activity and, consequently decreases cAMP levels. Decreased cAMP levels in adipocytes leads to inactivation of PKA and accordingly decreases phosphorylation of HSL and perilipin, which are obligatory enzymes for TAG hydrolysis. Reducing these enzyme activities leads to decrease of TAG synthesis, and consequently reduction of FFA production. The decreased plasma FFA levels, induced by nicotinic acid, results in a substrate shortage for hepatic TAG synthesis. Accordingly, less TAG and VLDL are produced in liver, and thus, plasma levels of TAG and VLDL as well as LDL-C are dropped. cAMP, cyclic adenosine monophosphate; FFA, free fatty acid; GPR109A, G protein-coupled receptor 109A; HSL, hormone-sensitive lipase; LDL-C, low-density lipoprotein cholesterol; PKA, protein kinase A; TAG, triacylglycerols; VLDL, very-low-density lipoprotein; ↓, decrease. (Adapted from Lukasova *et al*., 2011).

In contrast to the recent study in Zucker rats (Ringseis *et al*., 2013), in which fiber distribution of only one muscle (*M. rectus femoris*) was studied, the present studies analyzed three different skeletal muscles in pigs (*M. longissismus dorsi*, LD; *M. quadriceps femoris*, QF; *M.*

gastrocnemius, G) in **study 1** and in sheep (LD; *M. semimembranosus*, SM; *M. semitendinosus*, ST) in **study 2**. All of these muscles contained predominantly type II fibers (the type II fiber percentage in LD, QF and G muscles in the control group pigs were 81%, 87% and 71%, respectively; in LD, SM, and ST muscles in the control group sheep were 81%, 81% and 82%, respectively), because an effect of NA was expected only in skeletal muscles with a high percentage of type II fibers. A similar fiber type composition was also found in rats in the study of Delp and Duan (1996). As expected, muscle fiber typing revealed that the type I fiber percentage in all three muscles investigated increased from approximately 13-29% in pigs of the control group up to 28-39% in pigs of the NA group, whereas the type II fiber percentage decreased from 71-87% to 61-72% in **study 1**. In **study 2**, the type I fiber percentage in all three muscles studied increased from about 18-19% in sheep of the control group up to 30-32% in sheep of the NA group, whereas the type II fiber percentage decreased from 81-82% to 68-70%. This suggests that the effect of NA on muscle fiber distribution is largely independent of the muscle type, providing that the muscle contains a sufficient number of type II fibers.

