Wissenschaftliche Reihe der Klinik für Pferde

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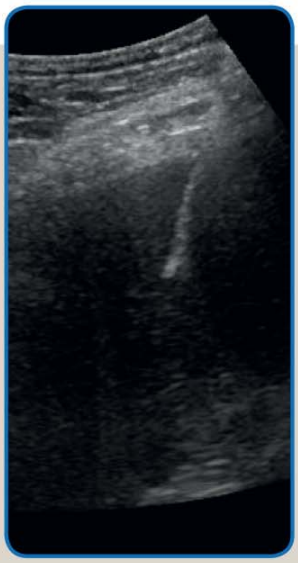
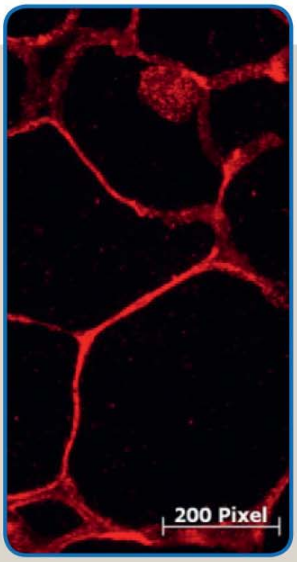
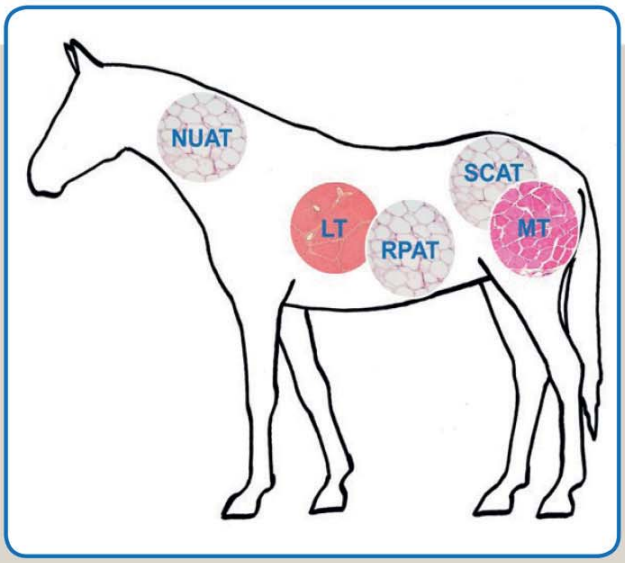
Equine Metabolic Syndrome

(Patho-)physiological variations in insulin sensitivity, glucose homeostasis and lipid metabolism in lean and obese horses







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THESIS

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Tobias Warnken

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MEINER FAMILIE

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ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
AT	adipose tissue
AMP	adenosine monophosphate
AUC	area under the curve
BCS	body condition score
BSA	bovine serum albumin
BW	body weight
cAMP	cyclic adenosine monophosphate
CEPT	cholesteryl ester transfer protein
CGIT	combined glucose-insulin test
CV	coefficient of variation
EDTA	ethylenediaminetetraacetic acid
EMS	equine metabolic syndrome
FAS	fatty acid synthase
FIA-MS/MS	flow-injection analysis and high-resolution/high-accuracy mass spectrometry
FSIGTT	frequently sampled IV glucose tolerance test
GIP	glucose dependent insulinotropic polypeptide
GLP-1	glucogon like peptide-1
GLUT	glucose transporter
GIT	gastrointestinal tract

GSK-3	glycogen synthase kinase-3
HDL	high-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI	hyperinsulinemia
HRP	horseradish peroxidase
HSL	hormone-sensitive lipase
HPLC	high-performance liquid chromatography
ID	insulin dysregulation / insulin-dysregulated
IDO	indoleamine-2-3-deoxygenase
IGF-1	insulin-like growth factor 1
InsR	insulin receptor
IRS	insulin receptor substrate
IR	insulin resistance / insulin-resistant
IS	insulin sensitivity / insulin-sensitive
kDa	kilo Dalton
LACT	lecithin cholesteryl acyltransferase
LCFA	long-chain fatty acids
LDL	low-density lipoprotein
LC-MS/MS	liquid chromatography analysis and high-resolution/high-accuracy mass spectrometry
LPL	lipoprotein lipase
MetS	metabolic syndrome (human)
MOI	metabolite of interest
mTOR	mechanistic target of rapamycin
Na	sodium
NEFA	non-esterified fatty acid

NUAT	nuchal adipose tissue
OGT	oral glucose test
OGTT	oral glucose tolerance test
OST	oral sugar test
PAK	primary antibody
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween
PDE-3	phosphodiesterase-3
PI3K	phosphatidylinositol-3-kinases
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PPID	pituitary *pars intermedia* dysfunction
p-AMPK	phosphorylated adenosine monophosphate-activated protein kinase
p-HSL	phosphorylated hormone-sensitive lipase
p-InsR	phosphorylated insulin-receptor
p-mTOR	phosphorylated mechanistic target of rapamycin
p-PKB	phosphorylated protein kinase B
RM	repeated measures
rmTWA	repeated measures two-way analysis of variance
ROS	reactive oxygen species
RPAT	retroperitoneal adipose tissue
SAK	secondary antibody
SCAT	subcutaneous adipose tissue
SCFA	short-chain fatty acids

SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
TBS-T	tris-buffered saline + Tween 20
TRG	triglyceride
VLDL	very low density lipoprotein
WB	Western blot

SUMMARY

Tobias Warnken

Equine Metabolic Syndrome – (Patho)-physiological variations in insulin sensitivity, glucose homeostasis and lipid metabolism in lean and obese horses

The Equine Metabolic Syndrome (EMS) is a severe endocrinopathy in equids. Alterations in insulin regulation in affected horses together with predisposition to laminitis and general or regional obesity are the leading laboratory findings (Frank et al. 2010, Frank and Tadros 2014). The obesity prevalence is generally high in the equine population (Wyse et al. 2008, Giles et al. 2014) and the incidence of endocrinopathic laminitis is emerging (Johnson et al. 2004, Karikoski et al. 2011). Veterinarian` and owners` awareness of the potential cross-link between hyperinsulinaemia (HI) and laminitis has been increasing recently. Therefore, owners` requests for EMS testing has increased. In practice, testing for impaired insulin regulation can be based on either assessment of basal HI, or dynamic diagnostic tests for insulin resistance (IR) or insulin dysregulation (ID) (Bertin and De Laat 2017). However, little is known about the underlying pathomechanisms of EMS and impaired insulin regulation. The objective of this PhD project was to investigate physiological and pathophysiological variations in insulin sensitivity, glucose homeostasis and lipid metabolism in healthy lean and obese horses on a clinically recognizable and molecular level.

The first part of this research project focuses on describing physiological principles during standard dosed oral glucose tests (OGT) and combined glucose-insulin tests (CGIT) which were performed consecutively in lean and obese horses to compare the endocrine and metabolic responses. In OGT, horses showed a highly individual increase of insulin concentrations in response to the glucose applied orally, whereas glucose concentrations showed similar dynamics. In the CGIT procedure, peak insulin concentrations of 493.98±86.84 µIU/mL were measured, followed by a continuous decline. Interestingly, concentrations of non-esterified fatty acids varied between

individual horses prior to testing but declined in a comparable manner to similar minimum concentrations of 93.82 ± 53.22 µmol/L in OGT and 91.97 ± 56.89 µmol/L in CGIT. Regarding the stress response of the test procedure cortisol concentrations remained unaffected during CGIT, while the OGT procedure was accompanied by a significant initial rise in cortisol concentrations. Summing up, OGT and CGIT mirror different facets of the metabolic response to a glycemic stimulus, highlighting different aspects of glucose homeostasis and insulin regulation and hence, reveal different applications in clinical settings. Moreover, insulin dynamics in CGIT, performed with porcine zinc insulin, differ from insulin dynamics described in reports published previously using short-acting insulins, providing not only potential advantages by reducing the risk of test induced hypoglycemia, but also limiting clinical usage, due to missing reliable reference ranges for test implementation with long-acting porcine zinc insulin, at least at the moment.

In the second part, the protein expression of key components of insulin signaling and their extent of phosphorylation were investigated in different equine tissue under basal conditions and stimulated conditions with HI and hyperglycemia provoked by intravenous injection of insulin and glucose in lean and obese horses. Injected insulin induced a high extent of phosphorylation of insulin receptor β in liver tissue but not in muscle tissue in all horses. Protein kinase B and mechanistic target of rapamycin expressed a higher extent of phosphorylation in all tissues under stimulated conditions. By contrast, adenosine monophosphate-activated protein kinase, as a component related to insulin signaling, expressed enhanced phosphorylation in muscle tissue and different adipose tissues, but not in liver tissue under stimulated conditions. In conclusion, tissue-specific variations in the acute response of insulin signaling to insulin injected intravenously were observed in horses. Insulin sensitivity in healthy horses is based on a complex concerted action of different tissues by their variations in the molecular response to insulin and we were able to identify hints which suggest that insulin and glucose homeostasis in horses is partially different to humans and rodents.

The third part of this research project focused on the dynamic metabolic profiles in response to a standard dosed OGT in horses of unknown insulin status. The results

indicated significantly higher insulin concentrations in horses with severe ID compared to insulin-sensitive horses. Moreover, analyses of samples with a targeted metabolomics approach revealed that 22 metabolites were affected by OGT, whereas most of the metabolites investigated (n = 163) did not show significant changes. Interestingly, the insulin status did not affect the metabolic profile during OGT. However, the short-term stimulation with oral glucose challenge resulted in metabolic and proinflammatory changes in both insulin dysregulated and insulin-sensitive horses. Interestingly, severe ID was announced even before OGT was indicated. The ID horses and ponies had lower trans-4-hydroxyproline and methioninesulfoxide concentrations compared to IS ones. Pathways associated with trans-4-hydroxyproline and methioninesulfoxide suggested that oxidative stress and impaired oxidant-antioxidant equilibrium are contributing factors to ID. The present findings provide new hypotheses for future research to understand pathophysiology in ID horses better.

ZUSAMMENFASSUNG

Tobias Warnken

Equines Metabolisches Syndrom – (Patho)-physiologische Variationen in der Insulinsensitivität, Glukosehomöostase und des Fettstoffwechsels in schlanken und obesen Pferden

Das Equine Metabolische Syndrom (EMS) ist eine bedeutende Endokrinopathie der Equiden. Erkrankte Pferde zeigen neben einer generalisierten Obesitas oder regionalen Adipositas mit Ausbildung krankheitstypischer Fettdepots im Bereich der Kruppe, der Schultern, der seitlichen Thoraxwand und des Halses, eine erhöhte Prädisposition für Hufrehe sowie Veränderungen in der Insulinregulation (Frank et al. 2010, Frank and Tadros 2014). Heutzutage ist die Obesitasprävalenz in der Pferdepopulation hoch (Wyse et al. 2008, Giles et al. 2014) und die Inzidenz der endokrinopathischen Hufrehe nimmt zu (Karikoski et al. 2011). Sowohl bei Tierärzten als auch bei Tierbesitzern steigt das Bewusstsein für einen Zusammenhang zwischen Hyperinsulinämie (HI) und Hufrehe. Daher steigen Besitzeranfragen zur Abklärungen einer EMS Erkrankung ihrer Pferde in der letzten Zeit an. In der Praxis kann eine gestörte Insulinregulation auf verschiedene Weise erfasst werden. Neben der Bestimmung einer basalen HI können auch dynamische Diagnostiktest angewendet werden um eine Insulinresistenz (IR) oder eine Insulindysregulation (ID) zu diagnostizieren (Bertin and De Laat 2017). Ziel des PhD Projektes war es die physiologischen und pathophysiologischen Variationen der Insulinsensitivität, der Glukosehomöostase und des Fettstoffwechsels in schlanken und obesen Pferden auf Grundlage von klinischen Parametern sowie auf molekularer Ebene zu untersuchen.

Im ersten Teil dieses Forschungsprojektes wurden aufeinanderfolgend ein oraler Glukose Test (OGT) und ein kombinierter Glukose-Insulin Test (CGIT) in schlanken und obesen Pferden durchgeführt, um die endokrinologischen und metabolischen Reaktionen zu vergleichen. Im OGT zeigten die Pferde deutliche individuelle Unterschiede in der Insulinkonzentration nach enteraler Absorption der mittels Nasenschlundsonde verabreichten Glukose. Im Gegensatz zu den Insulinkonzentrationen zeigten die Pferde vergleichbare Glukosekonzentrationen. Im

CGIT konnten initiale Peak Insulinkonzentrationen von 493,98 ± 86,84 µIU/ml gemessen werden; gefolgt von einem kontinuierlichen Abfall. Interessanterweise zeigten die basalen Konzentrationen an nicht-veresterten freien Fettsäuren (NEFA) eine hohe individuelle Schwankung zwischen den einzelnen Pferden. Im weiteren Verlauf der beiden Stimulationstests zeigte sich jedoch ein vergleichbarer Abfall der NEFA Konzentrationen bis hin zu vergleichbaren Minimalkonzentrationen im OGT von 93,82 ± 53,22 µmol/l und 91,97 ± 56,89 µmol/l im CGIT. Während im CGIT kein stressinduzierter Kortisolanstieg zu beobachten war, zeigten die Pferde im OGT einen signifikanten, initialen Anstieg im Serumkortisolgehalt. Zusammenfassend lässt sich feststellen, dass OGT und CGIT unterschiedliche Facetten einer metabolischen Antwort auf einen glykämischen Stimulus reflektieren. Die beiden Tests erfassen unterschiedliche Aspekte der Glukosehomöostase und der Insulinregulation und erlauben aufgrund Ihrer zugrundeliegenden Stimulationsmechanismen differenzierte Aussagen in Bezug auf IR und ID und eigenen sich für unterschiedliche klinische Fragestellungen. Es zeigte sich eine verzögerte Insulinkinetik bei der Durchführung des CGIT mit porzinem Zink Insulin im Vergleich zu den bisher eingesetzten sofortwirksamen Insulinanaloga. Diese Unterschiede können zum einen einen klinischen Vorteil darstellen und das Risiko einer testinduzierten Hypoglykämie reduzieren, erlauben jedoch zum anderen bisher keinen routinemäßigen Einsatz in der Pferdepraxis, da für das verzögert wirksame Zink Insulin noch keine verlässlichen Referenzwerte etabliert sind.

Im zweiten Teil der Studie wurde die Expression und Phosphorylierung mehrerer in der Insulinsignalkaskade relevanter Proteine unter basalen und stimulierten, hyperinsulinämischen und hyperglykämischen Bedingungen in unterschiedlichen Geweben dünner und adipöser Pferde untersucht. Alle Pferde zeigten eine deutliche Zunahme der Phosphorylierung des Insulinrezeptors-β (InsR- β) im Lebergewebe unter der Stimulation mit Insulin. Im Muskelgewebe zeigte sich jedoch keine Zunahme der Phosphorylierung des InsR-β. Protein Kinase B (PKB/AKT) und mechanistic Target of Rapamycin (mTOR) zeigten in allen untersuchten Geweben eine Zunahme der Phosphorylierung unter der Stimulation. Im Gegensatz dazu zeigte die AMP-aktivierte Proteinkinase α (AMPK-α), ein Protein, das mit der Insulinsignalkaskade in Verbindung

steht, eine gesteigerte Phosphorylierung im Muskel- und Fettgewebe. Im Lebergewebe jedoch fand sich keine Veränderung im Phyosphorylierungsgrad dieses Proteins zwischen basalen und stimulierten Bedingungen. Zusammenfassend konnten nach intravenöser Stimulation mittels Insulin gewebeabhängige Unterschiede in der Phosphorylierung verschiedener Proteine der Insulinsignalkaskade festgestellt werden. Insulinsensitivität in gesunden Pferden ist das Ergebnis einer komplexen Signaltransduktion in verschiedenen Geweben. Im Rahmen dieser Studie zeigten sich zudem im Pferdegewebe Hinweise auf partielle Unterschiede in der Insulin- und Glukosehomöostase im Vergleich zu Menschen und Nagern.

Im dritten Teil dieser Forschungsarbeit wurden die OGT induzierten metabolischen Reaktionen verschiedener Pferde und Ponies mit unbekanntem Insulinsensitivitätsstatus untersucht. Die Ergebnisse dieses Studienteils zeigten wie erwartet signifikant höhere Seruminsulinkonzentrationen in ID Pferden und Ponies im Vergleich zu den IS Tieren. Die Untersuchungen der Blutproben mittels zielgerichteter Metabolomanalyse ergab, dass 22 Metabolite durch die orale Glukosestimulation im OGT beeinflusst werden, wohingegen die restlichen analysierten Metabolite (n=163) keine signifikanten Veränderungen unter der OGT Stimulation zeigten. Der Insulinstatus der Tiere hatte dabei keinen direkten Einfluss auf das metabolische Profil der Tiere während des OGTs. Es zeigte sich jedoch, dass die kurzzeitige Stimulation mit einer oralen Glukosestimulation, wie sie im OGT vorgenommen wird, bereits ausreicht, um im Organismus einen proinflammatorischen Zustand hervorzurufen, unabhängig von IS oder ID. Interessanterweise gelang es, Pferde und Ponies, die unter einer erheblichen ID litten bereits aufgrund des basalen Metabolom-Profils zu identifizieren. Insulindysregulierte Pferde und Ponies wiesen im Vergleich zu IS Pferden und Ponies niedrigere Trans-4-hydroxyprolin- und Methioninsulfoxidkonzentrationen auf. Stoffwechselwege, die mit Trans-4-hydroxyprolin und Methioninsulfoxid assoziiert sind, lassen vermuten, dass oxidativer Stress und ein Ungleichgewicht zwischen oxidativen und antioxidativen Stoffwechselvorgängen relevante Faktoren im Zusammenhang mit equiner ID sein können. Die zugrundeliegenden Ergebnisse liefern neue Hypothesen für folgende

Forschungsarbeiten und tragen dazu bei, die Pathomechanismen der equinen ID zu entschlüsseln.

1 INTRODUCTION

1.1 The Equine Metabolic Syndrome

The Equine Metabolic Syndrome (EMS) was first introduced into veterinary medicine by Johnson (2002). The term was adopted from human medicine. The human metabolic syndrome describes a disease pattern in which obesity, diabetes type 2 and cardio-vascular diseases are the major symptoms. Similar symptoms in equids compared to the situation in human medicine had been observed (Johnson 2002). The EMS was defined by the American College of Veterinary Internal Medicine consensus statement in 2010 (Frank et al. 2010). According to this statement, affected horses show a cluster of the following signs:

a) generalized obesity or regional adiposity
b) altered insulin regulation characterized by insulin resistance (IR) represented by hyperinsulinemia (HI) or abnormal glycemic and insulinemic responses to oral or intravenous (IV) glucose and/or insulin challenges; and
c) a predisposition towards laminitis or laminitis that has developed in the absence of other recognized causes.

Additional clinical conditions must be considered in association with EMS. Hypertriglyceridemia or dyslipidemia (Frank et al. 2006, Treiber et al. 2006, Carter et al. 2009c), hyperleptinemia (Cartmill et al. 2003), arterial hypertension (Bailey et al. 2008), altered reproductive cycling in mares (Vick et al. 2006) and increased proinflammatory markers (Vick et al. 2007) have been described in horses and ponies suffering from EMS.

Despite concurrent conditions, equine laminitis is the most common result (Karikoski et al. 2011) and is a severe disease which causes an acutely painful condition of the feet, often resulting in acute and/or chronic lameness. Laminitis is defined as failure of the hoof lamellar-distal phalangeal attachment apparatus (Pollitt 2004). Although multiple inducing factors and etiologies have been identified, the exact pathogenesis is still not known in depth. However, acute and recurrent pasture-associated laminitis

is most frequently encountered and associated with HI or IR (Carter et al. 2009c, Karikoski et al. 2011, Patterson-Kane et al. 2018). In accordance with these findings, Walsh et al. (2009) demonstrated that enhanced insulin concentrations correlate with laminitis severity.

1.1.1 Obesity and assessment of body condition in horses

Obesity is a pathological condition associated with altered adipokine production and IR and is common in the equine population (Johnson et al. 2009). Generalized obesity in horses and ponies, which is simply defined as an expanded mass of adipose tissue (AT) in the body, is observed. Furthermore, regional adiposity occurs, which is characterized by an accumulation of fat in certain locations of the body. The body regions mainly affected are the neck, the shoulder region and the tail head (Carter et al. 2009a, Frank et al. 2010). Assessment of body condition and fat mass can be performed by multiple methods. Henneke et al. (1983) developed a body condition scoring (BCS) system with a nine-point scale, one described as “poor,” representing an emaciated horse, and nine as “extremely fat,” representing a pathologically obese horse. The score can be easily used by visual appraisal and palpation of six specific body locations, including the rib area, shoulder region, the area along the withers, the tail-head region, the neck region and along the back of the horse (Henneke et al. 1983). Despite this scoring system, six-point scores (Webb and Weaver 1979) and multiple new scoring systems or adaptations have been described to meet current demands and take into account breed-specific variations in the exterior (Kienzle and Schramme 2004, Dugdale et al. 2012). Regional adiposity can be evaluated in horses by using a five-point cresty neck scoring system (CNS) (Treiber et al. 2006).

The prevalence of obesity in horses and ponies has been investigated in multiple studies. Exemplarily, 32 out of 319 pleasure riding horses in Scotland were classified as obese with an BCS of 6/6 and 112 out of the 319 horses attracted attention with an BCS of 5/6 and were assessed as overweight (Wyse et al. 2008). Studies performed in the USA provided similar results regarding overweight and obese horses (Thatcher et al. 2008, Stewart-Hunt et al. 2010). Furthermore, Giles et al. (2014) compared the BCS in a cohort of ponies determined at the end of the summer period with the BCS

assessed at the end of winter and found out that only 28 % of the horses were obese at the end of the winter period compared to 36 % at the end of the summer period, indicating physiological variations and seasonal changes in BCS in horses (Giles et al. 2014). In addition to limited storage in all cell types, white AT is the major site for the storage of triglycerides (TRG). The AT is a central organ and key player in whole body energy regulation and is responsible for the release of free fatty acids as an energy supplier for other tissues.