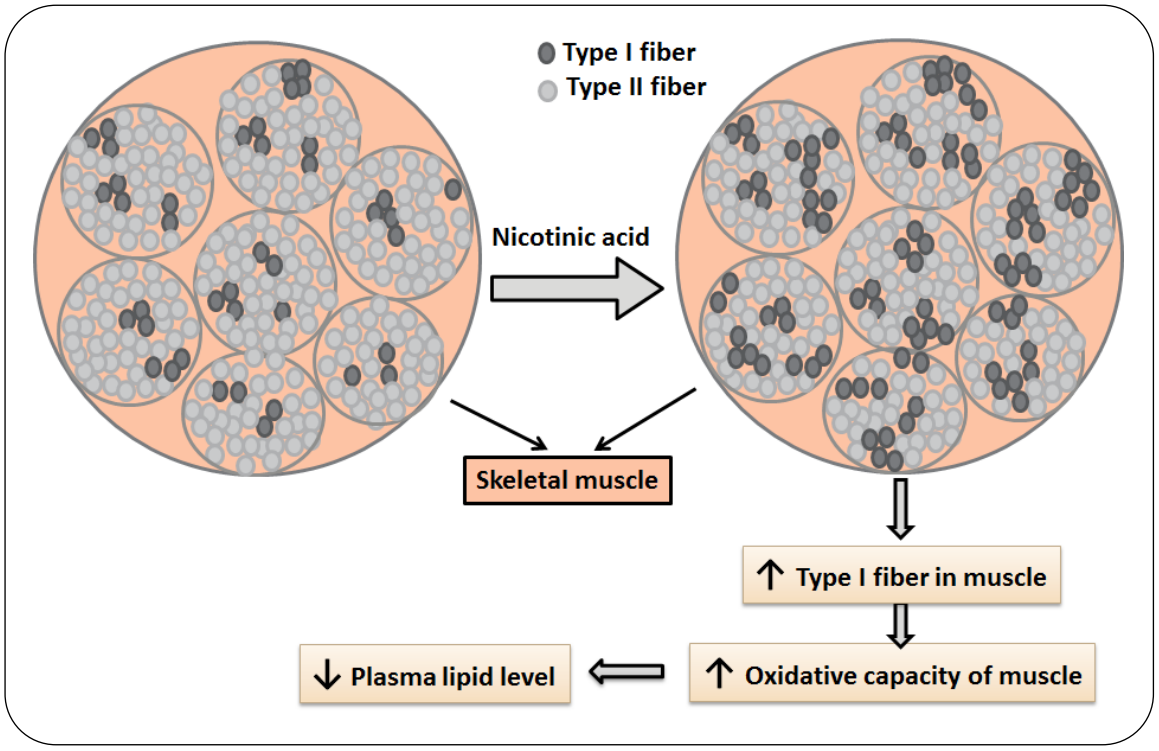

Figure 3: Diagrammatical picture of potential mechanisms underlying the lipid-lowering effect of nicotinic acid. Administration of nicotinic acid induces type II to type I muscle fibers transition in skeletal muscle, which increases the whole body oxidative capacity, and ultimately decreases the level of plasma lipids. ↑, increase; ↓, decrease.

Myosin is one of the most abundant proteins in the body and is essential for the body movement. It is a large protein molecule, which is responsible for muscle contraction, composed of six amino acid chains: two myosin heavy chains (MHC) and four myosin light chains (MLC) (Sciote and Morris, 2000). Skeletal muscle fiber can be distinguished by their MHC isoforms. In line with this, it was observed that the NA-induced fiber switching is also reflected by changes in the transcript levels of genes encoding fiber-specific MHC isoforms. Relative mRNA level of the type I fiber-specific MHCI isoform (encoded by MYH7 gene) tended ($P < 0.15$) to be higher and type II fiber-specific MHCIIA isoform (encoded by MYH2 gene), and MHCIIB isoform (encoded by MYH4 gene) were reduced or tended ($P < 0.15$) to be reduced, respectively, in LD muscle in the NA group compared to the control group of pigs in **study 1**. Similarly in **study 2,** relative mRNA level of the type I-specific MHCI was higher in SM and ST muscles and tended ($P < 0.15$) to be higher in LD muscle, but the relative mRNA levels of type II-specific MHC isoforms (MHCIIA, MHCIIX) in LD and SM muscle were lower in the NA group compared to the control group of sheep.

It is known that muscle fiber switching is initiated through the upregulation of key regulators of muscle fiber distribution and muscle metabolic phenotype (Lin *et al*., 2002b; Wang *et al*., 2004; Lin *et al*., 2005; Schuler *et al*., 2006) and recently it has been shown that NA supplementation results in an upregulation of three of these key regulators, namely PPARδ, PGC-1α and PGC-1β in *rectus femoris* muscle of rats (Ringseis *et al*., 2013). PPARδ is the most abundant PPAR isotype in skeletal muscle (Braissant *et al*., 1996; Muoio *et al*., 2002; de Lange *et al*., 2006) and has a higher expression in oxidative type I muscle fibers than glycolytic type II muscle fibers (Wang *et al*., 2004). PPARδ is involved in many different biological activities such as lipid and lipoprotein metabolism (Leibowitz *et al*., 2000; Risérus *et al*., 2008), skeletal muscle lipid oxidation (Wang *et al*., 2004), mitochondrial respiration (Luquet *et al*., 2003), thermogenesis (Guardiola-Diaz *et al*., 1999), and skeletal muscle fiber type distribution (Wang *et al*., 2004). Short-term exercise (Watt *et al*., 2004; Mahoney *et al*., 2005), endurance training (Russell *et al*., 2003; Fritz *et al*., 2006) and supplemental NA (Ringseis *et al*., 2013) lead to increased relative mRNA levels of PPARδ in human and rodent skeletal muscle. Similarly in sheep there was a significant effect of NA on relative mRNA levels of PPARδ in LD and ST muscles, and tended ($P < 0.15$) to be increased in SM muscle (**study 2)**. To determine the translation level of PPARδ relative mRNA into protein, the protein levels were analyzed of three muscles by western blot analysis, but the protein level of PPARδ in any muscle of sheep was not different between groups. It is not clear, why there was no effect of NA on PPARδ protein levels, but this may be due to the comparatively small

sensitivity of the western blotting technique making it difficult to detect slight differences between groups. It was observed, that the transcript levels of relative mRNA of PPARδ in all three muscles were also not so abundant in both groups. So, the unaltered protein level of PPARδ does not exclude that its DNA-binding activity was increased because it is known that PGC-1α and PGC-1β, whose relative mRNA expressions was clearly increased, act as coactivators of PPARδ and enhance the transactivation activity of PPARδ (Yu and Reddy, 2007).

PGC-1α is a transcription coactivator that coactivates a broad range of transcription factors that are involved in a wide variety of biological responses including adaptive thermogenesis, glucose/fatty acid metabolism and fiber type switching in skeletal muscle (Lin *et al*., 2002b; Puigserver and Spiegelman, 2003; Lin *et al*., 2005; Liang and Ward 2006). PGC-1α is also known as a master regulator of mitochondrial biogenesis (Wu *et al*., 1999; Fernandez-Marcos and Auwerx, 2011). PGC-1α is preferentially expressed in type I-rich and type IIA-rich muscle beds (Lin *et al*., 2002a). Like PGC-1α, PGC-1β is another member of PGC-1 family (Puigserver and Spiegelman, 2003; Lin *et al*., 2002b), also a key regulator of fatty acid oxidation, oxidative phosphorylation, mitochondrial biogenesis (Liang and Ward 2006, Arany *et al*., 2007). PGC-1β is also involved in the regulation of skeletal muscle fiber transition (conferred a switch toward a more slow myofibers phenotype) (Mortensen *et al.*, 2006). Both PGC-1α and PGC-1β are more highly expressed in oxidative fibers (Lin *et al*., 2002a; Arany *et al*., 2007), and particularly PGC-1α expression in human and rodent skeletal muscle is strongly induced by short-term exercise and endurance training (Baar *et al*., 2002; Russell *et al*., 2003; Norrbom *et al*., 2004; Koves *et al*., 2005). Though PGC-1β is highly expressed in skeletal muscle, it does not seem to be regulated by endurance exercise and/or have not been studied extensively (Meirhaeghe *et al*., 2003; Arany, 2008). However, in another study it was demonstrated that PGC-1β knockout causes a decrease of mitochondrial volume, a reduced expression of genes of the electron transport chain, and a mitochondrial respiration defect in skeletal muscle of rats (Mortensen *et al*., 2006). As expected, the relative mRNA levels of PGC-1α in LD muscle of pigs were numerically increased in NA group in **study 1**, even though this effect was not significant, which is attributed to the relatively high standard deviation of this parameter in both groups of pigs. Moreover, the relative mRNA level of PGC-1α in all three muscles and protein levels in two skeletal muscles (LD and SM) of sheep (**study 2**) were elevated in the NA group compared to control group. As predicted, it has been observed in **study 1** and **2** that, the transcript level of PGC-1β was higher or tended ($P < 0.15$) to be higher in all analyzed muscle of pigs and sheep of the NA group compared to control

group. Thus, the findings of the present studies, suggest that NA supplementation increases the transcriptional activity of these critical key regulators (PPARδ, PGC-1α and PGC-1β) of muscle fiber transition, and thus provides an explanation for the increased type I fiber content in skeletal muscles of NA-treated animals.