1.1.2 Hyperinsulinemia, insulin resistance and insulin dysregulation in horses

Glucose homeostasis is closely regulated by insulin to maintain essential homeostasis of the organism. Following food intake, plasma glucose concentrations rises due to enteral absorption. Insulin is secreted by the pancreas as counter-regulatory response to promote glucose uptake by insulin-sensitive (IS) tissues. Therefore, a postprandial increase in circulating plasma insulin concentrations is essential for glucose homeostasis. However, pathophysiologically enhanced postprandial or even fasting HI can occur if insulin regulation is impaired. The HI occurs in horses in an IR state (Frank et al. 2010). In contrast to humans who develop hyperglycemia under IR conditions, horses usually maintain glucose homeostasis with normoglycemia (Divers 2008).

Insulin resistance is defined as a decreased ability of IS tissues to respond adequately to insulin (Muniyappa et al. 2008). In humans, IR is generally a reflection of mainly skeletal muscle IR, as skeletal muscle is responsible for approximately 85 % of glucose disposal in a euglycemic, hyperinsulinemic state (Defronzo et al. 1981). Increased pancreatic beta cell secretion of insulin compensates for impaired tissue sensitivity, resulting in high circulating concentrations of insulin and HI. In humans, the pancreas loses its ability to compensate as the disease processes and will secrete insufficient amounts of insulin in response to hyperglycemia, resulting in a hyperglycemic, hypoinsulinemic state, which is known as type II diabetes mellitus (Shanik et al. 2008). By contrast, horses are rarely reported to develop type II diabetes (Durham et al. 2009). The IR can be caused by several impairments (Kahn 1980). Under physiological conditions, IR can occur during gestation as gestational diabetes in humans and horses (Fowden et al. 1984, Maresh 2001). Studies in horses have shown that IR can

occur in pregnant mares up to 270 days of gestation, indicated by higher levels of insulin release in response to exogenous and endogenous glucose (Fowden et al. 1984). Multiple mechanisms leading to IR have been proposed such as fewer insulin receptors (InsRs) due to downregulation, the decreased function of receptors themselves or a breakdown of insulin-signaling mediators (Kahn 1980, Kahn and Flier 2000, Shanik et al. 2008). Despite the fact that the mechanisms are not completely known so far, alterations in post-receptor signaling are discussed most.

Generalized obesity in humans is associated with the development of IR (Kahn and Flier 2000). Similarly, equine obesity is negatively correlated with IS (Hoffman et al. 2003, Carter et al. 2009b) and associated with an increased risk of HI (Carter et al. 2009c). Experimental studies in horses and ponies have revealed a significant cross-link between equine HI and the occurrence of laminitis. Prolonged HI for 48 hours induced by hyperinsulinemic clamps resulted in laminitis in previously healthy Standardbred horses and normal ponies (Asplin et al. 2007, De Laat et al. 2010, De Laat et al. 2015). Moreover, horses presented to a first opinion hospital for evaluation of laminitis were hyperinsulinemic in 86 % of cases (Karikoski et al. 2011). A recent study showed the direct link between a pathological high insulin response assessed with a glucose challenge test and the occurrence of experimentally induced laminitis by a dietary challenge high in nonstructural carbohydrates. Ponies with insulin concentrations higher than 65 µIU/mL developed laminitis after consuming 12 g NSC/kg BW/d for a period of up to 18 days (Meier et al. 2017). This is in accordance with the identification of high basal serum insulin concentrations as a risk factor for the development of laminitis (Menzies-Gow et al. 2017).

The IR and alterations in insulin regulation in horses have been extensively studied in the last decade and provided new information on potential pathophysiological mechanisms. Recently altered insulin regulation, including tissue IR and basal or postprandial HI, have been subsumed under the term insulin dysregulation (ID) (Frank and Tadros 2014, The Equine Endocrinology Group 2016, Bertin and De Laat 2017). De Laat et al. (2016) showed that ID can occur independently of tissue IR and that IV and oral tests did not supply similar results regarding the insulinemic state of ponies. Since then, equine HI has been considered a counter-regulatory response to IR.

However, there is growing evidence in recent research to support a gastrointestinal etiology by incretin hormones released from the proximal intestine, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide, which may augment insulin secretion (De Laat et al. 2016).

1.1.3 Assessment of disturbances in insulin regulation in horses

Although, several studies postulated a link between BCS or CNS and basal HI or an increased risk of laminitis (Carter et al. 2009a, Carter et al. 2009c), disturbances in insulin regulation may not be identified correctly by phenotype in all cases (Firshman and Valberg 2007, Bertin and De Laat 2017). Clinical experience indicated that high BCS or CNS does not necessarily implicate disturbed insulin regulation, alternatively, low BCS does not preclude ID or IR.

Basal measures of insulin and/or glucose are often used to assess disturbed insulin regulation. Nevertheless, single measurements of both or even further calculation of indices or ratios (Treiber et al. 2005, Carter et al. 2009c) based on these measures may not be sufficient in all patients (Dunbar et al. 2016). In addition to these simple and static diagnostic procedures, dynamic stimulation tests are proposed for the assessment of IR and ID (Frank and Tadros 2014, The Equine Endocrinology Group 2016, Bertin and De Laat 2017). Research in recent years has established several testing protocols for stimulation tests based on either oral challenge tests performed by the application of sugar or glucose formulations or IV testing protocols with injections of glucose or insulin, or even both to assess disturbed insulin regulation.

The most accurate gold standard test for the assessment of tissue IS is the hyperglycemic or hyperinsulinemic clamp (Defronzo et al. 1979). Patients with normal IS require more glucose to maintain euglycemia than an individual with IR. Put simply, the amount of glucose required to maintain basal concentrations is equal to the amount of glucose taken up by the tissues and, therefore, represents a measure of peripheral tissue sensitivity to insulin. Two types of clamping procedures can be distinguished. The euglycemic hyperinsulinemic clamp (EHC) provides supra-physiological steady-state insulin concentrations, during which the rates of glucose infusion required to maintain euglycemia are used to measure the IS of muscle and AT. By contrast, the

hyperglycemic clamp fixes plasma glucose at an acutely elevated level. Consequently, endogenous hepatic glucose production is suppressed and glucose infusion rates reflect pancreatic insulin secretion, allowing quantification of the sensitivity of the pancreatic beta cells to glucose. The EHC is ideal for assessing IS, as it assesses tissue IS isolated from the impact of pancreatic insulin secretion or enteral glucose absorption on glucose homeostasis (Defronzo et al. 1979). In addition, endogenous glucose production is largely suppressed by the insulin infusion. Several studies performed in horses used EHC procedures as the gold standard to test insulin-dependent tissue sensitivity (Rijnen and Van Der Kolk 2003, Kronfeld et al. 2005, Pratt et al. 2005, Pratt-Phillips et al. 2015). However, these tests are usually reserved for research approaches due to their complex implementation. Furthermore, EHCs do not reflect disturbed insulin regulation in addition to tissue IR or impaired insulin clearance.

Oral glucose challenge tests allow the assessment of postprandial HI under standardized conditions. The oral glucose tolerance test (OGTT) was initially designed for use in horses to evaluate small intestinal malabsorption (Roberts and Hill 1973). Subsequently, the OGTT was used to evaluate glucose tolerance in equids by administration of 1 g/kg body weight (BW) of glucose (Jeffcott et al. 1986). Physiologically, a peak in blood glucose concentrations can be observed 90-120 minutes after the administration of glucose and should decline and return to normal pre-stimulation baseline concentrations within 4-6 hours (Roberts and Hill 1973). More profound or prolonged hyperglycemia is reported to be indicative of impaired pancreatic insulin secretion, decreased tissue IS or enhanced enteral absorption.

The oral testing protocol has undergone profound changes over time and several variations have been described for the indirect assessment of IR or direct assessment of ID. Protocols differ regarding the different application routes and dosages of glucose or other sugar formulations used. Most oral tests protocols are subsumed under the term OGT.

Nowadays, OGT is most often performed as an in-feed OGT, by introducing 0.5, 0.75 or 1.0 g/kg BW glucose or dextrose powder mixed in low-glycemic meal followed by blood sampling after 120 minutes (Smith et al. 2016, De Laat and Sillence 2017). The

analysis of insulin with a commercially available chemiluminescent immunoassay in samples collected allows the classification of patients as ID during standard dose in-feed OGT with 1 g/kg BW glucose when the insulin concentration is > 85 µIU/mL and > 68 µIU/mL in the case of 0.5 g/kg BW glucose used (The Equine Endocrinology Group 2016). However, incomplete ingestion and prolonged consumption times can preclude reliable results for interpretation (Kronfeld et al. 2005, De Laat and Sillence 2017). The oral sugar test (OST), using commercially available corn syrup as a glucose substrate, was established to simplify the application of glucose (Schuver et al. 2010). In order to implement the OST, 0.15 or 0.25 mL/kg BW corn syrup is administered via syringe into the oral cavity of the horse, followed by the measurement of insulin and glucose (Schuver et al. 2014, Jacob et al. 2017). Insulin concentrations of > 45 µIU/mL are generally suggested as being indicative of ID (The Equine Endocrinology Group 2016). The most invasive but most precise oral test approach is the OGT via nasogastric tubing (Ralston 2002). The substantial benefit of this protocol is the exact intragastric administration of a defined glucose dosage in a short time. Standard dose OGT is performed with 1 g/kg BW glucose dissolved in water and administered via nasogastric tubing directly into the stomach of the horse. Application is followed by analyses of glucose and insulin concentrations at specific time points, usually 120 minutes. Although this remains the most precise procedure, it requires nasogastric tubing and often raises debates about the impairment of clinically relevant test results by activation of the hypothalamic-pituitary-adrenal axis.

In addition to these oral stimulation protocols, IV challenge tests have been developed. Protocols range from a simple IV glucose tolerance test (IVGTT) (Garcia and Beech 1986, Giraudet et al. 1994) or insulin response tests (IRT) (Caltabilota et al. 2010, Bertin and Sojka-Kritchevsky 2013) to more complex combined procedures using glucose and insulin stimuli. The combined glucose-insulin test (CGIT) (Eiler et al. 2005) and the frequently sampled IV glucose tolerance test (FSIGTT) (Hoffman et al. 2003, Pratt et al. 2005, Treiber et al. 2005) can be used to assess the capacity of exogenous insulin to shift glucose into the IS tissues.

Regarding the CGIT, glucose solution is administered intravenously to the horses, followed directly by a second injection of insulin. Blood samples are taken following a

specific protocol for at least 150 minutes, including analyses of glucose and insulin. Insulin is measured in the initial sample before the glucose administration and after 45 minutes, whereas glucose is measured in all samples. Typical healthy horses show a biphasic blood glucose curve during the CGIT procedure. The first phase shows a positive hyperglycemia and the second one a negative hypoglycemia, in which glucose concentrations drop below the baseline. In IR horses, the first positive phase is prolonged with a slower return to baseline (Eiler et al. 2005). The 45-minute value is used as a clinical cut-off value to distinguish between IS and IR individuals. Horses should achieve normal glucose concentrations, return to previous baseline levels and have insulin concentration under 100 µIU/mL within 45 minutes (Frank and Tadros 2014). Horses with insulin concentration above 100 µIU/mL are considered to secrete more insulin than normal or are clearing the hormone from the circulation at a slower rate. Therefore, values above these ranges are interpreted as an indication of reduced IS (Eiler et al. 2005).

1.2 Glucose homeostasis and insulin action

Glucose is an important energy source for mammalian cells, and glucose homeostasis is essential for survival and metabolic health. The blood glucose in healthy mammals is derived from enteral absorption, gluconeogenesis in the liver and kidneys, and glycogenolysis in cases of hypoglycemia. Postprandial blood glucose concentration is normally regulated primarily by pancreatic insulin secretion and insulin-mediated glucose uptake by IS tissues. Insulin-independent tissues account for a lesser amount of glucose uptake. Plasma glucose concentration in horses is tightly controlled within physiological ranges. Depending on which literature is consulted, physiological ranges have been reported to be between 3.3 and 5.0 mmol/L (Ralston 2002).

Glucose absorption in the equine small intestine occurrs mainly in the proximal to mid small intestine and is directed via two types of insulin-independent glucose transporters: sodium-glucose linked transporter 1, a sodium/glucose cotransporter, on the luminal membrane and glucose transporter 2 on the basolateral membrane (Shirazi-Beechey 2008, Shirazi-Beechey et al. 2011). Both hyperinsulinemia and hyperglycemia, as postprandial consequences, suppress hepatic glucose production

by gluconeogenesis, inhibit the breakdown of glycogen to glucose in the liver, and stimulate the uptake, storage and use of glucose in tissues, such as skeletal muscle and AT, to restore normoglycemia.

High luminal glucose concentrations in the gut trigger signaling pathways in endocrine cells causing the secretion of gastrointestinal hormones, so-called incretins. Incretins are synthesized in the endocrine cells of the gastrointestinal tract and promote the release of insulin under hyperglycemic conditions (Marks et al. 1991, Shirazi-Beechey et al. 2011). The glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are the incretins most investigated in horses (Duehlmeier et al. 2001, Chameroy et al. 2010, Bamford et al. 2015, Chameroy et al. 2016, De Laat et al. 2016). While increased plasma GIP concentrations occurred in horses and ponies during an OGTT, GIP concentrations remained normal in these animals during an IV glucose tolerance testing procedure (Duehlmeier et al. 2001).

Insulin controls the activities of several metabolic enzymes by phosphorylation and dephosphorylation and regulates the expression of genes involved in gluconeogenesis and glycolysis (Pilkis and Granner 1992).

Insulin is a peptide hormone synthesized by the β cells within the islets of Langerhans of the pancreas in response to hyperglycemia. It is synthesized in the ribosomes of the rough endoplasmic reticulum (RER) as pre-pro insulin, consisting of an A-chain, a B-chain and a connecting peptide (C-peptide) (Wahren et al. 2000). Pre-pro insulin is cleaved to pro-insulin by the removal of a signal peptide. Thus, pro-insulin acquires the characteristic tertiary structure in the RER. Pro-insulin is transported to the Golgi apparatus in secretory vesicles and forms soluble pro-insulin hexamers containing zinc (Dodson and Steiner 1998). The C-peptide is removed by enzymes during secretion of the pro-insulin vesicle from the Golgi, resulting in the conversion of pro-insulin to insulin and C-peptide (Steiner 2004). Consequently, insulin forms insoluble hexamers containing zinc, precipitating as chemically stable crystals at a pH of 5.5 stored in granules.

Insulin and C-peptide are co-secreted by exocytosis of mature granules into circulation in equimolar amounts. Cell membrane depolarization and opening of voltage-

dependent calcium channels force the influx of extracellular calcium, which, in turn, triggers the exocytosis of insulin granules (Prentki and Matschinsky 1987). Insulin secretion in humans is characteristically pulsatile and biphasic. In response to stimulation, for example, by glucose, the initial rapid phase of insulin secretion is followed by a less pronounced but sustained insulin release (Bratanova-Tochkova et al. 2002). However, monophasic and biphasic secretion patterns have been observed and discussed in equids (Hoffman et al. 2003, Bamford et al. 2014, Smith et al. 2016). It has been shown in humans that GIP is suggested to primarily amplify initial insulin secretion by promoting the exocytosis of previously docked insulin granules in humans, while GLP-1 also stimulates second-phase insulin secretion in nondiabetic subjects (Schou et al. 2005).

If secreted into the blood stream, insulin binds to its receptor in IS tissues, allowing the activation of the insulin signaling cascade and glucose uptake by target tissues. Insulin-mediated glucose disposal occurs primarily in the skeletal muscle, AT and liver. Despite insulin-mediated glucose uptake, glucose can also be transported by using insulin-independent glucose transporters (GLUT). This is also essential for glucose homeostasis and the maintenance of essential organ functions.

The first pass through the liver clears approximately 50 % of the insulin in humans. Insulin in liver tissue (LT) is degraded by insulinase (Valera Mora et al. 2003), whereas insulin in systemic circulation is removed mainly by glomerular filtration in the kidney together with C-peptide (Rabkin et al. 1984).

The counter-regulatory hormones to insulin, which increase the concentration of glucose in the blood, include glucagon, epinephrine and, to a lesser extent, growth hormone and cortisol. These hormones promote glycogenolysis in terms of negative energy balance and, therefore, provide glucose as a potent energy supplier.

1.3 Lipid metabolism and insulin actions

Adipose tissue metabolism is essential in the regulation of energy balance and lipid utilization. Lipids can be divided, based on their chemical structure, into TRG, phospholipids, glycolipids and steroids. The TRG are the most important energy

reservoir of the organism and consist of a glycerol molecule esterified with three fatty acids. Lipid metabolism is generally the synthesis by lipogenesis and degradation by lipolysis of lipids in cells (Ahmadian et al. 2009). These lipids are derived mainly from digestion and enteral absorption. Hepatic TRG in humans are generated from fatty acids derived from plasma and *de-novo* synthesis (Ginsberg et al. 2005). The TRG are transported in plasma by chylomicrons and very low-density lipoproteins (VLDL). Hepatic synthesis and the release of TRG as VLDL is normally decreased by insulin (Koo and Montminy 2006). There are also low-density lipoproteins (LDL) and high-density lipoproteins (HDL). The latter were found to dominate the equine lipoprotein spectrum (Watson et al. 1991). Lipoprotein lipase (LPL) – bound to capillary endothelial cells – is responsible for the release of free fatty acids from VLDL and from chylomicrons. Non-esterified cholesterol from peripheral tissues can be taken up by HDL and is esterified by the enzyme lecithin cholesteryl acyltransferase.

Humans and most animal species can transfer HDL cholesteryl esters to VLDL and LDL by plasma cholesteryl ester transfer protein (CETP). Interestingly, horses have a lack of plasma CETP activity (Watson et al. 1993). However, horses and ponies seem to have a CETP-independent pathway for the transfer of HDL cholesteryl esters to the LDL and the magnitude of this transfer seems to be related to the levels of HDL cholesteryl esters (Geelen et al. 2001).

In physiological conditions, AT releases non-esterified fatty acids (NEFA) if energy has to be provided elsewhere in the organism (Ahmadian et al. 2010). A dynamic balance is maintained between TRG lipolysis and NEFA release, as is the control of their uptake and oxidation by other tissues, especially the muscle (Guilherme et al. 2008). Accumulation of energy stores is regulated by the anabolic action of insulin, and the inhibition of lipolysis by insulin was demonstrated by *ex vivo in vitro* studies on equine adipocytes (Breidenbach et al. 1999).

The utilization of stored fat is controlled by proinflammatory cytokines, stress hormones and glucagon during starvation, infection, injury or stress (Jaworski et al. 2007). Release from the AT supplies fatty acids for utilization by muscle if energy is needed (Carmen and Victor 2006). Moreover, fatty acids are taken up by the liver and are

esterified into TRG for assembly into VLDL, which, in turn, deliver fatty acids to muscle tissues (MT) with a high activity of LPL in the surrounding capillaries. High activity of LPL in the AT ensures sufficient TRG amounts in these tissues under feeding conditions.

In addition to energy storage, AT is an active endocrine organ producing and releasing several important hormones, metabolites and factors that regulate appetite, inflammatory processes and the balance of metabolic pathways (Hajer et al. 2008). Leptin, an adipocyte hormone, indicates the energy balance to the brain. Plasma levels increase concurrently to weight gain, suggesting that leptin is responsive to short-term changes in the body condition (Houseknecht et al. 1998). Leptin is primarily produced and secreted from the AT in concentrations that are proportional to fat mass; this was also shown in horses (Buff et al. 2002, Kearns et al. 2006). Leptin secretion in obese horses is stimulated by insulin and inversely related to IS (Cartmill et al. 2003, Kearns et al. 2006).

Another hormone produced by AT is adiponectin. It forms trimers, hexamers or high molecular weight multimers in the circulation. The effects of adiponectin include the enhancement of IS, anti-inflammatory properties and inhibition of the development of atherosclerosis (Radin et al. 2009). Insulin sensitizing properties may be mediated by stimulating phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and, subsequently, the increase of glucose uptake (Hopkins et al. 2007). In contrast to leptin, adiponectin concentrations in horses are negatively correlated with fat mass and BCS (Kearns et al. 2006, Gordon et al. 2007).

1.4 Insulin signaling

Insulin initiates its functions by binding to the InsR in IS target tissues. The InsR is a heterotetrameric transmembrane receptor protein located within the cell membrane and is composed of two extracellular α-subunits and two transmembrane β-subunits. The transmembrane domain conveys a signal to the intracellular domain that insulin has bound to the receptor, and this signal involves tyrosine kinase (Patti and Kahn 1998). When insulin binds to its receptor, tyrosine kinase is activated; this leads to autophosphorylation of the β-sub-unit at the multiple tyrosyl residue, starting an intracellular signaling pathway that causes phosphorylation of cytosolic protein substrates, such as insulin receptor substrate-1 (IRS-1) (Saltiel and Kahn 2001).