Type I fibers, also called slow-twitch oxidative fibers, contain a high number of mitochondria, have a high oxidative capacity, and preferentially use fatty acids for energy production (Barnard *et al*., 1971; Peter *et al.*, 1972). This oxidative metabolic phenotype of type I fibers is reflected by an elevated expression of genes involved in fatty acid transport and uptake, β-oxidation, carnitine shuttle, TCA cycle, respiratory chain and oxidative phosphorylation. So, relative mRNA levels of genes involved in mitochondrial fatty acid uptake and oxidation (CACT/ SLC25A20- **in study 1 and 2;** CPT1B- in **study 2**), mitochondrial fatty acid transport (FATP1- in **study 1**), carnitine uptake (OCTN2- in **study 1**), oxidative phosphorylation (COX4/1- in **study 1;** COX5A- in **study 2;** COX6A1- in **study 1 and 2**), citrate cycle (SDHA- in **study 1 and 2**) and thermogenesis (UCP2, UCP3- in **study 1**) were determined in different skeletal muscle of pig and sheep. All of these genes are abundantly expressed in type I fibers, which is responsible for the oxidative metabolic phenotype and preferred oxidative phosphorylation for energy production (Peter *et al*., 1972; Pette and Staron, 1990). As expected, the relative mRNA levels of all of these genes were higher in the NA group compared to control group [only UCP2 had tendency ($P < 0.15$) to increase] in both pig and sheep of the present studies. Thus, abovementioned changes in skeletal muscle gene expression induced by NA indicate that as the oxidative type I fibers are increased, the capacity of the skeletal muscle for oxidative utilization of fatty acids has also increased by NA administration.

As VEGFA is one of the most potent inducers of angiogenesis and vasculogenesis, a key regulator of both physiological and pathological angiogenesis (Ferrara *et al*., 2003; Roy *et al*., 2006) and type I fibers exhibit a higher expression of this angiogenic factor, the relative mRNA level of this gene was measured in **study 2**. As expected, the relative mRNA level of VEGFA was higher in the NA group compared to control group in all three muscles of sheep. This suggests that angiogenesis was stimulated by NA leading to a higher capillary density which also contributes to an increased utilization of fatty acids in skeletal muscle (Ringseis *et al*., 2013). Indeed, VEGFs increase capillary density as well as the expression of endothelial cell fatty acid transport proteins and thus enhancing fatty acid uptake from blood into skeletal muscle (Hagberg *et al*., 2010).

In contrast to the recent study in obese Zucker rats (Ringseis *et al*., 2013), NA supplementation did not induce the well-documented plasma TAG-lowering effect in pigs, while TAG levels tended ($P < 0.15$) to be lower in the NA group than in the control group of sheep. It is not quite surprising because with similar dose of NA there were also no effect of NA on plasma TAG in healthy human subjects (Poynten *et al*., 2003). The lack of effect, however, is probably not due to an insufficient NA dose because the dose was similar as used in the obese Zucker rat study (Ringseis *et al*., 2013) and the administered NA dose caused a significant increase in plasma NA, particularly NAM levels, indicating sufficient bioavailability in both studies. It is more likely that plasma TAG concentration of pigs was not lowered because it was yet within the normal range making a further reduction unlikely.

In opposite to the well-documented antilipolytic effect of NA (Gille *et al*., 2008; Morey *et al*., 2011) the plasma FFA concentration of NA group was also not reduced in sheep, but even increased in pigs, at least numerically. The potential explanation for the non-reduced plasma FFA levels of NA treatment is rebound of plasma FFA. The increased FFA levels of pigs (**study 1**) and the baseline plasma FFA levels in sheep (**study 2**) are in agreement with the observations that chronic NA administration for at least 2 weeks results in elevated plasma FFA levels (Alvarsson and Grill, 1996; Poynten *et al*., 2003), and this rebound increased up to fasting FFA levels have been observed in case of more than 2 weeks of NA treatment (Poynten *et al*., 2003). It is well recognized that, both oral and intravenous administration of NA lead to dramatic and acute reductions of plasma FFA, followed by a rebound and subsequent return to baseline (Pereira, 1967; Waterman and Schultz, 1972; Waterman *et al*., 1972; Jaster *et al*., 1983; Carlson, 2005; Pires and Grummer, 2007). The basis for this rebound phenomenon on lipolysis during long-term NA treatment is not clear, but recent findings indicate that chronic NA treatment in rats resulted in the significant decrease of phosphodiesterase-3B (PDE-3B) gene expression, which might have led to increase cAMP level (a major regulator of lipolysis in adipocytes) and thus increase lipolysis to cause the FFA rebound (Oh *et al*., 2011). Moreover, the relative mRNA expression of several key enzymes of TAG synthesis was markedly decreased, suggesting the possibility that decreased TAG synthesis contributes to the FFA rebound (Oh *et al*., 2011). A meta-analysis of multiple studies involving NA feeding to dairy cows showed no statistical effects of NA in plasma FFA and BHBA concentrations (Schwab *et al*., 2005).