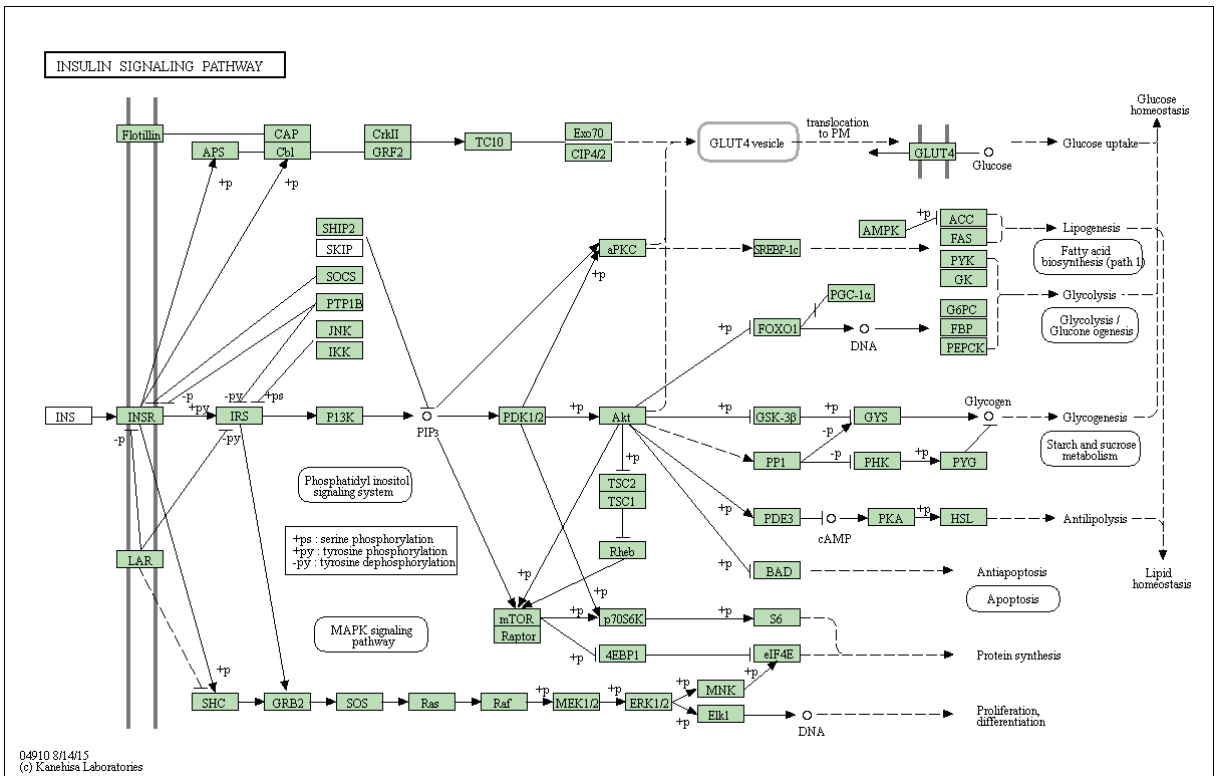

Fig. 1: Key components of insulin signaling (according to http://www.genome.jp/kegg/pathway.html; Kanehisa et al. 2017). InsR - Insulin receptor; Pi3K - Phosphoinositide 3 kinase; PKB/AKT - Protein kinase B; mTOR - Mechanistic target of rapamycin; Foxo - Forkhead box protein; HSL - Hormone-sensitive lipase; GSK-3β - Glycogen synthase kinase 3β; FAS - Fatty acid synthase; PKC ζ - Protein kinase C ζ; AMPK-α - Adenosine monophosphate-activated kinase α; GLUT4 - Glucose transporter 4.

The binding of IRS1 and IRS 2 to an InsR leads to an activation of the phosphatidylinositol-3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathway (Ogawa et al. 1998). The activation of the MAPK pathway mediates the effect of insulin on mitogenesis and cell growth, whereas activation of the PI3K pathway mediates the effect of insulin on metabolism. The PI3K catalyzes the phosphorylation of phosphatidylinositoldiphosphat (PIP2) to phosphatidylinositoltriphosphat (PIP3), an important key in signaling.

The protein kinase B (PKB/AKT) which is activated by phosphorylation, is a central hub of this signaling cascade. Activation of PKB/AKT leads to phosphorylation of several important downstream effectors. Exemplarily, it phosphorylates and inhibits glycogen synthase kinase 3β (GSK-3β), which, in turn, dephosphorylates and activates glycogen synthase (GS) (Saltiel and Kahn 2001). Furthermore, PKB/AKT regulates metabolism and survival by controlling the expression of genes through transcription factors, such as Forkhead box protein (FOXO). The latter is phosphorylated by PKB/AKT and suppresses glucose production in the liver (Guo et al. 1999, Zhang et al. 2012).

Stimulation of the mechanistic target of rapamycin (mTOR) by PI3K activation results in the direct control of mechanistic translation machinery by phosphorylation and activation of p70 ribosomal S6 kinase and the inhibition factor 4E. Therefore, cell growth and metabolism, in response to nutrients, and growth factors and energy status are controlled by mTOR (Sengupta et al. 2011).

The adenosine monophosphate-activated protein kinase (AMPK) is one of the most important regulators in general cell metabolism. It monitors cellular energy status, increases glucose uptake, and initiates glycolysis and β-oxidation as catabolic pathways or inhibits gluconeogenesis as an anabolic pathway (Kahn et al. 2005). This enzyme consists of three different subunits (α, β and γ). The catalytic α-subunit is further differentiated into an α1 and α2 form. The latter is a more specific form, expressed predominantly in skeletal muscle, cardiac muscle and LT (Musi et al. 2001). Activation of AMPK is mediated by lowered adenosine monophosphate (AMP) concentrations and cellular hypoxia and is, therefore, an energy status sensor of the cell. Due to the lack of energy, AMPK inhibits energy consuming processes, such as

lipogeneses and glycogensynthesis, and stimulates energy supplying processes, such as glucose uptake, glycolysis, glycogenolyses, lipolysis and fatty acid oxidation (Winder and Hardie 1999). Activation of AMPK stimulates the translocation of glucose-transporter 4 (GLUT4) into the plasma membrane (Murgia et al. 2009). Glucose transporter 4 is one of the insulin-dependent glucose transporters which carries glucose along the glucose concentration gradient into the cell. Translocation to the plasma membrane is regulated directly by PKC ζ activation in the insulin signaling cascade due to insulin stimulation and, thereby, increases glucose uptake (Bevan 2001, Saltiel and Kahn 2001).

In adipose metabolism, AMPK inhibits the activity of the enzyme acetyl-coA-carboxlase (ACC) and, thereby, the transformation from acetyl-coA to malonyl-coA, which is the end product of fatty acid synthesis in humans (Kahn et al. 2005). Hormone-sensitive lipase (HSL) is one of the key enzymes in AT regarding the regulation of adipose metabolism. Lipolysis is activated in adipocytes mainly by protein kinase A (PKA)-mediated phosphorylation of HSL and perilipin. The HSL catalyzes the hydrolysis of TRG and is responsible for supplying the liver NEFA for hepatic VLDL synthesis.

The antilipolytic effect of insulin on the adipose metabolism is mediated by low cyclic adenosine monophosphate (cAMP) levels. Inhibition of cAMP production via activating phosphodiesterase 3 (PDE3) by PKA results in decreased NEFA and glycerol concentrations and inhibition of the hepatic glucose production (Sindelar et al. 1997).

Fatty acid synthase (FAS) is a multi-enzyme complex which synthesizes malonyl-CoA and acetyl-CoA, is counter regulatory to HSL and promotes the synthase of fatty acids in AT. Insulin stimulates lipogenesis by increasing the FAS expression and activity, a pathway inhibited AMPK (Volpe and Vagelos 1974).

1.4.1 Current knowledge on insulin signaling in horses

Insulin signaling has been studied in multiple species, but only afew studies have been performed in horses to detect components of insulin signaling on a protein or mRNA level and to evaluate their modulation by insulin.

Campolo et al. (2016) studied InsR and insulin-like growth factor 1 receptor protein expression in cardiac and skeletal muscle in healthy horses and found no significant differences between the muscle types. However, analysis on an mRNA level revealed that hyperinsulinemic horses expressed higher amounts of PKB/AKT, GSK3β, GLUT1 and GLUT4 in the cardiac muscle compared to the skeletal muscle. Interestingly, IR horses had lower GLUT4 in the plasma membrane of muscle cells compared to IS horses in another study (Waller et al. 2011).

The HI established by clamp procedures provoked an increase in the extent of phosphorylation in PKB/AKT in MT, which is in accordance with the study mentioned previously. Moreover, 4E binding protein 1 and riboprotein S6, two further downstream targets of mTOR, showed an increased extent of phosphorylation in the MT (Urschel et al. 2014b). Selim et al. (2015) investigated the effect of grazing season and IR on the expression of genes associated with obesity and IR in subcutaneous AT and found a significant downregulation of InsR and upregulation of adiponectin receptor 1 gene expressions in healthy mares and mares suffering from EMS.

2 HYPOTHESIS AND AIMS

Disturbed insulin regulation has been identified as a central part of the EMS. Variations in insulin regulation reflected either as tissue IR or ID result in moderate to severe HI. Despite extensive research in the field of EMS, the HI is the only sign that has been clearly linked to the most important disease sequel: laminitis.

Several pathomechanisms provoking IR in horses have been suggested, but insulin signaling in healthy horses has not been studied in detail so far. However, detection of potential pathophysiological conditions in insulin signaling require knowledge of physiological insulin signaling and possible equine-specific variations.

Thus the objective of the present PhD project was to investigate equine IS, glucose homeostasis and lipid metabolism by combining examinations of insulin signaling on a protein level in different main metabolic tissues and analyses of dynamic hormone and metabolite changes during acute stimulations.

Furthermore, systemic markers of IR were analyzed by an innovative targeted metabolomics approach to provide better understanding of potential pathomechanisms involved in impaired insulin regulation.

Central hypotheses:

Hypothesis #1:

Differences in body condition will lead to variable changes in endocrine and metabolic responses to oral glucose or IV glucose and consecutive insulin stimulation in healthy horses.

Obesity and IS are negatively correlated. Therefore, it was expected that obese horses would respond with more profound changes in blood parameters than lean horses. Moreover, we expected OGT and CGIT to mirror different aspects of IS, glucose

homeostasis and lipid metabolism and not to be equally suitable in assessment of IS based on their different physiological mode of action.

Hypothesis #2: *Acute short-term stimulation with IV insulin will provoke changes in the phosphorylation of key components involved in insulin signaling in various equine tissues.*

Little is known about insulin signaling in horses. However, IR and HI are considered to be a major condition in horses and ponies suffering from EMS. It was expected that IV insulin would activate and phosphorylate InsR and, thereby, induce further insulin signaling in the AT, MT and LT, reflected by the phosphorylation of key proteins and, finally, insulin-mediated glucose uptake. Moreover, it was expected that anti-lipolytic effects of insulin would be reflected by the downregulation of lipolysis and dephosphorylation of HSL and decreased NEFA concentrations. It was expected that horses with different body condition would react with variable responsiveness in the insulin signaling cascade.

Hypothesis #3: *Insulin resistance affects metabolic profiles under the basal and under stimulated conditions provoked by OGT.*

It was expected that IS and ID horses and ponies would have diverse metabolic profiles. Due to impaired insulin regulation and the metabolic consequences, we expected ID individuals to respond differently to an oral glucose challenge compared to IS individuals. We speculate that the analyses of different metabolite changes will provide further information to define metabolic pathways involved in ID. Furthermore, we expected ID horses and ponies to react with more profound changes towards a proinflammatory response compared to ID ones.

The aims of the study:

1. The first aim of the study was to perform a standard dosed OGT and CGIT consecutively in lean and obese horses to compare and describe the endocrine and metabolic responses.

2. The second aim of the study was to determine the protein expression of key components of insulin signaling and their extent of phosphorylation in different equine tissues under basal and stimulated conditions with HI and hyperglycemia provoked by IV injection of insulin and glucose in lean and obese horses.

3. The third aim of the study was to analyze the metabolic profiles in horses of unknown insulin status under basal conditions and in response to a standard dosed OGT with a targeted metabolomics approach to identify novel metabolites associated with ID in horses.

The novelty of this research project was to investigate the protein expression and phosphorylation status of important key components of insulin signaling in multiple equine tissues at the same time under basal and hyperinsulinemic conditions with concordant analyses of blood samples. This allows the linking of molecular changes on a protein level to activation or deactivation of specific metabolic pathways reflected by dynamic variations in central blood metabolites and hormones.

By including innovative metabolomics into the research project, it was expected to provide further information about specific metabolic pathways associated with EMS and ID, to draw conclusions concerning potential pathomechanisms and enforce the generation of new hypotheses for future research projects.

3 MANUSCRIPT I

Comparison of endocrine and metabolic responses to oral glucose test and combined glucose-insulin test in horses

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Contribution to the manuscript

DR, KF and KH designed the study; TW, KF and KH performed the experiments, TW analyzed and interpreted the data; TW, DR, KF and KH drafted and revised the data; TW wrote the manuscript; DR, KF and KH approved the final version of the manuscript.

Abstract

Different diagnostic tests to determine the insulin sensitivity in horses are commonly used in veterinary practice. However, physiological processes provoked by the respective test procedures are not well described. In the present study oral glucose test (OGT) and combined intravenous glucose-insulin test (CGIT) were employed under standardized conditions. OGT and CGIT were performed in twelve healthy warmblood horses of different sex, age (15±6.5 years), weight (567±81 kg) and body-condition-score (BCS) (4.8±1.6). Horses were tested under fasting conditions. OGT was performed with 1 g/kg BW glucose administered via naso-gastric-tubing and CGIT was performed with injection of 150 mg/kg BW glucose solution and 0.1 IU/kg BW porcine zinc-insulin. Blood samples were taken for 3 hours in at least 15-min intervals and were analyzed for insulin, glucose, triglyceride, non-esterified-fatty-acids (NEFA), fructosamine and cortisol concentrations. Glucose concentrations increased in OGT and CGIT directly after administration. Insulin concentrations increased significantly in OGT within 30 minutes and stayed elevated for 3 hours. In CGIT peak concentrations of 493.98±86.84 µIU/mL were measured, followed by a continuous decline. Baseline NEFA concentrations varied between individual horses, and declined in comparable manner to similar minimum concentrations of 93.82±53.22 µmol/L in OGT and 91.97±56.89 µmol/L in CGIT. With respect to the stress response of the test procedure cortisol concentrations remained unaffected during CGIT, while the OGT procedure was accompanied by a significant initial rise in cortisol concentrations. To conclude, OGT and CGIT mirror different facets of the metabolic response to a glycemic stimulus, highlighting different aspects of glucose homeostasis and insulin regulation. Moreover, oral testing protocols are superior to intravenous protocols to assess ID because of their physiological mode of action. During CGIT, insulin dynamics with porcine zinc-insulin differ from insulin dynamics described in previously published reports using short acting insulins. Furthermore, insulins antilipolytic effects during OGT and CGIT via endogenous secretion or exogenous injection resulted in similar reduction of NEFA concentrations and unaffected triglyceride concentrations. This indicates a saturation of the suppression of lipolysis by insulin with already low concentrations and no induction of re-esterification in liver tissue.

Keywords: Horse, Insulin, Insulin dysregulation, Insulin resistance, Oral glucose test, Combined glucose-insulin test, Diagnostic test, Physiology

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Comparison of endocrine and metabolic responses to oral glucose test and combined glucose-insulin tests in horses

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Summary: Different diagnostic tests to determine the insulin sensitivity in horses are commonly used in veterinary practice. However, endocrine and metabolic responses provoked by physiological processes during the respective test procedures are not well described. In the present study, oral glucose tests (OGTs) and combined iv glucose-insulin tests (CGITs) were employed under standardized conditions. The OGTs and CGITs were performed in twelve healthy warmblood horses of different sex, age (15 ± 6.5 years), weight (567 ± 81 kg) and body condition score (4.8 ± 1.6). Horses were tested under fasting conditions. The OGT was performed with 1 g/kg BW glucose administered via nasogastric intubation and CGIT was performed by injection of 150 mg/kg BW glucose solution and 0.1IU/kg BW porcine zinc-insulin. Blood samples were taken for three hours at intervals of 15 minutes and were analysed for insulin, glucose, triglyceride, non-esterified fatty acids (NEFA), fructosamine and cortisol concentrations. Glucose concentrations increased in the OGTs and CGITs directly after administration. Insulin concentrations increased significantly in OGTs within 30 minutes and stayed elevated for three hours. Peak concentrations of 493.98 ± 86.84 µIU/mL were measured in the CGITs, followed by a continuous decline. Baseline NEFA concentrations varied between individual horses and declined in a comparable manner to similar minimum concentrations of 93.82 ± 53.22 µmol/L in OGTs and 91.97 ± 56.89 µmol/L in CGITs. Regarding the stress response of the test procedure, cortisol concentrations remained unaffected during CGITs, while the OGT procedure was accompanied by a significant initial rise in cortisol concentrations. To conclude, OGT and CGIT mirror different facets of the metabolic response to a glycemic stimulus, highlighting different aspects of glucose homeostasis and insulin regulation. During the CGIT, insulin dynamics with porcine zinc-insulin differ from insulin dynamics described in reports published previously using short-acting insulins. Furthermore, the antilipolytic effects of insulin during OGTs and CGITs via endogenous secretion or exogenous injection resulted in similar reduction of NEFA concentrations and unaffected triglyceride concentrations. This indicates a saturation of the suppression of lipolysis by insulin with already low concentrations and no induction of re-esterification in liver tissue.

Keywords: horse, insulin, insulin dysregulation, insulin resistance, oral glucose test, combined glucose-insulin test, diagnostic test, physiology

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Introduction

Equine metabolic syndrome (EMS) has been growing steadily in importance in the equine population over the last few years and has been the subject of many studies, workshops and discussions. Alterations in insulin regulation in affected horses are one of the leading laboratory findings, together with predisposition to laminitis and general or regional obesity (*Frank* et al. 2010, *Johnson* et al. 2010, *Frank* and *Tadros* 2014). Obesity prevalence is high in the equine population and studies performed in the UK and the USA published obesity prevalence rates ranging between 30 and 48% (*Thatcher* et al. 2008, *Wyse* et al. 2008, *Giles* et al. 2014). In addition, horses presenting to a first opinion hospital for evaluation of laminitis were hyperinsulinemic in 86% of cases (*Karikoski* et al. 2011), indicating the importance of endocrinopathic laminitis. Easy and quick to perform approaches to diagnose insulin dysregulation (ID) or insulin resistance (IR) are required from clinicians. Complex hyperinsulinemic euglycemic clamp tests, which were considered to be the gold standard for assessment of IR (*Rijnen* and *van der Kolk* 2003), are usually reserved for research approaches due to their complex and expensive implementation. In practice, assessment of ID is often based on the measurement of single resting glucose and insulin concentrations, but dynamic diagnostic tests are also performed. Dynamic testing is currently recommended and offers additional information about metabolic responses to certain glucose or insulin, or even combined glucose and insulin stimulations (*Frank* et al. 2010, Equine Endocrinology Group 2016). To date, clinicians have various options of testing protocols for assessment of alterations in insulin regulation, and can chose between protocols based on oral or intravenous (iv) procedures.

The aim of this study was to compare the oral glucose test (OGT) (*Ralston* 2002) and the combined iv glucose-insulin test (CGIT) (*Eiler* et al. 2005) in healthy horses and to obtain physiological information about adaptive metabolic and endocrine processes, with a main focus on insulin and glucose dynamics and their relation to the lipid homeostasis.

It was hypothesized that the OGT and CGIT would give different results in the same horse due to the activation of different

endocrine and metabolic pathways, and addressed the suitability of the tests to be "easy and quick to perform" to diagnose ID or IR in practice.

Materials and Methods

Animals

Twelve healthy warmblood breed horses were included in the study. There were seven mares, two geldings and three stallions, aged (mean ± SD) 15 ± 6.5 years and weighed 567 ± 81 kg. The horses included covered a wide range of body condition scores (BCSs) from 1.9 to 7.5 with a mean BCS of 4.8 ± 1.6 (Table 1). The BCS was determined as the average of five independent assessors according to the scoring system of *Henneke* et al. (1983). The horses were fed average mixed grass/hay twice daily and no supplementary feeding was provided for two weeks prior to the start of the experiment. They were stabled in individual boxes, under standardized feeding and management conditions, and were not exercised, with the exception of two hours on a paddock daily. All horses underwent clinical examinations, laboratory screenings and radiographically examinations of the hooves prior to the start of the study. None of the study horses showed clinical signs of pituitary pars intermedia dysfunction (PPID) or acute or chronic laminitis.

Study design

The horses were fed hay the evening before the test, but food was withheld overnight and in the morning for about 14 hours prior to testing. The horses were muzzled to avoid excessive straw or flex bedding uptake. Water was available continuously before and during the test procedure. Testing of each horse started between 10 and 11a.m. to avoid daily variations in serum insulin concentrations (*Firshman* and *Valberg* 2007). Regarding the seasonal variation of the plasma insulin concentrations described (*Place* et al. 2010, *Funk* et al. 2012), all horses underwent the study examination in autumn and winter during October through January. The horses completed both tests over a two-week period. The trial started for each horse with the OGT, followed by a recovery phase of nine days before the CGIT was performed.

Oral Glucose Test (OGT)

An iv indwelling catheter size 12G (EquiCath™ Fastflow, Braun Vet Care GmbH, Tuttlingen, Germany) was aseptically implanted in the left or right jugular vein of the horses for the collection of blood samples twelve hours before the start of the OGT. An amount of 1g/kg body weight (BW) glucose powder (Glukose, WDT, Garbsen, Germany) was dissolved in two litres of water and administered by nasogastric intubation (*Ralston* 2002). Prior to the administration of glucose solution, a first basal blood sample was extracted via a jugular vein catheter. After administration of the glucose solution, blood samples were obtained at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 minutes. The catheter was flushed with saline solution (NaCl; 0.9%; B. Braun Melsungen AG, Germany) after each blood sample removal.

Combined Glucose-Insulin Test (CGIT)

The horses underwent a modified version of the CGIT procedure first described by *Eiler* et al. (2005). Two iv indwelling catheters size 12G (EquiCath™ Fastflow, Braun Vet Care GmbH, Germany) were aseptically implanted in each jugular vein of the horses twelve hours before the start of the CGIT. One catheter was used for the administration of glucose solution and insulin, and the second one for the collection of blood samples during the testing procedure. An amount of 150mg/kg BW glucose solution (Glucose 500mg/mL, B. Braun Melsungen AG, Germany) were injected intravenously as a bolus within 1 minute, immediately followed by 0.1IU/kg BW porcine zinc-insulin (Caninsulin® 40I.E./ml, MSD, Unterschleißheim, Germany). Due to legal restrictions, veterinarians in Germany are legally bound to pharmaceuticals labelled for veterinary use before drug formulations for human purposes can be used, with the exception that the human formulation provides an essential advantage for the specific application. Therefore, we decided to test porcine zinc-insulins suitability for implementation of the CGIT. In contrast to

Table 1 Age, sex, body weight and body condition score of all study horses

Horse	Age (years)	Sex	Body Weight (kg)	Body Condition Score (BCS)
1	19	mare	465	2.9
2	19	mare	440	1.9
3	24	gelding	480	3
4	4	stallion	540	6
5	15	mare	619	5.1
6*	16	mare	663	6.5
7	15	stallion	515	5.1
8	25	mare	570	4.5
9	4	stallion	560	4
10	11	gelding	640	5.9
11	20	mare	537	4.7
12	13	mare	695	7.5

* no combined glucose-insulin test (CGIT) due to technical reasons

the original protocol developed by *Eiler* et al. (2005) that employed a fast-acting human recombinant insulin formulation. An amount of 20 mL saline solution (NaCl; 0.9%; B. Braun Melsungen AG, Germany) were used to flush the catheter after the insulin injection. A first blood sample was taken via the jugular catheter prior to the administration of glucose and insulin. Subsequently, blood samples were taken at 3, 6, 9, 12, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 minutes. The catheter was flushed with saline solution (NaCl; 0.9%; B. Braun Melsungen AG, Germany) after each blood sample removal.

Blood samples and analyses

An amount of 5 mL of blood were withdrawn through the catheter and discarded before 20 mL blood were taken for measurements. The catheter was flushed with 10 mL saline solution after each sampling step. Blood samples were placed into tubes containing fluoride oxalate for the determination of glucose concentrations and into plain tubes for serum preparation. Blood samples for serum preparation were incubated at room temperature for sixty minutes, centrifuged at 1000 × g for 6 minutes, and stored at -80°C until further analysis. Glucose concentrations were measured immediately after finishing the diagnostic test by using a colorimetric assay (GLUC3, Cobas, Roche Diagnostics GmbH, Mannheim, Germany) on an automated discrete analyser (Cobas Mira, Roche Diagnostics GmbH, Mannheim, Germany). Serum insulin concentrations were analysed by using a commercially available equine-optimized enzyme-linked immunosorbent assay (ELISA; Equine Insulin ELISA, Mercodia, Uppsala, Sweden) previously validated for use in horses (*Öberg* et al. 2012, *Warnken* et al. 2016). Samples with insulin concentrations greater than the highest value of the standard curve from the ELISA (>1.5 µg/L) were diluted with Diabetes Sample Buffer (Diabetes Sample Buffer, Mercodia, Uppsala, Sweden). The conversion factor 115 is suggested by the manufacturer (*Ab* 2017) to convert the results supplied in µg/L by the ELISA into the SI unit mU/L or µIU/mL used commonly. Triglycerides (TRG) and non-esterified fatty acids (NEFA) concentrations were measured with commercial kits (ABX Pentra Triglycerides CP, HORIBA ABX, Montpellier, France; Wako NEFA-HR(2), Wako Chemicals GmbH, Neuss, Germany) for enzymatic colorimetric measurements on an automated discrete analyser (ABX Pentra 400, HORIBA ABX SAS). Cortisol was measured by a commercial chemiluminescent immunoassay kit with an automated analyser (IMMULITE Cortisol, Siemens Medical Solutions, Bad Nauheim, Germany) and fructosamine concentrations were measured with a commercially available kit (FRA, Roche Diagnostics GmbH, Mannheim, Germany) for automatic analysers (Cobas Mira, Roche Diagnostics GmbH, Mannheim, Germany).

Statistics and calculations

Data analysis was performed using GraphPad Prism software (version 7.02; GraphPad Inc. La Jolla, CA, USA). Data were tested for normality using the Shapiro-Wilk normality test. Time courses were compared with RM One-Way ANOVA followed by Tukey's multiple comparisons test, as appropriate. Comparisons between basal metabolite and hormone concentrations on OGT and CGIT days were performed with student's paired t-tests. Areas under the curve (AUC) were calculated for insulin, glucose, NEFA, TRG and cortisol with the trapezoid method. Pearson and Spearman correlations were used to compare the laboratory parameter with individual background data, i.e. gender, age, BW and BCS. Maximal and minimal concentrations (Cmax and Cmin) for insulin, glucose, NEFA, TRG and cortisol were calculated, as well as metabolite and hormone concentrations at specific time points. Comparison between tests and groups was performed with RM two-way ANOVA or two-way ANOVA followed by Sidak's multiple comparisons test. Statistical significance was accepted at $P < 0.05$. All values are expressed as mean ± SD, unless otherwise indicated.

Results

All horses tolerated the experiments well. Neither clinical signs of hypoglycaemia were observed during CGIT, nor did horses discover signs of laminitis during the whole study period. One of the twelve horses exhibited slight colic symptoms during the washout phase between OGT and CGIT, whereby the CGIT was postponed by one week. One horse (horse 6)

Table 2 Hormone and metabolite concentrations during oral glucose test (OGT). Mean±SD, n = 12.

	insulin (µIU/mL)	glucose (mmol/L)	NEFA (µmol/L)	TRG (mmol/L)	cortisol (ng/mL)
baseline	6.42 ± 3.72	4.61 ± 0.31	402.08 ± 216.30	0.28 ± 0.22	41.37 ± 17.53
C_{min}	6.23 ± 3.81	4.59 ± 0.31	93.82 ± 53.22	0.20 ± 0.24	34.35 ± 17.10
C_{max}	69.10 ± 39.07	8.46 ± 0.89	469.08 ± 224.24	0.34 ± 0.27	80.35 ± 29.85
C_{120min}	54.05 ± 32.02	7.98 ± 1.09	133.86 ± 61.57	0.24 ± 0.26	47.59 ± 25.88

Table 3 Hormone and metabolite concentrations during combined glucose-insulin test (CGIT). Mean±SD, n=11.

	insulin (µIU/mL)	glucose (mmol/L)	NEFA (µmol/L)	TRG (mmol/L)	cortisol (ng/mL)
baseline	5.33 ± 2.07	4.63 ± 0.23	492.00 ± 230.26	0.27 ± 0.11	41.57 ± 14.71
C_{min}	5.12 ± 2.20	3.81 ± 0.65	91.97 ± 56.89	0.17 ± 0.08	33.24 ± 14.38
C_{max}	493.98 ± 86.84	10.49 ± 0.93	613.91 ± 224.69	0.29 ± 0.10	57.04 ± 23.21
C_{45min}	51.96 ± 34.78	6.35 ± 1.07	205.30 ± 139.99	0.28 ± 0.26	
$C_{60min\ cortisol}$					45.38 ± 26.56

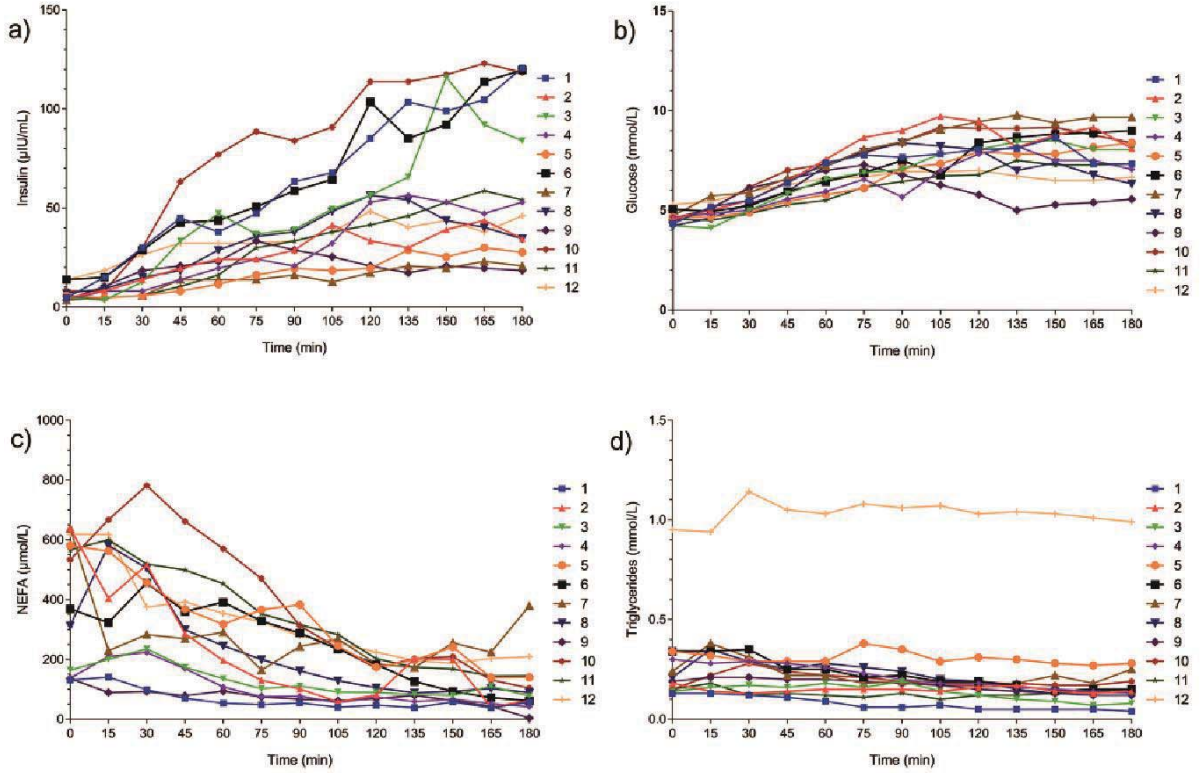

Fig. 1 Hormone and metabolite concentrations of twelve study horses during oral glucose tests (OGTs). a) Insulin (µIU/mL), b) glucose (mmol/L), c) non-esterified fatty acids (NEFA) (µmol/L) and d) triglycerides (TRG) (mmol/L).

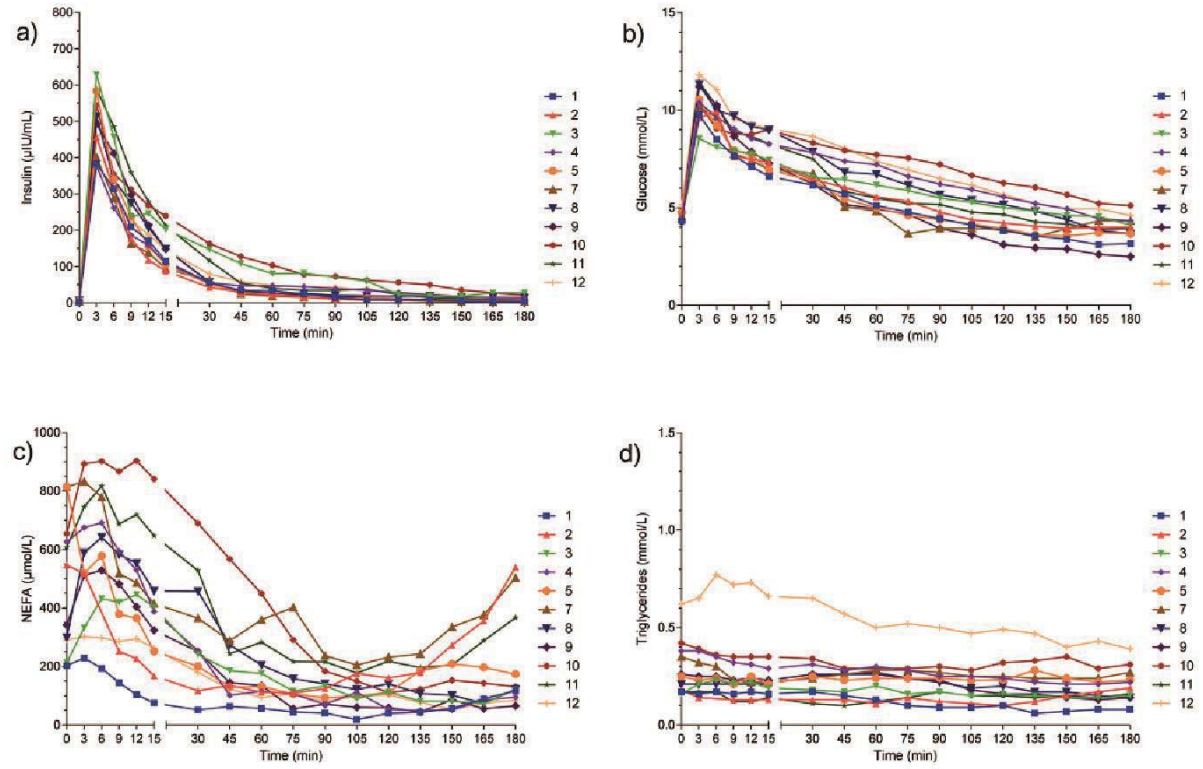

Fig. 2 Hormone and metabolite concentrations of eleven study horses during CGITs. a) Insulin (µIU/mL), b) glucose (mmol/L), c) NEFA (µmol/L) and d) TRG (mmol/L).

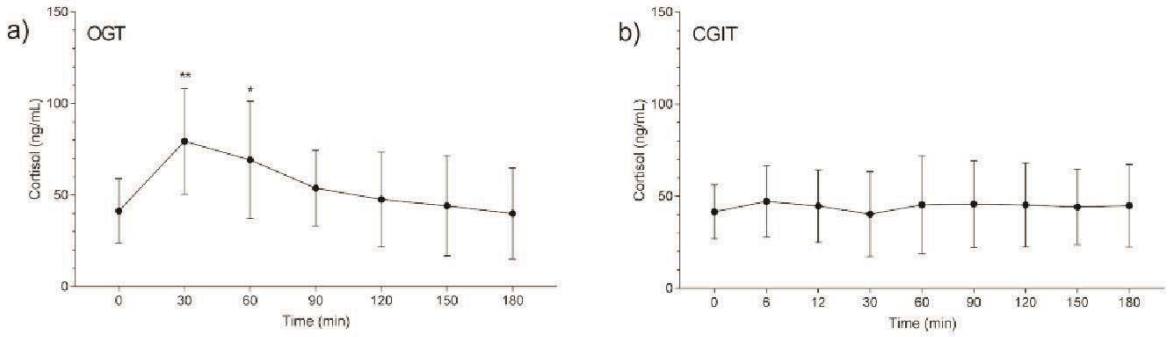

Fig. 3 Cortisol concentrations (ng/mL) during a) OGT and b) CGIT. Given are means ± SD; one-way ANOVA; * P < 0.05 ** P < 0.01.

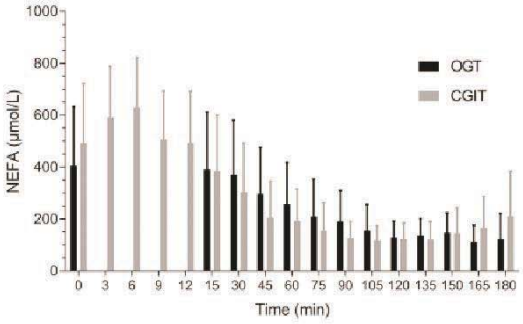

Fig. 4 NEFA concentrations during OGT (black) and CGIT (grey). Means ± SD are given.

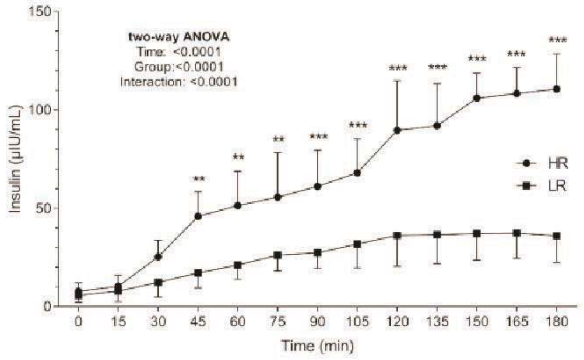

Fig. 5 Serum insulin concentration (µIU/mL) during the OGT in high responding (HR) and low responding (LR) animals. Means ± SD are given; two-way ANOVA; ** P < 0.01 *** P < 0.001. HR: n = 4; LR: n = 8.

was excluded from the analysis of CGIT because of technical reasons.

Baseline concentrations in all metabolites and hormones examined did not differ significantly between OGT and CGIT days (Tab. 2 and 3; Fig. 1 and 2). The fructosamine concentration was 323.73 ± 25.2 µmol/L prior to OGT and 319.6 ± 26.0 µmol/L prior to CGIT and remained unaffected during both testing procedures.

The horses had basal cortisol concentrations of 41.37 ± 17.53 ng/mL prior to OGT and responded to glucose administration via nasogastric intubation with an increase in cortisol to 79.34 ± 28.95 ng/mL after 30 minutes (P = 0.0020), the first sample which was analysed for cortisol. Cortisol concentration decreased consistently during the test procedure which followed and did not differ significantly from basal concentrations 90 minutes after intubation (Fig. 3, Tab. 2). Basal cortisol concentrations were 41.57 ± 14.71 ng/mL prior to CGIT and remained unaffected during the testing procedure (Fig. 3b, Tab. 3).

Insulin, glucose and NEFA dynamics during OGT and CGIT

OGT

Insulin concentration started to increase after 30 minutes (P = 0.0330) during the OGT, with a huge variation in individual horses, and remained elevated until the end of the sampling period, i.e. 180 minutes (P = 0.0141) (Fig. 1a). A $C_{max\ insulin\ OGT}$ of 69.10 ± 39.07 µg/L was reached after 75 to 180 minutes (mean: 148 ± 32 minutes), but C^{max} insulin OGT varied from 23 to 123.05 µIU/mL. Four out of twelve horses exhibited maximal insulin concentrations above 100 µIU/mL, whereas the remaining eight horses ranged between 23 µIU/mL and 58.65 µIU/mL (Fig 1a). The $C_{120min\ insulin\ OGT}$

Table 4 Hormone and metabolite area under the curves (AUC) during oral glucose test (OGT) and combined glucose-insulin test (CGIT). Mean±SD; OGT n = 12; CGIT n = 11.

area under the curve (UC)	OGT	CGIT
insulin (µIU/mL x min)	6991.62 ± 3773.34	10362.75 ± 4242.51
glucose (mmol/L x min)	1249.42 ± 120.13	1006.27 ± 156.40
NEFA (µmol/L x min)	40428.42 ± 19791.13	36807.73 ± 17147.87
TRG (mmol/L x min)	46.32 ± 45.51	41.71 ± 20.51
cortisol (ng/mL x min)	10349.45 ± 4549.50	8269.90 ± 3935.52

was 54.05 ± 32.02 µIU/mL (Tab. 2). Blood glucose concentration increased immediately after the administration of glucose in all horses and no decrease back to baseline levels was observed during the whole OGT testing period in any horse (Fig. 1b). The increase in serum glucose concentration was significantly different after 30 minutes compared to baseline levels (P = 0.0051). The basal NEFA concentration (402.08 ± 216.30 µmol/L) had a wide variety after fasting conditions at the beginning of the test prior to glucose application, and declined during OGT procedure in all horses (P < 0.0001) (Fig. 1c). The NEFA concentration (199.60 ± 116.20 µmol/L) decreased significantly (P < 0.05) within 90 minutes after glucose administration. The mean $C_{min\ NEFA\ OGT}$ was 93.82 ± 53.22 µmol/L and was reached in 153 ± 33 minutes (range 75 to 180 minutes). The horses reached a plateau phase with only marginal changes in NEFA concentration towards the end of the sampling period from 120 to 180 minutes.

CGIT

Serum insulin concentration increased from 5.33 ± 2.07 µIU/mL at baseline to $C_{max\ insulin\ CGIT}$ 493.98 ± 86.84 µIU/mL three minutes after injection in the CGIT (Fig. 2a, Tab. 3). All horses showed an even decline in insulin concentration immediately after the initial peak. The peak serum insulin concentration was nearly halved (243.80 ± 64.40 µIU/mL) after nine minutes. Only three out of twelve horses returned to baseline insulin concentrations after 150 or 165 minutes (Fig. 2a). The mean serum insulin concentration ($C_{45min\ insulin\ CGIT}$) was 51.96 ± 34.78 µIU/mL 45 minutes after injection of glucose and insulin. The glucose concentration increased from physiological baseline concentrations of 4.63 ± 0.23 mmol/L to peak concentrations of 10.49 ± 0.93 mmol/L at the first sampling time point three minutes after injection (P < 0.0001) (Fig. 2b, Tab 3). Thereafter, plasma glucose concentration declined and returned to baseline concentrations 123 ± 37 minutes after the beginning of insulin and glucose injection. Decline continued slightly, with minimal glucose concentrations of 3.81 ± 0.65 mmol/L (Fig. 2b). Clinical signs of hypoglycaemia were not observed in any of the horses during the test procedure. The $C_{45min\ glucose\ CGIT}$ was 6.35 ± 1.07 mmol/L.

The basal NEFA concentration varied greatly, similar to that already observed prior to OGT. The baseline concentrations were 492.00 ± 230.26 µmol/L and NEFA concentrations decreased in all horses with the first statistically significant change compared to baseline concentrations after 75 minutes (P = 0.0186). The $C_{min\ NEFA\ CGIT}$ was 91.97 ± 56.89 and was reached after 123 ± 30 minutes (Fig. 2c, Tab. 3). The NEFA concentrations during OGTs and CGITs were comparable with similar decrease and comparable $C_{min\ NEFA}$ concentrations (OGT 93.82 ± 53.22 µmol/L; CGIT 91.970 ± 56.89 µmol/L) (Fig. 4).

Individual variations in endocrine and metabolic response

The twelve horses were clustered into two groups based on these findings and the underlying variations in insulin concentrations (absolute values, AUC) in the OGT. Four horses (high responder – HR) exhibited significantly higher insulin concentrations over time (P < 0.0001) in response to the oral glucose load compared to the eight horses remaining (low responders – LR) (Fig. 5). The four HR horses exhibited significantly higher values for AUCinsulin OGT (10289.05 ± 1070.65 µIU/mL × min) compared to the LR horses (3685.75 ± 424.35 µIU/mL × min), while comparing AUC-glucose OGT did not reveal any differences. The HR and LR did not differ either in basal insulin concentrations, nor in any other metabolite or hormone concentrations analysed at any time. None of the hormone or metabolite concentrations or responses to the test procedures showed any correlation with gender, age or BCS of the horses.