The underlying mechanism how NA switches fiber types cannot be resolved from the present thesis studies, but the possibility is that the effect of NA involves NA receptor-independent

mechanisms, because the skeletal muscle does not express NA receptor. In this line it is remarkable that NA has been reported to induce several humoral changes, such as increases in the plasma levels of epinephrine, corticosterone and glucagon (Quabbe *et al*., 1983). Furthermore, NA supplementation also causes an elevation in the plasma levels of growth hormone, adiponectin and leptin, all of which are recognized to influence gene expression and cellular signalling in different tissues (Westphal *et al*., 2007; Plaisance *et al*., 2008). Hence, future studies remain to figure out whether these NA-induced humoral changes are responsible for the observed muscle fiber switching.

In conclusion, the results of the present thesis studies show that NA supplementation to pig and sheep induces a switch from type II to type I fibers with profound changes in the skeletal muscle metabolic phenotype. Thus, NA supplementation induces type II to type I muscle fiber switching, and thereby an oxidative metabolic phenotype of skeletal muscle in pigs. It is recognized that muscle fiber's composition affects the energy metabolism during postmortem conversion of muscle to meat, hence affecting ultimate meat quality (Karlsson *et al*., 1999; Monin and Ouali, 1992). Given that oxidative muscle types tend to develop dark, firm and dry pork in response to intense physical activity and/or high psychological stress levels preslaughter, a NA-induced change in the muscle fiber type distribution may improve meat quality of pigs. Furthermore, the NA-induced switch from type II to type I in skeletal muscle indicates an increased capacity of skeletal muscle for oxidative utilization of fatty acids. Therefore, the results suggest that an increased utilization of fatty acids by increased type I fibers, which significantly contributes to whole-body fatty acid utilization, and ultimately contributes to the lipid-lowering effects of NA treatment. Thus, findings of the **study 2** might be most appropriate in the circumstances in which farms are experiencing unusually high incidence rates of metabolic disorders like ketosis and/or fatty liver of high yielding dairy cows.

5. SUMMARY

Nicotinic acid (NA) is the oldest lipid-lowering drug and has been used for more than five decades in the treatment of atherosclerosis and metabolic disorders. The lipid-lowering mechanism of NA has been extensively investigated and traditionally attributed to its antilipolytic effect on adipocytes through binding with the NA receptor G-protein-coupled receptor 109A (GPR109A). However, the circulating free fatty acid (FFA) level rebound and over-shoot the baseline due to long-term NA treatment even though its lipid-lowering effects persist. Thus, despite a long history of clinical use, to date the precise mechanism by which NA lowers plasma lipid is far from clear. In such a context, in the present thesis it has been hypothesized that NA induces type II to type I muscle fiber transition, thereby increasing the oxidative capacity of overall skeletal muscle, which may be the background mechanism of lipid-lowering properties of NA. It has been already found that NA supplementation counteracts the obesity-induced muscle fiber transition from oxidative type I to glycolytic type II and increases the number of type I fibers in skeletal muscle of obese Zucker rats. These effects were likely mediated by the induction of key regulators of fiber transition, peroxisome proliferator-activated receptor δ (PPARδ), PPARγ coactivator-1α (PGC-1α) and PPARγ coactivator-1β (PGC-1β), leading to type II to type I fiber transition and upregulation of genes involved in fatty acid oxidation, mitochondrial oxidative phosphorylation, and angiogenesis. So, the main intention of the present thesis studies was to investigate the hypothesis that, whether NA administration also influences fiber type distribution and thereby the metabolic phenotype of different skeletal muscles in two farm animal species, namely in pig as a model of non-ruminants (**study 1**) and in sheep as a model of ruminants (**study 2**).