Discussion

Testing horses for ID is challenging work in routine practice. Obesity prevalence is high in the equine population (*Thatcher* et al. 2008, *Wyse* et al. 2008, *Giles* et al. 2014) and owners are becoming more aware of the potential cross-links between obesity or reginal adiposity, ID and laminitis. Therefore, owners' request for EMS testing has increased in recent times. No standard testing protocol has been established to date in equine medicine to determine ID in equine patients. However, recently published recommendations (Equine Endocrinology Group 2016) provide useful information for targeted diagnostic approaches. Insulin resistance is generally defined as a pathologic condition in which the biological tissue responsiveness to insulin is decreased (*Kahn* 1979). Tissue IR, especially in ponies, is often compensated by an increased pancreatic secretion of insulin, resulting in hyperinsulinemia (*Jeffcott* et al. 1986). Recent research has highlighted the importance of incretins, gastrointestinal tract hormones that effect insulin regulation and glucose homeostasis (*de Laat* et al. 2016). The term ID is used more commonly to consider the complexity related to variations in insulin dynamics and to shift the focus from only peripheral IR to a more complex pattern of impairments of insulin and glucose homeostasis. The term ID highlights the combination of fasting hyperinsulinemia and postprandial hyperinsulinemia assessed, for example, by oral glucose challenge procedures (Equine Endocrinology Group 2016).

Variations in dynamic insulin responses and plasma insulin clearance

Oral stimulation tests include assessment of functions of the gastrointestinal tract. It has recently been shown that, similar to other mammals, glucagon-like peptide-1 (GLP1) and gastric inhibitory peptide, also known as glucose-dependent insulinotropic polypeptide (GIP), are secreted in response to oral glucose or food and may enhance the glucose-dependent insulin secretion in the horse (*Chameroy* et al. 2010, *Bamford* et al. 2014, *de Laat* et al. 2016). The importance of the gastrointestinal tract, i.e. incretins, has been shown by several studies where insulin secretion after oral glucose intake or application was higher than after isoglycaemic iv glucose application (*Dühlmeier* et al. 2001, *de Laat* et al. 2016). Furthermore, it has been shown that ID can occur independently from tissue IR and that iv and oral tests gave different results regarding the insulinemic state being abnormal or nor-

mal in ponies being obese and/or laminitic (*de Laat* et al. 2016). Measuring glucose and insulin increase in plasma and time-dependent disappearance from plasma is the net result of intestinal absorption, glucose uptake by insulin-sensitive and insulin-independent tissues, and urinary glucose spilling. This rate of disappearance is influenced by all glucose-related adaptive physiological responses of peripheral tissues. Therefore, results from oral testing protocols could be influenced by several physiological pathways, thus, oral testing protocols can be used to assess ID and are considered to be indirect methods to assess IR. Oral glucose tests simulate the physiological way of ingestion of meals containing starches and reflect the physiological conditions followed by food intake. The way of application and dosages of glucose or other sugars in OGTs vary between protocols and range from administration of corn syrup with low glucose content (*Carter* et al. 2009, *Schuver* et al. 2014) through in-feed variations, with 0.5 or 1.0 g glucose powder mixed in low-glycemic meals (*Smith* et al. 2015), to glucose application via nasogastric intubation (*Ralston* 2002). In contrast to oral stimulation tests, none of the gastro-intestinal tract-related functions are assessed during iv stimulation tests, such as the CGIT, where glucose and insulin were applied directly into the bloodstream (*Eiler* et al. 2005). Therefore, no absorption from the GIT is necessary, the enteroinsular axis is bypassed and even pancreatic response is thought to be depressed due to the immediate application of supra-physiological doses of insulin. Consequently, besides urinary glucose spilling and insulin clearance by liver passage, only the capacity of exogenous insulin to shift the injected glucose into the insulin-sensitive tissues is assessed with this test.

The OGT in the present study could identify four horses that had a clearly exaggerated insulin response (HR) compared to the other eight horses (LR). The AUCinsulin OGT differed, whereas the AUCglucose OGT did not differ between HR and LR animals, suggesting that HR horses secrete more insulin to maintain their glucose homeostasis after the oral glucose challenge. According to recently published reference ranges for OGT procedures performed by nasogastric intubation and quantification of equine insulin with the ELISA used in the present study, insulin concentrations above 110 µIU/mL insulin at 120 minutes are indicative for ID (*Warnken* et al. 2018). If this cut-off is supplied to the oral sugar test (OGT) data only one horse (horse 10) has slightly elevated insulin response in OGT with 114 µIU/mL. It has been reported that insulin responses after OGT show wide variation and fluent transition from IS to ID (*Warnken* et al. 2018). However, it remains unclear whether these clear differences between horses were normal physiological variation or already indicative of subclinical abnormalities which may predispose these four individuals to develop metabolic pathologies. Differences between animals may also be explained by variations in gastric emptying rate, intestinal glucose absorption, hepatic extraction or urinary glucose spilling if renal threshold was exceeded after enteral glucose absorption or iv glucose.

Variations between the twelve horses due to variable feeding and management regimes were unlikely, since all horses were kept and fed under equal conditions for a fourteen-day acclimatization period in the clinic. Several studies investigated the effect of feeding and different diets on basal glucose and insulin, as well as their dynamics during diagnostic procedure with increased insulin concentrations and decreased insulin sensitivity (*Pratt* et al. 2006, *Bailey* et al. 2007, *Borer* et al. 2012). However, the horses in the present study were all maintained on average grass-hay diet without additional supplement feeding for at least 14 days prior to the start of the study. Besides variations due to feeding managements, seasonal changes in blood glucose and insulin concentrations and dynamics have also been reported (*McIntosh* 2006, *Bailey* et al. 2008, *Borer* et al. 2010, *Banse* and *McFarlane* 2014). In the present study, all horses underwent the study examination in autumn and winter during October through January to minimize seasonal variations in insulin and glucose dynamics.

Frank and colleagues (2006) showed that horses with a moderate BCS of 4 to 6 on a nine-point scale had lower insulin levels compared to horses with a BCS of 7 to 9. *Pleasant* and colleagues (2013) detected that horses aged 17 to 20 years had higher insulin levels and lower insulin sensitivity compared to younger horses in a random sample of 300 light breed horses. Comparably, *Vick* and colleagues (2007) found that age was negatively correlated with insulin sensitivity in a population of horses aged 3 to 29 years. Moreover, this negative correlation was not influenced by BCS, leading to the conclusion that older horses might be generally at a higher risk of developing IR. In this study, we were not able to show associations between BCS or age and insulin concentrations, either in basal insulin concentrations or in dynamic insulin response in OGTs or insulin clearance in CGITs.

Influence of insulins formulation used for CGITs

In contrast to the original protocol developed by *Eiler* et al. (2005) due to legal restrictions, a porcine zinc-insulin formulation was used in the present study to perform the CGIT instead of a fast-acting human recombinant insulin formulation. The porcine zinc-insulin formulation is an intermediary insulin consisting of highly sanitized amorphous insulin and crystalline insulin. According to the results of the present study, this formulation results in higher concentrations and a slower elimination of the insulin after injection compared to short-acting insulins. *Fleeman* et al. (2009) reported two-step responses in blood insulin concentration after subcutaneous porcine zinc-insulin injection in dogs. Mean peak concentrations where observed after three and nine hours, whereas median duration of insulins action was approximately 14 hours. By contrast, blood insulin concentration following subcutaneous injection of regular human insulin peak faster in humans and are reported to reach mean peak concentration after 117 minutes (*Hompesch* et al. 2011). However, reliable pharmacokinetic and pharmacodynamics data following iv or subcutaneous injection in horses are lacking and subject of current research. In the present study, none of the twelve horses returned back to baseline glucose concentrations at the suggested clinical-relevant time-point 45 minutes (C^{45min}) after glucose and insulin administration. Moreover, two horses (horse 3 = 108 µIU/mL and horse 10 = 127 µIU/mL) remained with insulin concentrations above the cut-off value of 100 µIU/mL at 45 minutes published previously (*Eiler* et al. 2005). Nonetheless, adjustment of reference ranges and cut-off values when used in clinical settings for assessment of IR by CGIT with porcine zinc-insulin in patients would be necessary.

A possible advantage of porcine zinc-insulin for CGITs is that the porcine insulin molecule and the equine insulin molecule only differ by one amino acid at position A9, whereas the human insulin molecule differs from the equine insulin molecule at position A9 and position B30 (*Ho* et al. 2008). Position B30 is especially considered to be important for the three-dimensional structure of the protein (*Conlon* 2001) and, thus, might influence receptor binding. Therefore, porcine insulin may act more comparably to the physiological endogenous equine insulin. It was noteworthy that none of the horses in our study exhibited a clinically relevant hypoglycaemia in response to the iv injection of exogenous insulin, either during the sampling period or after sampling had finished. This is a commonly observed adverse effect in CGITs (*Funk* et al. 2012). Thus, the use of a porcine-zinc insulin may be a safer and more physiological alternative to the use of short-acting human insulin preparations in CGITs.

Variations in insulin-dependent NEFA suppression

The concert of maintaining an organism's metabolism is complex. In addition to major aspects in the control of glucose homeostasis, insulin also affects lipid metabolism. Results of the present study clearly illustrate insulin effects on lipid metabolism. Basal NEFA concentrations, determined prior to both tests, showed high interindividual variation. The TRG stored in adipose tissue undergo lipolysis in a state of fasting and NEFA and glycerol are released into the circulation. Previous studies investigating the effect of withholding feed on insulin and very low-density lipoprotein concentrations found significantly increased NEFA concentrations in horses which were subjected to 36 hours of withholding feed compared to fed control horses. The mean NEFA concentrations of 513 µmol/L observed in the study by *Frank* et al. (2002) were comparable to the values of the present study. Fasting horses prior to assessment of IS is discussed controversial. Recent research investigated the optimal fasting period prior to dynamic testing for ID and IR and reported different periods for specific tests. Knowles et al. (2017) reported significant higher insulin responses in oral sugar test (OST) after fasting compared to non-fasted conditions. Currently, a three hour fast is recommended for OST (*Bertin* et al. 2016), whereas overnight fasting is recommended for in-feed OGT and a 14 hours fast has been described prior to CGIT (*Bröjer* et al. 2013). In order to provide similar metabolic conditions prior to testing, horses included in the present study were fasted for 14 hours before OGT and CGIT were started. Euglycemic hyperinsulinemic clamp studies in horses have reported reduced NEFA concentrations during clamp procedures (*Suagee* et al. 2011, *Urschel* et al. 2014). Similar reduction of NEFA during clamp studies in humans occurred and is due to a reduced plasma rate of appearance of fatty acids (*Shadid* et al. 2007). The reduction of plasma NEFA concentration is believed to be due to the inhibitory effects of insulin on the lipolytic enzyme hormone-sensitive lipase, resulting in reduced release of NEFA from adipose tissue (*Meek* et al. 1999). It was shown recently that insulin in the horse also seems to deactivate hormone-sensitive lipase (*Warnken* et al. 2017). Furthermore, recent literature suggested that, in addition to the suppression of lipolysis in adipose tissue, insulin also stimulates free fatty acid uptake from the plasma into peripheral tissues in humans (*Ramos-Roman* et al. 2012). However, whether similar conditions occur in horses is not known so far.

Interestingly, the NEFA dynamics were very similar in OGTs and CGITs, despite the different insulin dynamics in both tests. In the OGT, the insulin concentrations continuously increased, whereas in CGIT, there was an initial increase followed by a continuous decrease. Despite these differences, both led to a comparable decrease in plasma NEFA concentrations. There was no statistically significant difference in NEFA concentration between OGT and CGIT. The C_{min} NEFA was nearly similar during the OGT and CGIT procedure with 93.82 ± 53.22 µmol/L and 91.97 ± 56.89 µmol/L, respectively. This might indicate a saturation of the suppression of lipolysis by insulin already with insulin concentrations, provoked by the OGT after enteral resorption of oral-applied glucose. In accordance with these findings, hyperinsulinemic euglycemic clamp studies indicated that insulin concentrations similar to postprandial insulin concentrations were sufficient for maximal antilipolytic effects (*Urschel* et al. 2014). Moreover, the present study revealed that the anti-lipolytic effect seems to be independent of the insulin's origin. The exogenous porcine zinc-insulin seems to be as equally effective as the endogenous equine insulin.

Absent increase in TRG concentrations during OGT and CGIT in our study indicates that re-esterification of NEFA in liver tissue is unlikely and, thus, insulin seems to suppress lipolysis predominantly in adipose tissues in the horse.

Several studies reported partly conflicting results of correlations between NEFA and/or TRG concentrations and weight gain, obesity or gender (*Frank* et al. 2006, *Carter* et al. 2009, *Pleasant* et al. 2013). In our study, lipid parameters or their response to the challenges did not correlate with age, gender, weight or BCS.

Effect of short-term hyperglycaemia on fructosamine

Fructosamine is used as a marker for the evaluation of abnormal glycaemic control in humans (*Goldstein* et al. 2004) and companion animals (*Reusch* et al. 1993, *Reusch* and *Haberer* 2001) suffering from diabetes for long-term monitoring. Hyperglycaemia, which may affect fructosamine concentrations, is often reported in horses diagnosed with pituitary pars intermedia dysfunction (PPID) (*McFarlane* 2011). However, the potential clinical applicability of fructosamine as a marker in horses is questionable due to a significant overlap of concentration ranges obtained from PPID horses and those horses not suspected of PPID (*Knowles* et al. 2014). In the present study, fructosamine concentrations did not differ significantly pre and post OGT or CGIT, or during the experimental time period. Horses exhibited higher or lower concentrations after glucose challenges with no consistent trend during the testing procedure or the experimental period. Thus, even repeated challenging with both tests and two times of unphysiological hyperglycaemia did not affect fructosamine concentrations in our twelve study horses.

Stress response in OGT and CGIT

Manipulation by nasogastric intubation for the administration of glucose solution into the stomach of the horse may lead to stress in most of the animals. Variability in blood glucose concentrations due to stress during nasogastric intubation is a

commonly discussed issue (*Firshman* and *Valberg* 2007). Furthermore, stress hormones can impact glucose dynamics and alter test results. A corresponding rise in serum cortisol concentrations after manipulations was observed. Nevertheless, horses returned to baseline levels after a short period of time. Protocols for oral glucose tests with oral application or feeding have been used successfully in several studies (*Schuver* et al. 2014, *Smith* et al. 2015, *de Laat* and *Sillence* 2016), but it has been shown that meal size and compositions as well as feed consumption time alter gastric emptying and small intestine motility (*Metayer* et al. 2004). Therefore, these disruptive factors, which may influence test results, are minimized when glucose is administered directly into the stomach of the patient. However, further studies are required to investigate stress response during OGT procedure with nasogastric intubation and examine the interference of test results.

The results of the present study underline that OGT and CGIT mirror different facets of glucose homeostasis and insulin regulation and sensitivity. While CGIT focuses on tissue insulin sensitivity, OGT reflects more facets of glucose homeostasis and insulin regulation, leading to the conclusion that oral testing protocols are superior to iv protocols to assess ID because of their physiological mode of action. Furthermore, insulin dynamics during CGIT with porcine zinc-insulin differ from insulin dynamics described in reports using short-acting insulins published previously, and necessitates adjustment of clinical interpretation when used for the assessment of IR in patients. On the other hand, our data indicate that intermediary insulins may have the advantage of being safer, i.e. have a lower risk of inducing hypoglycaemia than short-acting insulins.

Abbreviations

BCS: body condition score; CGIT: combined glucose-insulin test; EMS: equine metabolic syndrome; Fig: figure; ID: insulin dysregulation; IR: insulin resistance; ELISA: enzyme-linked immunosorbent assay; NEFA: non-esterified fatty acids; OST: oral sugar test; PPID: pituitary pars intermedia dysfunction; SD: standard deviation; Tab: Table; TRG: triglycerides

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Animal Welfare Statement

The study has been approved by the ethics committee within the University of Veterinary Medicine, Hannover, and the State Office for Consumer Protection and Food Safety in accordance with the German Animal Welfare Law (LAVES – Reference number: 33.14 42502-04-13/1259).

Competing interests

The authors declare that they have no competing interests.

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Erweiterte Zusammenfassung

Vergleich der endokrinen und metabolischen Reaktion gesunder Pferde auf einen oralen Glukose-Test und auf einen kombinierten Glukose-Insulin Test

Das Equine Metabolische Syndrom (EMS) ist eine bedeutende endokrinologisch-metabolische Erkrankung bei Pferden und Ponies. In Anlehnung an das humane metabolische Syndrom (MetS), welches mit Obesitas, Diabetes Typ 2, Gefäßerkrankungen und Hypertonie einhergeht, hat sich dieser Begriff in den letzten Jahren immer mehr etabliert und die ursprüngliche Bezeichnung als „Peripheres Cushing Syndrom" ersetzt. Erkrankte Pferde zeigen neben einer generalisierten oder regionalen Obesitas mit Ausbildung krankheitstypsicher Fettdepots im Bereich der Kruppe, der Schultern, der seitlichen Thoraxwand und des Halses, eine chronisch rezidivierende Hufrehe sowie eine Insulindysregulation (ID). Verschiedene Untersuchungen zeigten eine hohe Obesitasprävalenz von 30 bis 48% in der Pferdepopulation in Großbritannien und in den USA. Die zugrunde liegenden Pathomechanismen die zur ID, dem Hauptsymptom des EMS führen sind nach wie vor weitgehend unbekannt. Eine Untersuchung bei Pferden, die aufgrund einer Hufrehe-Erkrankung in einer Klinik vorgestellt wurden, zeigte, dass in ca. 86% dieser Fälle eine Hyperinsulinämie vorlag. Eine Studie aus 2007 zeigte, dass eine experimentell induzierte, anhaltende Hyperinsulinämie durch exogene Insulinzufuhr bei gesunden Ponies eine Hufrehe auslösen kann. Darüber hinaus zeigte eine kürzlich publizierte Studie den direkten Zusammenhang zwischen einer im oralen Glukosetest (OGT) diagnostizierten postprandialen Hyperinsulinämie und einer experimentell induzierten Hufrehe. Derzeit stehen verschiedene diagnostische Tests zur Feststellung einer veränderten Insulinregulation beim Pferd zur Verfügung. Die physiologischen und endokrinen Veränderungen, die durch das jeweilige Testverfahren beim zu untersuchenden Pferd hervorgerufen werden, wurden bisher jedoch nicht eingehend untersucht. Ziel dieser Studie war es, die verschiedenen metabolischen und endokrinologischen Prozesse zu untersuchen, die durch zwei verschiedene Stimulationstests beim gesunden Warmblutpferd induziert werden. In der vorliegenden Studie wurden ein oraler Glukose Test (OGT) und ein kombinierter Glukose-Insulin Tests (CGIT) unter standardisierten Bedingungen an zwölf gesunden Warmblutpferden unterschiedlichen Geschlechts, Alters (15±6.5 Jahre), Körpergewichts (567±81 kg) und Body-Condition-Scores (BCS) (4.8±1.6) durchgeführt. Die Studienpferde wurden 14 Tage vor dem ersten dynamischen Diagnostiktest unter standardisierten Fütterungs- und Haltungsbedingungen gehalten. Keines der Studienpferde zeigte klinische oder röntgenologische Anzeichen einer Hufrehe oder klinische Anzeichen einer Pituitary Pars Intermedia Dysfunction (PPID). Vor der Durchführung der jeweiligen Tests wurde den Pferden für 12 Stunden das Futter entzogen. Im Rahmen des OGT wurde den Pferden 1g/kg KGW Glukose (Glukose, WDT, Garbsen, Deutschland) in 2 Litern Wasser gelöst mittels Magenschlundsonde verabreicht. Zur Durchführung des CGIT wurde den Pferden 150mg/kg KGW einer 50%igen Glukoselösung (Glukose 500mg/mL, B. Braun Melsungen AG, Deutschland) injiziert, gefolgt von einer zweiten Injektion von 0,1IU/kg KGW porzinem Zink-Insulin. Es folgte eine Blutentnahme über 3 Stunden im mindestens 15-minütigen Intervall zur Bestimmung der Konzentrationen an Insulin, Glukose, Triglyceriden, nicht veresterten Fettsäuren (NEFA), Fruktosaminen und Kortisol.