In order to investigate the hypotheses of **study 1,** twenty five male, 11 weeks old crossbred pigs (Danzucht x Pietrain) with an average body weight of 32.8 ± 1.3 (mean ± SD) kg were randomly allocated to two groups of 12 in control group and 13 pigs in NA group which were fed either a control or a diet supplemented with 750 mg NA/kg diet for 3 weeks. Fiber typing was performed in three different skeletal muscles (*M. Longissimus dorsi*, LD; *M. Quadriceps femoris,* QF; *M. Gastrocnemius,* G) and quantitative polymerase chain reaction (qPCR) was performed in LD muscle only. The percentage numbers of type I fibers in three different skeletal muscles were higher in the NA group and the percentage numbers of type II fibers were lower in the NA group compared to the control group. In line with this, the relative mRNA level of the type I fiber-specific myosin heavy chain, MYH7 gene tended ($P < 0.15$) to be higher; type II fiber-specific MYH2 and MYH4 genes were reduced or tended ($P < 0.15$) to be reduced, respectively, in LD muscle in the NA group compared to the control group of

pigs. The relative mRNA levels of key regulators of muscle fiber transition, PGC-1β was increased and PGC-1α was numerically increased by NA treatment. Genes involved in mitochondrial fatty acid utilization and thermogenesis [carnitine acylcarnitine translocase, fatty acid transport protein1, novel organic cation transporter 2, succinate dehydrogenase subunit A (SDHA), cytochrome c oxidase 4/1 and 6A1, (COX4/1 COX6A1) and uncoupling proteins 3] measured in LD muscle were higher in the NA treated pigs compared to control pigs.

In order to investigate the hypotheses of **study 2,** sixteen male, 11 weeks old Rhoen sheep with an average body weight of 29.6 ± 3.0 (mean ± SD) kg were randomly allocated to two groups of 8 sheep each and treated either without (control group) or with 1 g NA per day (NA group) for 4 weeks. After 4 weeks, the percentage numbers of type I fibers in three different skeletal muscles (LD; *M. Semimembranosus*, SM; *M. Semitendinosus*, ST) were higher in the NA treated sheep, whereas the percentage numbers of type II fibers were lower in the NA group compared to the control group of sheep. This effect was also reflected by the NA induced increase in the transcript level of fiber type I specific myosin heavy chain I, (MHCI) isoform in SM and ST muscles or tended ($P < 0.15$) to be increased in LD muscle; the fiber type II specific MHCIIA isoform was lowered in LD and SM muscles; fiber type II specific MHCIIX isoform was lowered in LD and tended ($P < 0.15$) to be lowered in SM muscle by NA treatment. The relative mRNA levels of the key regulators of muscle fiber transition, PGC-1α, PGC-1β and PPARδ, in all three muscles were higher or tended ($P < 0.15$) to be higher in the NA treated group compared to the control group sheep. Moreover, the protein level of PGC-1α was elevated in two muscles (LD and SM) of the NA group compared to the control group. In line with this, it was observed that the relative mRNA levels of genes involved in fatty acid oxidation and angiogenesis [SDHA, carnitine palmitoyltransferase 1B, solute carrier family 25 member 20, COX5A, COX6A1 and vascular endothelial growth factor A] in all three skeletal muscles of sheep were elevated by NA treatment.

In conclusion, the overall finding of the present PhD thesis is that NA causes type II (fast-glycolytic) to type I (slow-oxidative) fiber switch and thereby increases the oxidative capacity in different types of skeletal muscle of pigs and sheep. This increased oxidative skeletal muscle capacity induced by NA might improve the pork quality because the oxidative muscle types tend to develop dark, firm and dry pork in response to intense physical activity and/or high psychological stress levels preslaughter. As well as the increased oxidative capacity of skeletal muscle to utilize fatty acids in ruminants could be particularly useful during

metabolic states in which fatty acids are excessively mobilized from adipose tissue, such as in ketosis and/or fatty liver of high yielding dairy cows.