Die Ausgangskonzentrationen der Laborparameter zeigten keine signifikanten Unterschiede zwischen den verschiedenen Versuchstagen. Keines der Studienpferde zeigte eine basale Hyperinsulinämie oder Hyperglykämie. Sowohl im OGT als auch im CGIT stiegen die Blutglukosekonzentrationen erwartungsgemäß direkt nach oraler oder intravenöser Verabreichung an. Während des OGT folgte die Insulinkonzentration der durch die enterale Resorption hervorgerufenen Hyperglykämie und stieg innerhalb von 30 Minuten signifikant ($p < 0.05$) an um dann über einen Zeitraum von 3 Stunden erhöht zu bleiben ($p = 0.0141$). Die untersuchten Pferde zeigten eine hohe individuelle Variation in der Insulinantwort auf die verabreichte Glukose, während die Plasmaglukosekonzentrationen keine signifikanten Unterschiede zeigten. Vier der zwölf Pferde (HR) zeigten im OGT signifikant höhere Insulinkonzentrationen ($p < 0.0001$) als die verbleibenden acht Pferde (LR). Die HR Pferde (10289.05±1070.65µIU/mL×min) zeigten eine signifikant höhere AUCinsulin OGT im Vergleich zu den LR Pferden (3685.75±424.35µIU/mL×min) ($p < 0.0001$), während die Berechnung der AUCglucose OGT keinen Unterschied zwischen den Gruppen zeigte. Im CGIT reagierten die Studienpferde vergleichbar, so dass keine deutliche Trennung von HR Pferden und LR Pferden möglich war. Nach Injektion des exogenen Insulins wurde eine durchschnittliche maximale Insulinkonzentration von 493.98±86.84µIU/mL erreicht, gefolgt von einem kontinuierlichen Abfall der Seruminsulinkonzentration. Nach 9 Minuten zeigten die Pferde bereits einen Abfall um die Hälfte der maximalen Insulinkonzentration. Es zeigte sich jedoch, dass die untersuchten Pferde im Mittel erst nach 123±37 Minuten die basalen Glukosekonzentrationen erreichten. Die basalen NEFA-Konzentrationen zeigten eine hohe individuelle Schwankung. Während des OGT und des CGIT zeigten die NEFA Konzentrationen einen sowohl zeitlichen als auch absolut vergleichbaren Abfall auf 93.82±53.22µmol/L im OGT und 91.97±56.89µmol/L im CGIT. Um eventuell bestehende Stresseinflüsse während des Tests zu erfassen, wurde die Kortisolkonzentrationen über den Zeitraum der Versuche bestimmt. Während die Kortisolkonzentrationen im CGIT unbeeinflusst blieben, zeigte sich im OGT ein initialer, signifikanter Anstieg ($p = 0.002$) der Serumkortisolkonzentration nach Verabreichung der Glukose mittels Magensonde. Bereits nach 90 Minuten zeigte die Kortisolkonzentration jedoch keine signifikanten Unterschiede zu den ursprünglichen Basalwerten. Als Langzeitmarker zur Überprüfung der Glukosehomöostase wird in der Humanmedizin, sowie in der Kleintiermedizin Fruktosamin genutzt. Die Fruktosaminkonzentrationen zeigten in der vorliegenden Studie keine signifikanten Veränderungen vor und nach OGT oder CGIT, sowie vor Studienbeginn und nach Studienende und mehrmaliger Glukose-Exposition.

Generell lässt sich feststellen, dass der OGT dem CGIT aufgrund seines der Physiologie näher stehenden Testprinzips in Bezug auf die Feststellung einer ID überlegen ist. Orale Testprotokolle haben intravenösen Protokollen gegenüber den Vorteil eine ID zu erfassen. Sie basieren auf einer Stimulation des physiologischen Systems und erlauben eine standardisierte Untersuchung einer postprandialen Hyperinsulinämie. Der mit dem Einführen der Magensonde verbundene Stress hat vermutlich keinen Einfluss auf die klinisch relevanten Testergebnisse. Die in Verbindung mit dem OGT gemessenen Kortisolspiegel waren lediglich initial geringgradig erhöht, nicht aber während der späteren Probenentnahmezeitpunkte, die für die klinische Interpretation des Testergebnisses herangezogen werden. Hinsichtlich der Auswirkungen auf den Lipidstoffwechsel unterscheiden sich beide Tests nur unwesentlich. Der antilipolytische Effekt des Insulins war im OGT und im CGIT vergleichbar. Unabhängig von der Stimulation sowie der absoluten Insulinkonzentration der endogenen Sekretion oder der Injektion eines exogenen Insulins zeigte sich eine vergleichbare Reduktion der NEFA-Konzentrationen bei gleichbleibender Triglyceridkonzentration. Dieses Ergebnis impliziert eine bereits früh eintretende Supression der Lipolyse bei mäßigen Insulinkonzentration und keinen Hinweisen auf eine Reveresterung in der Leber. In der vorliegenden Studie wurde porzines Zink-Insulin zur Durchführung des CGIT genutzt um ein auf dem veterinärmedizinischen Markt zugelassenes Präparat für den Einsatz in diesem Testverfahren zu untersuchen. Besonders hervorzuheben sind die veränderten Dynamiken im CGIT bei Durchführung mit porzinem Zink-Insulin im Vergleich zu den in der Originalarbeit beschriebenen Insulindynamiken eines schnell-wirksamen rekombinanten Humaninsulins. Unter Praxisbedingungen sollten die veränderten Kinetiken des jeweils eingesetzten Insulinanalogons bei der Befunderhebung einer durch einen CGIT diagnostizierten IR berücksichtigt werden.

Schlüsselwörter: Pferd, Insulin, Insulindysregulation, Insulinresistenz, Insulinsensitivität, oraler Glukose Test (OGT), kombinierter Insulin-Glukose Test (CGIT), Diagnostiktest, Physiologie

4 MANUSCRIPT II

Insulin signaling in various equine tissues under basal conditions and acute stimulation by intravenously injected insulin

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Contribution to the manuscript

KF and KH designed the study; TW, KF and KH performed the experiments, TW analyzed and interpreted the data; TW, RB, KF and KH drafted and revised the data; TW wrote the manuscript; RB, KF and KH approved the final version of the manuscript.

Abstract

The aim of the study was to analyze key proteins of the equine insulin signaling cascade and their extent of phosphorylation in biopsies from muscle tissue (MT), liver tissue (LT), and nuchal AT, subcutaneous AT, and retroperitoneal adipose tissues. This was investigated under unstimulated (B1) and intravenously insulin stimulated (B2) conditions, which were achieved by injection of insulin (0.1 IU/kg bodyweight) and glucose (150 mg/kg bodyweight). Twelve warmblood horses aged 15 ± 6.8 yr (yr), weighing 559 ± 79 kg, and with a mean body condition score of 4.7 ± 1.5 were included in the study. Key proteins of the insulin signaling cascade were semiquantitatively determined using Western blotting. Furthermore, modulation of the cascade was assessed. The basal expression of the proteins was only slightly influenced during the experimental period. Insulin induced a high extent of phosphorylation of insulin receptor in LT ($P < 0.01$) but not in MT. Protein kinase B and mechanistic target of rapamycin expressed a higher extent of phosphorylation in all tissues in B2 biopsies. Adenosine monophosphate protein kinase, as a component related to insulin signaling, expressed enhanced phosphorylation in MT ($P < 0.05$) and adipose tissues (nuchal AT $P < 0.05$; SCAT $P < 0.01$; retroperitoneal adipose tissue $P < 0.05$), but not in LT at B2. Tissue-specific variations in the acute response of insulin signaling to intravenously injected insulin were observed. In conclusion, insulin sensitivity in healthy horses is based on a complex concerted action of different tissues by their variations in the molecular response to insulin.

Keywords: Equine, Insulin, Signaling, Adipose tissue, Muscle, Liver

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Insulin signaling in various equine tissues under basal conditions and acute stimulation by intravenously injected insulin

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ABSTRACT

The aim of the study was to analyze key proteins of the equine insulin signaling cascade and their extent of phosphorylation in biopsies from muscle tissue (MT), liver tissue (LT), and nuchal AT, subcutaneous AT, and retroperitoneal adipose tissues. This was investigated under unstimulated (B1) and intravenously insulin stimulated (B2) conditions, which were achieved by injection of insulin (0.1 IU/kg bodyweight) and glucose (150 mg/kg bodyweight). Twelve warmblood horses aged 15 ± 6.8 yr (yr), weighing 559 ± 79 kg, and with a mean body condition score of 4.7 ± 1.5 were included in the study. Key proteins of the insulin signaling cascade were semiquantitatively determined using Western blotting. Furthermore, modulation of the cascade was assessed. The basal expression of the proteins was only slightly influenced during the experimental period. Insulin induced a high extent of phosphorylation of insulin receptor in LT ($P < 0.01$) but not in MT. Protein kinase B and mechanistic target of rapamycin expressed a higher extent of phosphorylation in all tissues in B2 biopsies. Adenosine monophosphate protein kinase, as a component related to insulin signaling, expressed enhanced phosphorylation in MT ($P < 0.05$) and adipose tissues (nuchal AT $P < 0.05$; SCAT $P < 0.01$; retroperitoneal adipose tissue $P < 0.05$), but not in LT at B2. Tissue-specific variations in the acute response of insulin signaling to intravenously injected insulin were observed. In conclusion, insulin sensitivity in healthy horses is based on a complex concerted action of different tissues by their variations in the molecular response to insulin.

1. Introduction

To date insulin action on insulin-sensitive tissues is not well studied in horses. In all mammalian species, insulin-dependent tissues are liver, skeletal muscle, and the adipose tissues. Insulin binds to its specific receptor (InsR) which activates the InsR substrate and the downstream signaling cascade (Fig. 1). A central hub of this signaling cascade is the protein kinase B (PKB/AKT) which is activated by phosphorylation. From there, the protein synthesis pathway is positively influenced by insulin. This is reflected by enhanced phosphorylation of mechanistic target of rapamycin (mTOR), a serine-threonine kinase which stimulates translation initiation. PKB/AKT inhibits lipolysis by activating phosphodiesterase 3 which inhibits cAMP production by protein kinase A and thereby reduces phosphorylation of hormone-sensitive lipase (HSL) and its lipolytic activity. Furthermore, insulin stimulates

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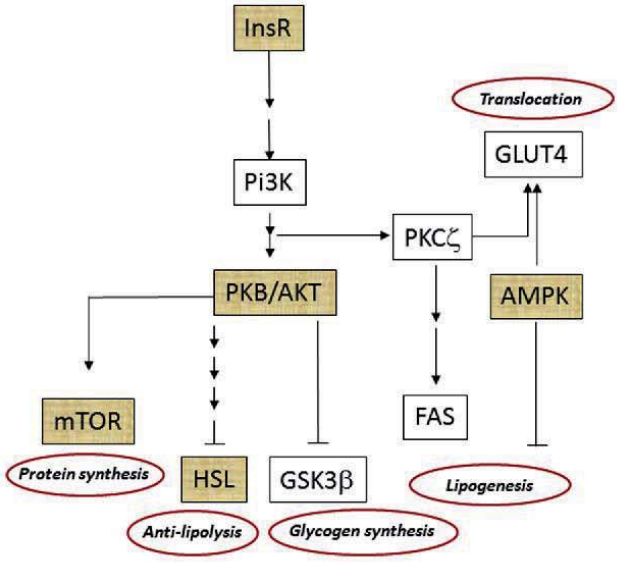

Fig. 1. Key components of insulin signaling examined in horses of protein level (according to http://www.genome.jp/kegg/pathway.html). All proteins highlighted in color were examined on their total protein content and their extent of phosphorylation under unstimulated basal conditions (B1) and under stimulated conditions (B2) with provoked hyperinsulinemia and hyperglycemia in equine adipose tissues, liver tissue, and muscle tissue. Arrows indicate activating effects and horizontal lines indicate inhibiting effects. InsR, insulin receptor; Pi3K, phosphoinositide 3 kinase; PKB/Akt, protein kinase B; mTOR, mechanistic target of rapamycin; HSL, hormone-sensitive lipase; GSK-3β, glycogen synthase kinase 3β; FAS, fatty acid synthase; PKC ζ, protein kinase C ζ; AMPK-α, adenosine monophosphate–activated kinase α; GLUT4, glucose transporter 4. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

lipogenesis by increasing fatty acid synthase (FAS) expression and activity, a pathway inhibited by adenosine monophosphate-activated kinase (AMPK). The glucose transporter 4 (GLUT4) translocation from cytosol stores into plasma membranes is stimulated by atypical protein kinase C zeta (PKC ζ) resulting in increased glucose uptake by insulin-dependent tissues. Insulin-regulated glycogen synthase kinase 3 beta (GSK3β) is a critical enzyme regulating glucose storage of cells [1]. Only few studies have been done in horses to detect components of insulin signaling on protein level and to evaluate their modulation by insulin. In equine cardiac and skeletal muscle, InsR and insulin-like growth factor 1 receptor protein expression was studied in healthy horses, revealing no differences between the muscle types [2]. However, on mRNA level, hyperinsulinemic horses expressed higher mRNA amounts of PKB/AKT, GSK3β, GLUT1, and GLUT4 in the cardiac, but not in the skeletal muscle [2]. Although it is difficult to interpret what functional consequences the changes in mRNA expression may have, the tissue-dependent sensitivity to high insulin concentrations appeared to be a physiological feature in horses. Hyperinsulinemia established by clamp procedures provoked an increase in the extent of phosphorylation in PKB/AKT, 4E binding protein 1, and riboprotein S6. The latter 2 proteins are downstream targets of mTOR in the gluteus medius muscle [3]. This indicates an insulin-driven stimulation of protein synthesis in this muscle in horses. Muscle GLUT4 expression and -translocation to the plasma membrane appeared not to be dependent on insulin, however insulin-resistant horses had lower GLUT4 in the plasma membrane of muscle cells [4]. To understand the regulation of insulin-dependent pathways it is necessary to increase the knowledge on physiological features of the insulin signaling pathway in horses. Therefore, the objectives of this study were to determine the protein expression of key components of insulin signaling and their extent of phosphorylation in the main peripheral insulin-sensitive tissues in horses under basal conditions and under hyperinsulinemic and hyperglycemic conditions provoked by injection of insulin and glucose.

2. Materials and methods

2.1. Animals

This study was approved by the ethics committee of the University of Veterinary Medicine, Hannover, and the Lower Saxony State Office for Consumer Protection and Food Safety, in accordance with the German Animal Welfare Law (File No. 33.14 42502-04-13/1259). The experiment was conducted at the Clinic for Horses, University of Veterinary Medicine Hannover, Germany. Twelve healthy warmblood breed horses were included in the study (Table 1). There were six mares, three geldings, and three stallions aged from 4 to 24 yr (15 ± 6.8 yr) and weighing 440 to 695 kg (559 ± 79 kg). The body condition score (BCS) of the horses covered a wide range from 1.9 to 7.5 with a mean BCS of 4.7 ± 1.5. The BCS was determined as the average of five independent assessors according to the scoring system of Henneke et al [5]. Starting 2 wk before the beginning of the experiment, the horses were fed average mixed-grass hay on maintenance requirement twice daily and no supplementary feeding was provided. They were stabled in individual boxes, under standardized feeding and management conditions and were not exercised, except for 2 h access to a large paddock, daily. All horses underwent clinical examinations and laboratory screenings before beginning the study. To exclude horses with insulin dysregulation (ID), all horses underwent an oral glucose testing procedure with 1 g/kg BW glucose administered via nasogastric tubing [6]. Furthermore,

Table 1
Horses background data.

Horse	Age (yr)	Sex	BW (kg)	Body condition score (BCS)
1	11	Gelding	650	5.3
2	19	Mare	465	2.9
3	19	Mare	440	1.9
4	24	Gelding	480	3.0
5	4	Stallion	540	6.0
6	15	Mare	619	5.1
7	15	Stallion	515	5.1
8	25	Mare	570	4.5
9	4	Stallion	560	4.0
10	11	Gelding	640	5.9
11	20	Mare	537	4.7
12	13	Mare	695	7.5

Abbreviation: BW, bodyweight.
Animals background data.

radiographic examinations of the limbs were performed to evaluate and exclude previous episodes of laminitis. None of the study horses showed clinical signs of pituitary pars intermedia dysfunction.

2.2. Biopsy sampling

Biopsies were collected under basal conditions (B1) and under stimulated conditions (B2) on 2 different days. All horses underwent the biopsy sampling trials within in a 3-wk period with recovery and a 16-day washout phase in between. Biopsy sampling from liver tissue (LT), adipose tissues (AT), and muscle tissue (MT) was performed while the horses were fixed in standing stocks and were sedated with propofol (initial bolus 0.35 mg/kg BW followed by 0.15 mg/kg BW when required; Narcofol, CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany). Horses were fasted overnight and received no further stimulation process during the B1 biopsy sampling. Stimulations to provoke variations in blood glucose and insulin concentrations for the B2 biopsy sampling were achieved by intravenous injection of porcine-zinc insulin (0.1 IU/kg BW) (Caninsulin 40 I.E./ml, MSD, Unterschleißheim, Germany). Glucose solution (150 mg/kg BW; Glucose 500 mg/mL, B. Braun Melsungen AG, Germany) was given simultaneously as a bolus through an intravenous jugular vein catheter to avoid the development of hypoglycemia. First biopsies were taken 12 min after injection of glucose and insulin. All biopsies were collected under local infiltration anesthesia with lidocaine (2–5 mL; Lidocainhydrochlorid 2%, Bela Pharm GmbH & Co KG, Lohne, Germany) and aseptic preparation of the surgical site. After biopsy collection, the skin incisions were closed using a sterile stapler (Weck Visistat 35 W, Teleflex Medical, Dublin, Ireland). Nuchal AT (NUAT) samples from the neck of the horses were taken in the mid region of the neck, 1 cm anterior to the mane line. Three punch biopsies (approximately 6 mm in diameter and 10 mm in depth, 80 mg of tissue each) were taken from each tissue and location with a biopsy sampler and a 14-G semi-automatic biopsy needle (Plus Speed, Peter Pflugbeil GmbH, Zorneding, Germany) after initial skin incision. The LT biopsies were taken under sonographic control in the 13th intercostal space of the right side of the horse. Subcutaneous AT (SCAT) biopsy sites were located a hand's width cranial to the tail line and a hand's width lateral to the midline. The second sample from this location was taken from the MT after the preparation of the incision line into the depth of the muscles. Retroperitoneal AT (RPAT) was sampled under sonographic control in the ventral midline at the lowest point of the abdomen along the linea alba. After skin incision, the RPAT biopsy sampling was performed with a perforated Grazl-needle taking care not to perforate the peritoneum. The order of biopsy sampling was the same for both sampling procedures and all horses, with NUAT being first, followed by SCAT, MT, LT and, finally, RPAT. Maximal time for collection of all 5 biopsies was 20 min (Fig. 2).

2.3. Western blot analyses

Directly after biopsy, the samples were trimmed of connective tissue, rinsed in sterile physiological saline solution to decrease blood contamination, immediately deep frozen in liquid nitrogen and stored at −80°C until further analysis. Tissues were homogenized using FastPrep (MP Biomedicals, Eschwege, Germany) for protein extraction. About 100–200 mg of tissue was homogenized in 400–600 μL of prechilled lysis buffer containing 50-mM HEPES (Carl Roth GmbH, Karlsruhe, Germany), 4-mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N'-tetraacetic acid (Sigma-Aldrich, St. Louis, MO, USA), 10-mM EDTA (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), 100-mM β-glycerol phosphate (Sigma-Aldrich), 15-mM sodium pyrophosphate (Sigma-Aldrich), 5-mM sodium orthovanadate (Sigma-Aldrich), 2.5-mM sodium fluoride (Sigma-Aldrich), a protease inhibitor cocktail (CompleteMini, Roche Diagnostics GmbH, Mannheim, Germany), and a phosphatase inhibitor cocktail (PhosStop, Roche Diagnostics GmbH). The homogenates were centrifuged at 1,000 × g for 10 min at 4°C to remove lipids and other particulate material. Supernatants were collected and frozen in aliquots and stored at −20°C until electrophoresis. Protein concentrations of the homogenates were measured using Bradford reagent (Serva Electrophoresis GmbH, Heidelberg, Germany). Samples in loading buffer (50 mmol/L of Tris-HCL [Sigma-Aldrich], 10% glycerol [Sigma-Aldrich], 2% SDS [Serva Electrophoresis GmbH], 0.1% bromophenol blue [Sigma-Aldrich], and 2% mercaptoethanol [Sigma-Aldrich]) were denatured by heating them for 5 min at 95°C before loading 20 μg (LT), 10 μg (AT), and 30 μg (MT) per lane onto a 5% stacking and 8.1% separation gel. Electrophoresis was carried out according to Laemmli [7]. Detection of specific proteins was performed after blocking membranes in a PBS-based solution containing 5% fat-free milk powder (Carl Roth GmbH) and 0.1% Tween 20 (Sigma-Aldrich) for 1 h at room temperature. Membranes were incubated overnight at 4°C in PBS-based solution containing 0.1% Tween 20 (Sigma-Aldrich) and 5% to 10% fat-free milk powder (Carl Roth GmbH) or 5% bovine serum albumin, with primary antibodies (Table 2). Detection of the primary antibodies and their phosphorylated forms was performed using secondary goat anti-rabbit-HRP antibody (Cell Signaling Technology Inc; 1:2,000) or secondary goat anti-rabbit antibody (Sigma-Aldrich; 1:50,000) for 60 to 90 min at room temperature and against β-actin using secondary goat anti-mouse antibody (Sigma-Aldrich; 1:100,000). After washing twice in PBST and once in PBS for 5 min each, immunodetection was performed by incubating the membranes with Pierce West Dura chemiluminescence substrate (Thermo Scientific, Braunschweig, Germany) or LumiGLO reagent (Cell Signaling Technology Inc), and chemiluminescence was detected by a ChemiDoc XRS + system (Bio-Rad Laboratories GmbH, Munich, Germany). The bands were quantified by densitometry using Image Lab 5.2 software (Bio-Rad Laboratories GmbH). Chemiluminescence signals were measured with at least 5 consecutive exposure times to determine the linear range of signal intensity of each antibody to ensure that quantitative data were obtained. Values of exposure times within the linear range were used for quantification and further analyses. Finally, membranes were stained with Indian ink (Pelikan PBS, Peine, Germany). Specific band signals were normalized to the β-actin signal (LT) and Indian ink signal (AT and MT) as an internal standard. Two standard linker

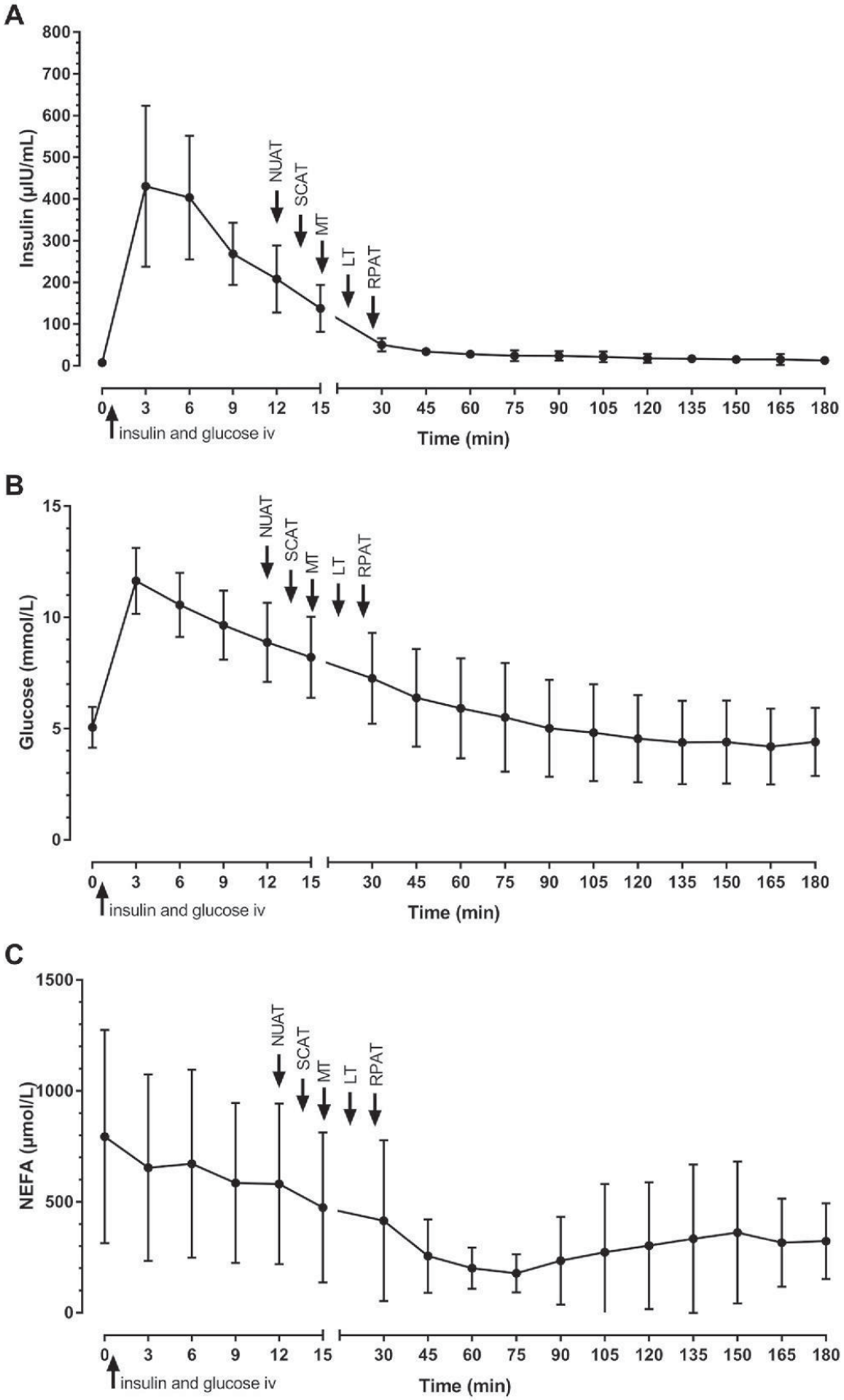

Fig. 2. Hormone and metabolite concentrations during B2 procedure. Arrows indicating biopsy sampling time points for specific tissues. Nuchal adipose tissue (NUAT), subcutaneous adipose tissue (SCAT), muscle tissue (MT), liver tissue (LT), and retroperitoneal adipose tissue (RPAT). (A) Insulin concentrations (µIU/mL); (B) glucose concentration (mmol/L), and (C) nonesterified fatty acid (NEFA) concentrations (µmol/L). Given are means ± SD. SD, standard deviation.