6. ZUSAMMENFASSUNG

Nikotinsäure (NA) ist das älteste lipidsenkende Medikament und wird bereits seit mehr als fünf Jahrzehnten bei der Behandlung von Atherosklerose und Stoffwechselkrankheiten eingesetzt. Der lipidsenkende Effekt der Nikotinsäure wurde intensiv untersucht und ist gekennzeichnet durch einen antilipolytischen Effekt auf Adipozyten, der durch die Bindung an den Nikotinsäurerezeptor GpR109A vermittelt wird. Während der Langzeittherapie mit Nikotinsäure kommt es zu einem Rebound-Effekt mit überschießenden Konzentrationen an freien Fettsäuren im Plasma, wobei jedoch der lipidsenkende Effekt erhalten bleibt. Trotz der langen Historie der klinischen Verwendung, ist der genaue Mechanismus der lidpidsenkenden Wirkung noch nicht eindeutig geklärt. In diesem Zusammenhang soll die vorliegende Dissertation die Hypothese überprüfen, dass Nikotinsäure den Übergang von Typ-II-Muskelfasern zu Typ-I- Muskelfasern induziert und somit die oxidative Kapazität des Skelettmuskels erhöht, was einen zugrundeliegenen lipidsenkenden Mechanismus der Nikotinsäure darstellten könnte. Es ist bereits bekannt, dass die Supplementierung von Nikotinsäure der Adipositas-induzierten Muskelfaserumwandlung von oxidativen Typ-I Muskelfasern zu glykolytischen Typ-II Fasern entgegenwirkt und die Anzahl der Typ-I Muskelfasern im Skelettmuskel von adipösen Zucker-Ratten erhöht. Dieser Effekt wird wahrscheinlich über die Aktivierung der Hauptregulatoren der Muskelfaserumwandlung erreicht, zu denen der *peroxisome proliferator-activated receptor gamma coactivator 1-alpha* (PGC-1α), der *peroxisome proliferator-activated receptor gamma coactivator 1-beta* (PGC-1β) und der *peroxisome proliferator-activated receptor delta* (PPARδ) gehören. Dessen Aktivierung führt zu einer Umwandlung von Typ-II Muskelfasern zu Typ-I Muskelfasern und einer Hochregulierung von Genen, die bei der Fettsäureoxidation, der oxidativen Phosphorylierung sowie der Angiogenese involviert sind. Das Ziel der vorliegenden Dissertation bestand darin, die Hypothese zu untersuchen, dass die Verabreichung von Nikotinsäure die Muskelfaserverteilung und den metabolischen Phänotyp ausgewählter Skelettmuskeln beim Schwein, als Model eines Nichtwiederkäuers (Studie 1), und beim Schaf (Studie 2), als Model eines Wiederkäuers, beeinflusst.

Um die Hypothese der ersten Studie zu untersuchen, wurden 25 männliche Schweine im Alter von 11 Wochen und einem durchschnittlichen Körpergewicht von 32,8 ± 1,3 kg (Mittelwert ± SD) zufällig 2 Gruppen zugeordnet. Die 12 Schweine der Kontrollgruppe bekamen ein Kontrollfutter, während den 13 Schweinen der Behandlungsgruppe das Kontrollfutter mit Zusatz von 750 mg Nikotinsäure/kg Ration verabreicht wurde. Die Muskelfasertypisierung wurde mit Proben des M. longissimus dorsi (LD), M. quadriceps femoris (QF) und M.

gastrognemicus (G) vorgenommen. Zusätzlich wurden quantitative PCR-Analysen mit LD-Proben durchgeführt. Durch die Gabe von Nikotinsäure erhöhte sich der prozentuale Anteil von Typ-I-Muskelfasern signifikant in allen ausgewählten Muskeln (LD, QF, G), während sich der prozentuale Anteil der Typ-II-Muskelfasern verringerte, im Vergleich mit der Kontrollgruppe.

In Übereinstimmung dazu waren die relativen mRNA-Konzentrationen des Typ-I-spezifischen Gens *myosin heavy chain*, MYH7 im LD der Behandlungsgruppe tendenziell höher ($P < 0.15$) und die der Typ-II-spezifischen Gene MYH2 und MYH4 signifikant bzw. tendenziell ($P < 0.15$) vermindert, im Vergleich zur Kontrollgruppe. Die relativen mRNA-Konzentrationen der Hauptregulatoren waren nach Behandlung mit Nikotinsäure im Falle von PGC1β signifikant erhöht und bei PGC1α numerisch erhöht. Für die Gene des mitochondrialen Fettsäurestoffwechsels [*carnitine acylcarnitine translocase*, *fatty acid transport protein1*, *novel organic cation transporter 2*, *succinate dehydrogenase subunit A*, (SDHA), *cytochrome c oxidase 4/1 und 6A1*, (COX4/1 und COX6A1) und der Thermogenese *uncoupling proteins 3*] konnte ein signifikanter Anstieg der relativen mRNA-Konzentrationen im LD in der Gruppe mit Nikotinsäuresupplementierung gezeigt werden.