Table 2
Primary antibodies used for Western blot analyses in equine tissues.

Target	Antibody	Dilution	Company
Insulin receptor β (InsR)	Rabbit anti InsR-β	1:250	Santa Cruz Biotechnology Inc
Phosphorylated insulin receptor-β (Try1162/1163) (p-InsR)	Rabbit anti p-InsR-β (Try1162/1163)	1:250	Santa Cruz Biotechnology Inc
Mechanistic target of rapamycin (mTOR)	Rabbit anti mTOR	1:250	Cell Signaling Technology Inc
Phosphorylated mechanistic target of rapamycin (Ser2448; p-mTOR)	Rabbit anti p-mTOR (Ser2448)	1:300	Cell Signaling Technology Inc
5′ adenosine monophosphate-activated protein kinase α (AMPK-α)	Rabbit anti AMPKα	1:500	Cell Signaling Technology Inc
Phosphorylated 5′ adenosine monophosphate-activated protein kinase α (Thr172) (p-AMPK-α)	Rabbit anti p-AMPKα (Thr172)	1:500	Cell Signaling Technology Inc
Protein kinase b (PKB)	Rabbit anti AKT	1:500	Cell Signaling Technology Inc
Phosphorylated protein kinase b (Ser473; p-PKB)	Rabbit anti p-AKT (Ser473)	1:500	Cell Signaling Technology Inc
Hormone-sensitive lipase (HSL)	Rabbit anti HSL	1:1,000	Cell Signaling Technology Inc
Phosphorylated hormone-sensitive lipase (Ser563; p-HSL)	Rabbit anti p-HSL (Ser563)	1:1,000	Cell Signaling Technology Inc
Fatty acid synthase (FAS)	Rabbit anti FAS	1:2,000	Sigma-Aldrich
Protein kinase c ζ (PKC ζ)	Rabbit anti PKC ζ	1:250	Santa Cruz Biotechnology Inc
Glycogen synthase kinase 3β (GSK-3β)	Rabbit anti GSK-3β	1:2,500	Sigma-Aldrich
Phosphatidylinositide 3-kinase p85 (PI3K)	Rabbit anti PI3-kinase p85	1:500	Cell Signaling Technology Inc
Glucose transporter 4 (GLUT4)	Rabbit anti GLUT4	1:300	Alpha Diagnostics Intl Inc
β-actin	Mouse anti β-actin	1:10,000	Sigma-Aldrich

samples from pooled equine tissue samples were blotted on each membrane to adjust signals from different membranes. This allowed the comparison of all the membranes. Omitting the specific signal by blocking with the respective antigenic peptide was considered to confirm the specificity of heterologous antibodies to detect marker proteins in equine tissues.

2.4. Immunofluorescence

Immunofluorescence (IF) was performed on Bouin solution fixated and deparaffinized sections from NUAT, SCAT, and RPAT biopsies. The staining slices were immersed for 20 min in 10 mM citrate buffer (pH 6.0) at 96°C–99°C to optimize immunohistochemical staining. After heating, the sections were left to cool for 15 min at room temperature. Then, sections were washed and subsequently blocked with blocking solution (PBS, Goat Serum, Triton X-100, NaN_3 [0.1%]) for 2 h and the immunostaining procedure was commenced. Sections were incubated overnight at 4°C in the presence of the primary antibody, insulin receptor-β (InsR-β) 1:100 (Table 2). Next, the secondary antibody, CY3 Anti-Rabbit (1:500), was applied and incubated for 2 h at room temperature. Finally, sections were examined with an AxioVert 200M microscope (Zeiss, Germany). A negative control was performed by using the same protocol without incubation with the primary antibody.

2.5. Blood samples and analyses

During B1, blood samples were collected before and during biopsy collection. In the B2, blood samples were collected before injection of glucose and insulin for determination of basal hormone and metabolite concentrations, followed by blood sampling for 3 h in 15-min intervals, during and after biopsy sampling in B2. The blood samples were collected via a jugular vein catheter (EquiCathTM Fastflow, Braun Vet Care GmbH, Tuttlingen, Germany) and were placed into tubes containing fluoride oxalate and plain tubes for serum preparation (Vacuette Greiner Bio One, Frickenhausen, Germany). The samples for serum preparation were incubated at room temperature for 60 min, centrifuged at 1,000 × g for 6 min, and stored at −80°C until further analysis. Glucose was analyzed with a colorimetric assay (GLUC3, Cobas, Roche Diagnostics GmbH, Mannheim, Germany; CV 0.68%). The insulin concentrations of the serum were analyzed by an equine-optimized ELISA (Equine Insulin ELISA, Mercodia, Uppsala, Sweden) previously validated for use in horses [8,9] (intra-assay CV 4.6%, interassay CV 5.3% [low range], 3.24% [medium range], and 3.2% [high-range]). Triglyceride and nonesterified fatty acid (NEFA) concentrations were measured with commercial kits for enzymatic colorimetric measurements (ABX Pentra Triglycerides CP, HORIBA ABX, Montpellier, France; Wako NEFA-HR(2), Wako Chemicals GmbH, Neuss, Germany) on an automated discrete analyzer (ABX Pentra 400, HORIBA ABX SAS; triglyceride CV 1.69%; NEFA CV 7.97%).

2.6. Data analysis

Data analysis was performed with GraphPad Prism software (version 7.00; GraphPad Inc, La Jolla, CA, USA). The Shapiro-Wilk normality test was used to assess data for normality. The extent of total protein and phosphorylated protein in the two biopsies was analyzed by paired Student *t* test. Statistical significance was accepted when $P < 0.05$. All values are expressed as mean ± SD unless indicated otherwise.

3. Results

3.1. Serum and plasma metabolite and hormone concentrations

Provoked hormone and metabolite concentrations and time courses are shown in Figure 2A–C. Injection of glucose- and insulin-provoked hyperglycemia and hyperinsulinemia in all horses. Insulin concentrations differed significantly ($P < 0.001$) between B1 and B2. During B1, the insulin concentration was 5.32 ± 3.16 μIU/mL compared

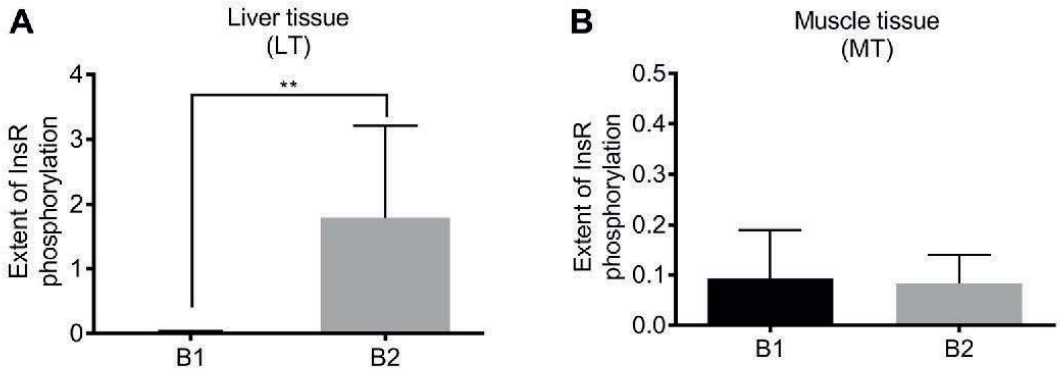

Fig. 3. Extent of phosphorylated insulin receptor β (InsR-β) in (A) liver tissue (LT) and (B) muscle tissue (MT) at unstimulated basal conditions (B1) and stimulated conditions (B2) with provoked hyperinsulinemia and hyperglycemia in healthy horses. Given are means ± SD; $^{**}P < 0.001$. SD, standard deviation.

with 207.96 ± 80.41 μIU/mL 12 min after injection of insulin at the beginning of B2. Furthermore, glucose concentrations differed significantly ($P < 0.001$) between both sampling times with 5.29 ± 0.78 mmol/L in B1 and 8.36 ± 1.16 mmol/L at the beginning of biopsy sampling in B2. The NEFA concentration was 795.6 ± 291.9 μmol/L in B1 compared with 641.4 ± 368.5 μmol/L in the beginning of biopsy sampling during B2. However, no statistically significant difference was observed between the NEFA concentrations of B1 and B2. Triglyceride concentrations were 0.35 ± 13 mmol/L in B1 and 0.26 ± 0.11 mmol/L in the beginning of B2 without a statistically significant difference between both time points.

3.2. Basal expression of insulin signaling proteins in tissues

Basal expression of InsR-β (LT, MT, NUAT, SCAT, RPAT), mTOR (LT, MT, NUAT), PKB/AKT (LT, MT, NUAT, SCAT), HSL (MT, NUAT, SCAT), and AMPK-α (LT, MT, NUAT, SCAT, RPAT) did not differ between B1 and B2 (data not shown). In SCAT, the basal expression of mTOR differed between B1 and B2 ($P < 0.01$) with higher protein expression in B2. In RPAT, the basal expression of mTOR ($P < 0.05$), PKB/AKT ($P < 0.01$), and HSL ($P < 0.01$) differed significantly between B1 and B2 with higher contents of total protein expression in B2.

3.3. Liver tissue signaling protein phosphorylation

In liver, the extent of phosphorylated InsR-β ($P \leq 0.01$; Fig. 3A), phosphorylated PKB/AKT ($P < 0.01$; Fig. 4A) and phosphorylated mTOR ($P < 0.001$; Fig. 4B) was significantly enhanced between basal conditions in the B1 setting and stimulated conditions in B2. The extent of phosphorylated AMPK-α did not differ between B1 and B2 conditions (Fig. 4C). The hormone-sensitive lipase (HSL) and

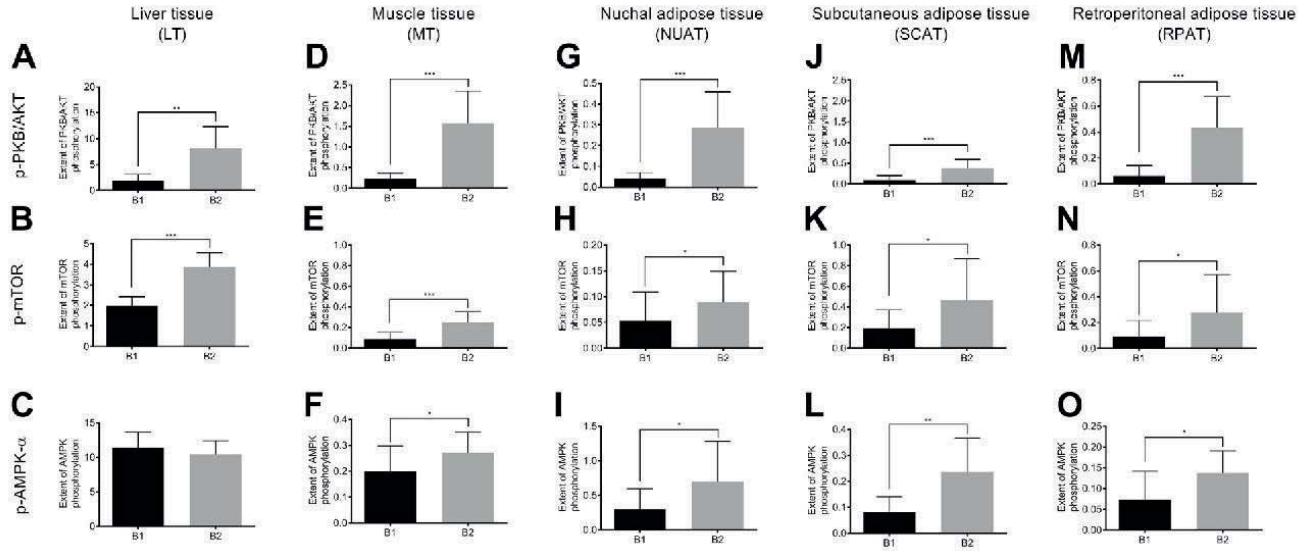

Fig. 4. Extent of phosphorylated (A) protein kinase b (PKB/AKT), (B) mechanistic target of rapamycin (mTOR) and (C) 5′ adenosine monophosphate-activated protein kinase α (AMPK-α) in liver tissue (LT). Extent of phosphorylated (D) protein kinase b (PKB/AKT), (E) mechanistic target of rapamycin (mTOR) and (F) 5′ adenosine monophosphate-activated protein kinase α (AMPK-α) in muscle tissue (MT). Extent of phosphorylated (G) protein kinase b (PKB/AKT), (H) mechanistic target of rapamycin (mTOR) and (I) 5′ adenosine monophosphate-activated protein kinase α (AMPK-α) in nuchal adipose tissue (NUAT). Extent of phosphorylated (J) protein kinase b (PKB/AKT), (K) mechanistic target of rapamycin (mTOR) and (L) 5′ adenosine monophosphate-activated protein kinase α (AMPK-α) in subcuatneous adipose tissue (SCAT). Extent of phosphorylated (M) protein kinase b (PKB/AKT), (N) mechanistic target of rapamycin (mTOR) and (O) 5′ adenosine monophosphate-activated protein kinase α (AMPK-α) in retroperitoneal adipose tissue (RPAT). All presented at unstimulated basal conditions (B1) and stimulated conditions (B2) with provoked hyperinsulinemia and hyperglycemia in healthy horses. Given are means ± SD; $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$. SD, standard deviation.

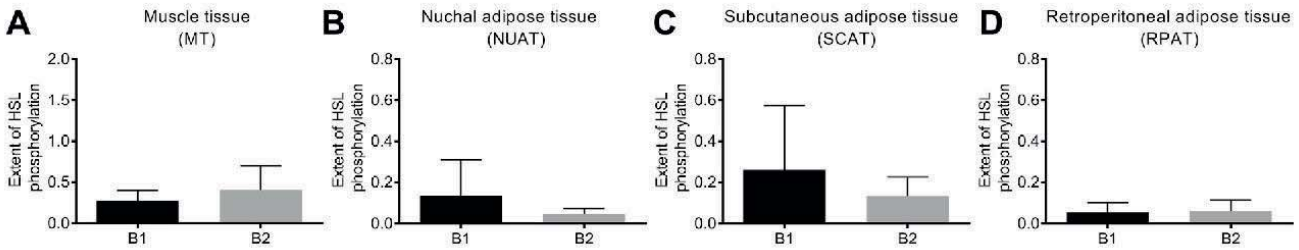

Fig. 5. Extent of phosphorylated hormone sensitive lipase (HSL) in (A) muscle tissue (MT), (B) nuchal adipose tissue (NUAT), (C) subcutaneous adipose tissue (SCAT), and (D) retroperitoneal adipose tissue (RPAT) at unstimulated basal conditions (B1) and stimulated conditions (B2) with provoked hyperinsulinemia and hyperglycemia in healthy horses. Given are means ± SD. SD, standard deviation.

phosphorylated-HSL (p-HSL) were not detectable in our LT samples.

3.4. Muscle tissue signaling protein phosphorylation

The extent of phosphorylated InsR-β and HSL did not differ between B1 and B2 (Figs. 3B and 5A), whereas the extent of phosphorylated PKB/AKT ($P < 0.001$; Fig. 4D), phosphorylated mTOR ($P < 0.001$; Fig. 4E), and phosphorylated AMPK-α ($P < 0.05$; Fig. 4F) were significantly higher in B2 compared with B1.

3.5. Nuchal adipose tissue signaling protein phosphorylation

The extent of phosphorylated PKB/AKT ($P < 0.001$; Fig. 4G), phosphorylated mTOR ($P < 0.05$; Fig. 4H), and phosphorylated AMPK-α ($P < 0.05$; Fig. 4I) differed significantly between B1 and B2 with enhanced phosphorylation under B2 conditions. The extent of phosphorylated HSL did not differ between B1 and B2 (Fig. 5B).

3.6. Subcutaneous adipose tissue signaling protein phosphorylation

The extent of phosphorylated mTOR ($P < 0.05$; Fig. 4K) differed significantly between B1 and B2, with higher extent of phosphorylation under stimulated conditions in B2. Furthermore, the extent of phosphorylated PKB/AKT ($P < 0.001$; Fig. 4J) and phosphorylated AMPK-α ($P < 0.01$; Fig. 4L) differed significantly between B1 and B2, expressing a higher extent of phosphorylation in B2. The extent of phosphorylated HSL did not differ significantly between B1 and B2 (Fig. 5C).

3.7. Retroperitoneal adipose tissue signaling protein phosphorylation

Statistically significant differences with a higher extent of phosphorylated PKT/AKT ($P < 0.001$; Fig. 4M), phosphorylated mTOR ($P < 0.05$; Fig. 4N), and phosphorylated AMPK-α ($P < 0.05$; Fig. 4O) were observed between B1 and B2. The extent of HSL phosphorylation did not differ between B1 and B2 in RPAT (Fig. 5D).

3.8. Further investigated proteins important to insulin signaling

Further proteins related to the insulin signaling cascade were examined in LT and AT. In LT, FAS, GSK-3β, PKC ζ, and PI3-K did not differ significantly in their total protein extent between prestimulation conditions in B1 and stimulated conditions in B2. Similar to LT, NUAT biopsies showed no significant difference in the extent of total protein between B1 and B2 for FAS, GLUT4, and PKC ζ. FAS did neither differ between B1 and B2 in SCAT nor in RPAT.

3.9. Insulin receptor localization in equine adipose tissues

IF staining illustrated the clear expression of InsR-β in all three investigated AT (Fig. 6). Slides showed heterogeneous distribution but did not differ significantly in fluorescent intensity between the 3 different AT.