Zur Überprüfung der Hypothese der zweiten Studie wurde ein vierwöchiger Versuch mit 16 männlichen, 11 Wochen alten Rhönschafen durchgeführt, welche ein durchschnittliches Körpergewicht von 29,6 ± 3,0 kg (Mittelwert ± SD) hatten und zufällig der Kontrollgruppe (Kontrollration ohne Nikotinsäure) oder der Behandlungsgruppe (Kontrollration plus 1 g Nikotinsäure/Tag) zugeordnet wurden.

Die vierwöchige Nikotinsäuresupplementierung der Schafe führte zu einer prozentualen Erhöhung der Typ-1-Muskelfasern in den ausgewählten Muskeln LD, M. semimebranosus (SM) und M. Semitendinosus (ST), wohingegen der prozentuale Anteil der Typ-II Muskelfasern im Vergleich zur Kontrollgruppe geringer war. Dieser Effekt äußerte sich auch in den erhöhten relativen mRNA-Konzentrationen der spezifischen *myosin heavy chain*, MHCI Isoform im Muskel SM und ST ($P < 0.05$) und LD ($P < 0.15$) sowie in den Transkriptleveln der MHCIIX-Isoform, die verringert waren in den Muskeln SM ($P < 0.05$) und LD ($P < 0.15$), verglichen mit der Kontrollgruppe. Auch die relativen mRNA-Konzentrationen der Hauptregulatoren der Muskelfaserumwandlung, PGC1α, PGC1ß und PPARδ, waren in den drei ausgewählten Muskeln signifikant oder tendenziell signifikant erhöht in der Gruppe mit Nikotinsäuresupplementierung. Darüber hinaus konnte auch ein Anstieg der Proteinkonzentration von PGC1α in den Muskeln LD und SM nach Gabe von Nikotinsäure mittels Western Blot Analysen nachgewiesen werden. Damit einhergehend

zeigten die ausgewählten Gene des Fettsäurestoffwechsels und der Angiogenese (SDHA, *carnitine palmitoyltransferase 1B*, *solute carrier family 25 member 20*, COX5A, COX6A1 und *vascular endothelial growth factor A*) in allen drei Muskeln der Schafe der Niacingruppe erhöhte relative mRNA-Konzentrationen.

Zusammenfassend bestätigt die vorliegende Dissertation, dass Nikotinsäure ursächlich für die Muskelfaserumwandlung von (schnellen-glykolytischen) Typ-II Fasern zu (langsamen-oxidativen) Typ-I Fasern ist und damit die oxidative Kapazität in den verschiedenen ausgewählten Muskeln von Schwein und Schaf erhöht. Diese gesteigerte oxidative Kapazität, die durch Nikotinsäure induziert wird, verbessert möglicherweise die Fleischqualität, da die oxidativen Muskelfasern aufgrund der intensiven körperlichen Aktivität und/oder des hohen psychischen Stresslevels unmittelbar vor der Schlachtung die Entstehung von dunklem, festem und trockenem Fleisch begünstigen. Die erhöhte oxidative Kapazität der Skelettmuskulatur könnte auch in Situationen, die mit einer erhöhten Stoffwechselrate assoziiert sind, wie zum Beispiel Ketose oder Fettleber bei der hochleistenden Milchkuh, vorteilhalt sein, da somit möglicherweise die Metabolisierung von Fettsäuren verbessert werden kann.

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Erklärung

„Ich erkläre: Ich habe die vorgelegte Dissertation „**Investigations on the effect of nicotinic acid supplementation on muscle fiber distribution and muscle metabolic phenotype in pig and sheep**“ selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Gießen, den …….

Muckta Khan

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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