4. Discussion

The results of the present study indicate that iv-injected insulin could induce phosphorylation of specific proteins involved in the insulin-signaling cascade in several equine

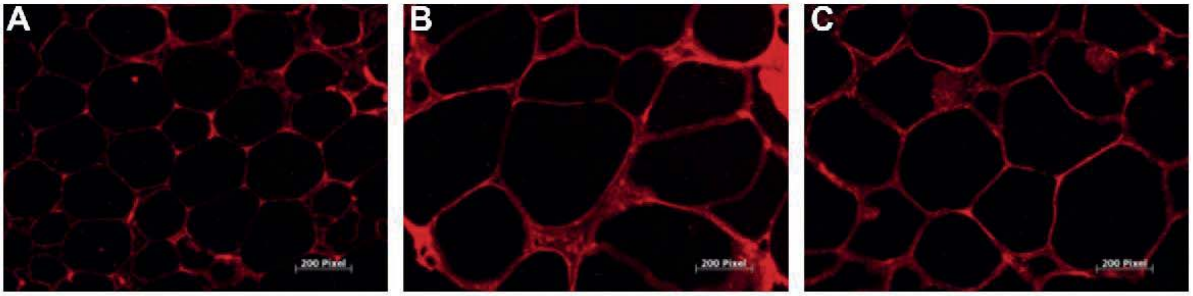

Fig. 6. Immunofluorescence staining of (A) RPAT, (B) NUAT, and (C) SCAT with primary antibody against insulin receptor β (1:100) and secondary antibody CY3 anti-rabbit (1:500). Microscopic enlargement: ×200. Measuring bar: 200 pixel.

tissues by stimulation under hyperglycemic and hyperinsulinemic conditions. However, this molecular response varied widely between tissues and horses. In this experiment, horses received a combined glucose and insulin injection to increase blood insulin concentrations, to avoid insulin-mediated hypoglycemia and to stimulate the insulin signaling cascade in insulin-dependent target tissues, such as LT, MT, and AT. An acute, short-term hyperinsulinemic state in healthy horses was induced and insulin-related molecular responses of various tissues were detected as indicated by an increased extent of phosphorylation of key proteins involved in the insulin-signaling cascade. A major finding of this basic research study was that phosphorylation of InsR-β was observed in LT, but not in MT after provoking hyperinsulinemia by injection of glucose and insulin. Furthermore, major downstream targets of the insulin signaling cascade, PKB/AKT, and mTOR were stimulated in all tissues examined, as indicated by a higher extent of phosphorylation. The AMPK-α, as a component related to insulin signaling, expressed enhanced phosphorylation after stimulation (B2) only in MT and AT. Biopsies were taken at the time when insulin was lowering the plasma glucose concentrations indicating a maximal activation of insulin signaling. One limitation of this study was the artificially high insulin concentrations provoked by iv injection of insulin. Furthermore, the insulin used was not equine-specific insulin, thus the efficacy of porcine insulin on equine insulin signaling might be different to endogenous equine insulin.

4.1. Insulin-related molecular responses

Hyperinsulinemia provoked by injection of 0.1 IU porcine zinc-insulin per kg BW resulted in supraphysiological insulin concentrations, which were significantly higher than previously reported postprandial concentrations. Urschel et al observed reduced tissue sensitivity to circulating insulin when insulin infusion rates during hyperinsulinemic euglycemic clamp were increased to supraphysiological concentrations compared with more sensitive tissue responses during infusion rates providing approximately postprandial insulin concentrations [10]. Therefore, the initial injection of high insulin doses used in our study could have had a negative influence on the peripheral insulin sensitivity in the first minutes after injection. Serum insulin concentrations decreased rapidly after intravenous injection and peak insulin concentrations were nearly bisected after 9 min, indicating high liver clearance rates. The serum insulin concentrations at the beginning of biopsy sampling in the B2 procedure were around 189 μIU/mL. This concentration is comparable to postprandial insulin concentrations observed in pony breeds [11].

Interestingly, the InsR-β, as the first component of the signaling cascade, was only affected in the liver, although the downstream target PKB/AKT was phosphorylated in all tissues after stimulation. We investigated the phosphorylated InsR-β in all tissues samples, also in our AT samples. Unfortunately, the antibody for the phosphorylated InsR-ß did not provide satisfying results in our AT samples, even though the selected antibody worked perfectly in muscle and liver tissues. Therefore, we were not able to detect the extent of phosphorylated InsR-β in theses AT tissue samples. It is possible that there is either a very low abundance of that phosphorylated protein in the equine AT or that the isoform of that protein in the equine AT is different than what is in the other equine tissues. One of the major functions of insulin is to increase glucose uptake into insulin-sensitive body cells. As a consequence of the activation of insulin signaling, glucose transporters of GLUT4 type were integrated in the plasma membrane, thereby increasing cellular glucose uptake [12]. An activation of InsR-β was not observed in equine MT; however, all tissues expressed an enhanced AMPK-α phosphorylation. This enzyme is known to modulate an alternative pathway to translocate GLUT4 into plasma membranes, eg, when it is activated by exercise [13]. It is not directly involved in the insulin signaling due to its catabolic role in tissues which are inverse to the anabolic role of insulin [14]. Horses were not exercised in this study; only 2 h of paddocks access with unrestricted movement was provided during experimental period. Thus, equine MT and AT appeared to respond to insulin simultaneously with an activation of AMPK-α, which should result in an increase of glucose uptake in these tissues. These data suggest that the insulin-glucose homeostasis in horses is partially different to humans and rodents. However, this finding needs to be confirmed by further studies.

Although the first step of insulin signaling was differently mediated at the receptor level, B2 conditions stimulated higher phosphorylation of PKB/AKT, as the central hub of intracellular signaling cascades [15], in all tissues. Since many other pathways apart from insulin also involve PKB/AKT, it is difficult to explain by which mechanisms other than insulin signaling this enzyme is activated in equine MT and AT. Confirmatively, Urschel et al also found increased activation of PKB/AKT in equine MT in response to increased insulin infusion rates in concordance with the already maximized the activation of downstream signaling factors at insulin concentrations which could be compared with postprandial levels of approximately 50–100 μIU/mL [3,16]. Along the same lines, McCutcheon also demonstrated that elevated insulin concentrations due to induced hyperinsulinemia were associated with PKB/AKT activation in equine MT [17]. However, if this increase in phosphorylation found in the study led to an increase in the activity of PKB/AKT in equine tissues, mTOR, was also activated (as indicated by the increased phosphorylation in all tissues examined), thereby stimulating protein synthesis and cellular proliferation [15]. Stimulation of protein synthesis and cell proliferation are basal insulin effects, which appeared to be fully conserved in horses, similar to other species [3]. Previous studies in horses also investigating the mTOR-signaling cascade in skeletal MT were based on feeding stimuli, which increase insulin and amino acid concentrations. Therefore, differentiation between the effects of insulin or amino acids on mTOR activation was not possible [18]. Urschel et al investigated the effect of insulin infusion on whole-body and muscle protein metabolism in horses. They found an increase in the activation of upstream (AKT) and downstream (rpS6) factors in the mTOR-signaling pathway in equine skeletal muscle under insulin infusion [3]. It was remarkable that

the amino acid concentrations were affected by the insulin infusion rates, which lead to decreased plasma concentrations with enhanced insulin infusion rates, suggesting insulin-mediated activation of mTOR signaling in horses as well [3]. In agreement with the study presented, insulin was suggested to activate mTOR solely in all tissues examined.

Furthermore, GSK-3α phosphorylation in equine MT enhanced by hyperinsulinemia, led to the deactivation of GKS-3α [19]. GSK-3α and GSK-3β, both isoforms of GSK3, are expressed in insulin-sensitive peripheral tissues, such as skeletal muscle. They are thought to contribute to insulin resistance when abnormal overexpression occurs [20]. In our study, we were not able to show LT-related differences between normo-insulinemic and hyperinsulinemic conditions in the total protein content of GSK-3β investigated. Moreover, the phosphorylated content of GSK-3β would have been of great interest but could not be studied due to unavailability of an appropriate antibody.

4.2. Insulin-related effects on lipid metabolism

The NEFA concentrations dropped after the injection of glucose and insulin and decreased to concentrations comparable to those in fed horses at the end of the experiment [21] (Fig. 2). The observed reduced serum NEFA concentrations may be due to the insulin-mediated suppression of lipolysis. However, the extent of phosphorylation of HSL was only slightly lower in NUAT and SCAT and did not reach a significant level of change, indicating that these 2 AT might be more insulin-sensitive than the RPAT. In RPAT, the extent of phosphorylation was equal at B1 and B2 conditions. The overall low molecular response of AT might be due to the kinetics of insulin effects. Biopsies were taken 12 min after the insulin injection. At this time, serum NEFA concentrations had just started decreasing. After 120 min, NEFA concentrations reached a nadir, indicating that lipolysis was maximally inhibited. Taking biopsies at this time might have resulted in higher extents of phosphorylation of HSL. Even if the molecular findings were not strong, one could suggest that an antilipolytic effect of insulin also exist in horses. In this context, the RPAT appeared to express a different molecular response to insulin compared with subcutaneous and NUAT. The biological role of the different AT in physiology and pathophysiology of equine metabolic diseases needs to be elucidated in further studies. The exact molecular localization of potential disruption of endocrine control in metabolic pathologies, such as ID which may be accompanied by tissue insulin resistance (IR), is unknown and is subject of ongoing research of our research group at the moment. Impaired insulin signaling, similar to that observed in humans with IR, is discussed as a pathophysiological condition in the Equine Metabolic Syndrome [22,23], but scientific evidence is missing due to a lack of knowledge about the physiological variations in insulin-signaling cascade in equine tissues. Moreover, further research focusing on new potential drug-based approaches for treatment of metabolic pathologies like ID caused by variations in insulin sensitivity and glucose homeostasis in horses and ponies is urgently required and assumes exact knowledge about insulin signaling and potential key proteins in equine insulin-dependent tissues.

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5 MANUSCRIPT III

Lower plasma trans-4-hydroxyproline and methionine sulfoxide levels are associated with insulin dysregulation in horses

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Contribution to the manuscript

TW, KF and KH designed the study; TW performed the experiments; ÁK and KH analyzed and interpreted the data; ÁK, TW, KF and KH drafted and revised the data; ÁK, TW, KF and KH approved the final version of the manuscript and ÁK, TW, KF and KH agree to be accountable for all aspects of the study.

Abstract

Background: Insulin dysregulation in horses is a metabolic condition defined by high insulin concentrations in the blood and peripheral insulin resistance. This hyperinsulinemia is often associated with severe damage in the hooves, resulting in laminitis. However, we currently lack detailed information regarding the potential involvement of particular metabolic pathways in pathophysiological causes and consequences of equine insulin dysregulation. This study aimed to assess the dynamic metabolic responses given to an oral glucose test (OGT) in insulin-sensitive and insulin-dysregulated horses by a targeted metabolomics approach to identify novel metabolites associated with insulin dysregulation.

Results: Oral glucose testing triggered alterations in serum insulin (26.28±4.20 vs. 422.84±88.86 µIU/mL, $p < 0.001$) and plasma glucose concentrations (5.00±0.08 vs. 9.43±0.44 mmol/L, $p < 0.001$) comparing basal and stimulated conditions after 180 minutes. Metabolome analyses indicated OGT-induced changes in short-chain acylcarnitines (6.00±0.53 vs. 3.99±0.23 µmol/L, $p < 0.001$), long-chain acylcarnitines (0.13±0.004 vs. 0.11±0.002 µmol/L, $p < 0.001$) and amino acids (2.18±0.11 vs. 1.87±0.08 µmol/L, $p < 0.05$). Kynurenine concentrations increased (2.88±0.18 vs. 3.50±0.19 µmol/L, $p < 0.01$), whereas spermidine concentrations decreased during OGT (0.09±0.004 vs. 0.08±0.002 µmol/L, $p < 0.01$), indicating proinflammatory conditions after oral glucose load. Insulin dysregulation was associated with lower concentrations of trans-4-hydroxyproline (4.41±0.29 vs. 6.37±0.71 µmol/L, $p < 0.05$) and methionine sulfoxide (0.40±0.06 vs. 0.87±0.13 µmol/L, $p < 0.01$; mean±SEM in insulin-dysregulated vs. insulin-sensitive basal samples, respectively), two metabolites which are related to antioxidant defense mechanisms.

Conclusion: Oral glucose application during OGT resulted in profound metabolic and proinflammatory changes in horses. Furthermore, insulin dysregulation was predicted in basal samples (without OGT) by pathways associated with trans-4-hydroxyproline and methionine sulfoxide, suggesting that oxidative stress and oxidant–antioxidant disequilibrium are contributing factors to insulin dysregulation. The present findings

provide new hypotheses for future research to better understand the underlying pathophysiology of insulin dysregulation in horses.

Keywords: Metabolome, Metabolomics, Insulin sensitivity, Insulin dysregulation, Oral glucose test, Horses

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RESEARCH ARTICLE Open Access

Lower plasma trans-4-hydroxyproline and methionine sulfoxide levels are associated with insulin dysregulation in horses

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Background: Insulin dysregulation in horses is a metabolic condition defined by high insulin concentrations in the blood and peripheral insulin resistance. This hyperinsulinemia is often associated with severe damage in the hooves, resulting in laminitis. However, we currently lack detailed information regarding the potential involvement of particular metabolic pathways in pathophysiological causes and consequences of equine insulin dysregulation. This study aimed to assess the dynamic metabolic responses given to an oral glucose test (OGT) in insulin-sensitive and insulin-dysregulated horses by a targeted metabolomics approach to identify novel metabolites associated with insulin dysregulation.

Results: Oral glucose testing triggered alterations in serum insulin (26.28 ± 4.20 vs. 422.84 ± 88.86 μIU/mL, $p < 0.001$) and plasma glucose concentrations (5.00 ± 0.08 vs. 9.43 ± 0.44 mmol/L, $p < 0.001$) comparing basal and stimulated conditions after 180 min. Metabolome analyses indicated OGT-induced changes in short-chain acylcarnitines (6.00 ± 0.53 vs. 3.99 ± 0.23 μmol/L, $p < 0.001$), long-chain acylcarnitines (0.13 ± 0.004 vs. 0.11 ± 0.002 μmol/L, $p < 0.001$) and amino acids (2.18 ± 0.11 vs. 1.87 ± 0.08 μmol/L, $p < 0.05$). Kynurenine concentrations increased (2.88 ± 0.18 vs. 3.50 ± 0.19 μmol/L, $p < 0.01$), whereas spermidine concentrations decreased during OGT (0.09 ± 0.004 vs. 0.08 ± 0.002 μmol/L, $p < 0.01$), indicating proinflammatory conditions after oral glucose load. Insulin dysregulation was associated with lower concentrations of trans-4-hydroxyproline (4.41 ± 0.29 vs. 6.37 ± 0.71 μmol/L, $p < 0.05$) and methionine sulfoxide (0.40 ± 0.06 vs. 0.87 ± 0.13 μmol/L, $p < 0.01$; mean ± SEM in insulin-dysregulated vs. insulin-sensitive basal samples, respectively), two metabolites which are related to antioxidant defense mechanisms.

Conclusion: Oral glucose application during OGT resulted in profound metabolic and proinflammatory changes in horses. Furthermore, insulin dysregulation was predicted in basal samples (without OGT) by pathways associated with trans-4-hydroxyproline and methionine sulfoxide, suggesting that oxidative stress and oxidant–antioxidant disequilibrium are contributing factors to insulin dysregulation. The present findings provide new hypotheses for future research to better understand the underlying pathophysiology of insulin dysregulation in horses.

Keywords: Metabolome, Metabolomics, Insulin sensitivity, Insulin dysregulation, Oral glucose test, Horses

Background

The equine metabolic syndrome (EMS) in horses is associated with severe disturbances in glucose and lipid homeostasis and adiposity. Equine insulin dysregulation reflected by hyperinsulinemia is a key symptom [1, 2]. However, the underlying causal pathomechanisms of EMS and metabolic consequences of insulin dysregulation are currently poorly understood. Laminitis, a chronic painful disease of the hooves, develops as a severe consequence and is often a cause for euthanasia [3]. Insulin and glucose concentrations in plasma as indicators of metabolic dysregulation are frequently measured parameters in the clinical routine of equine medicine. Oral glucose tests and other challenges were used to assess insulin dysregulation exactly [1]; however, these tests assay only the disturbed insulin response, but do not provide information about potential causal pathways leading to insulin dysregulation

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in horses. Furthermore, data on the metabolic consequences of equine insulin dysregulation, other than hyperinsulinemia, are still limited.

Metabolomics can be used as a powerful tool to depict the phenotypic image of metabolism under the current conditions [4]. This is provided by a snapshot-like quantification of an extensive set of metabolites which are products or substrates of various metabolic pathways. Due to the known links between metabolites and pathways, hypotheses about underlying (patho)physiological processes can be proposed. Furthermore, new hypotheses concerning affected pathways can be established as a basis for new experimental designs by introducing novel associations between certain metabolites and phenotypic observations. These new scientific studies will help to identify pathophysiological causes or consequences of equine insulin dysregulation. The identification of potential biomarkers capturing the acute health status is another benefit of metabolomics approaches.

Therefore, the aims of the present study were to (1) elucidate the dynamic metabolic response to a defined oral glucose challenge in horses previously classified as insulin-sensitive (IS) or -dysregulated (ID), and (2) to identify novel metabolites associated with the ID state. To accomplish these aims, the blood plasma metabolome of IS and ID horses was analyzed using a targeted metabolomics approach, important metabolites were selected by multivariate statistical methods, and pathways associated with the selected metabolites were described based on previous knowledge in the context of insulin homeostasis and metabolic disorders.

Methods

Animals and sample collection

Twenty horses of various breeds, ages and body weights (BW) were included in the study. Body condition scoring (BCS) (according to Henneke et al. [5]), (BW) and ages were recorded for each horse. The ages of the horses ranged from 6 to 23 years, and their BW ranged from 147 to 695 kg. Horses had unknown insulinemic status prior to testing and blood samples were collected during routine diagnostic procedures for the assessment of insulin dysregulation in the Clinic for Horses (University of Veterinary Medicine, Hannover). Informed consent was obtained from the owners for scientific use and publication. Horses were fasted overnight before sampling and, in connection with the sample collection, an oral glucose testing (OGT) procedure [6] was conducted. The OGT was carried out by administering 1.0 g/kg BW glucose powder dissolved in 2 L of water by a nasogastric tube. Blood samples were collected from each horse three times: immediately before starting the OGT (BASAL), 120 min after starting the OGT (OGT-120) and 180 min after starting the OGT (OGT-180). Samples were collected via a jugular vein catheter, transferred into plain tubes (Vacuette® Greiner Bio One, Frickenhausen, Germany) for serum and EDTA tubes (Vacuette® Greiner Bio One, Frickenhausen, Germany) for plasma preparation. Blood for serum was incubated at room temperature for 1 h before centrifugation at 3000 g for 6 min at room temperature and blood for plasma was immediately centrifuged at 3000 g for 6 min. Serum and plasma samples were shock-frozen in liquid nitrogen and subsequently continuously stored at − 80 °C until analysis.

Blood serum and plasma analysis

Plasma glucose concentrations were analyzed with a colorimetric assay (GLUC3, Cobas, Roche Diagnostics GmbH, Mannheim, Germany) on an automated discrete analyzer (Cobas Mira, Roche Diagnostics GmbH, Mannheim, Germany). Serum insulin concentrations were analyzed by an equine-optimized ELISA (Equine Insulin ELISA, Mercodia, Uppsala, Sweden) previously validated for use in horses [7].

Plasma metabolome analysis was performed on all samples by the AbsoluteIDQ p180 Kit (Biocrates Life Science AG, Innsbruck, Austria) in the laboratory of Biocrates Life Science AG, according to the manufacturer's standard protocol. This kit format-targeted metabolomics measurement was used to identify and quantify 188 metabolites belonging to five compound classes: acylcarnitines (40), proteinogenic and modified amino acids (19), glycerophospho- and sphingolipids (76 phosphatidylcholines, 14 lyso-phosphatidylcholines, 15 sphingomyelins), biogenic amines (19) and hexoses (1). A detailed list of the compounds is shown in the supplementary material (Additional file 1: Online Resource 1). The fully automated assay was based on phenylisothiocyanate derivatization in the presence of internal standards followed by FIA-MS/MS [acylcarnitines, (lyso-) phosphatidylcholines, sphingomyelins, hexoses] and LC-MS/MS (amino acids, biogenic amines) using a SCIEX 4000 QTRAP® (SCIEX, Darmstadt, Germany) or a Xevo TQ-S Micro (Waters, Vienna, Austria) instrument with electrospray ionization. The experimental metabolomics measurement technique is described in detail by patent US 2007/0004044 [8]. All pre-analytical and analytical procedures were performed, documented and reviewed according to the ISO 9001:2008 certified in-house quality management rules and guidelines of Biocrates Life Sciences AG.

Data analysis and visualization

Plasma glucose and serum insulin concentrations of BASAL, OGT-120 and OGT-180 samples were used as a marker of insulin sensitivity status to assign horses to either of the two experimental groups: IS or ID. This

was conducted by a hierarchical clustering algorithm using the Euclidean distance measure and Ward's clustering method in MetaboAnalyst 3.0 [9].

Plasma metabolome data (absolute concentrations of compounds) were analyzed in MetaboAnalyst after normalization by Pareto scaling. All data were tested and confirmed to be normally distributed by use of the Shapiro-Wilk test in GraphPad Prism (GraphPad Prism, Version 6.07 for Windows, Graph-Pad Inc. La Jolla, CA). Principal component analysis (PCA), repeated measures two-way ANOVA (rmTWA) and heatmap generation were conducted in MetaboAnalyst to visualize and evaluate differences in the metabolic profiles due to classification according to insulin sensitivity status and during OGT. The heatmap was created for the top 22 significant metabolites identified by rmTWA; clustering of the metabolites by 'average' clustering algorithm and plotting the tree by Euclidean distance measure were also included. The RmTWA was performed for the factor 'OGT' (BASAL vs. OGT-120 vs. OGT-180) and 'insulin sensitivity status' (IS vs. ID), also considering interactions and applying false discovery rate correction. $P \leq 0.05$ was considered as statistically significant. A volcano plot was created separately for the non-challenged condition (BASAL) to identify metabolites of interest, searching for metabolites having significantly different concentrations between IS and ID horses, even without any OGT influence. Metabolites with a fold change of at least 1.5 and a t-test p value lower than 0.05 were considered to have high importance. Concentrations of important metabolites were plotted in GraphPad Prism. These were tested statistically for the effect of 'insulin sensitivity status' at different levels of the OGT by rmTWA combined with a pairwise Bonferroni post hoc test in GraphPad Prism.

Results

All horses tolerated the OGT procedure without complications. Plasma glucose and serum insulin increased significantly as a response to oral glucose administration observed after 120 and 180 min of the OGT (Fig. 1a+b; both $p < 0.001$). The clustering algorithm applied to these data divided the horses clearly into two groups, which was the basis to assign them to either the IS ($n = 10$) or ID ($n = 10$) group. The IS group consisted of horses that had low to medium insulin concentrations in response to OGT, while the ID group consisted of horses that had high insulin concentrations in response to OGT. After creating the groups by clustering, the insulin threshold between the two groups was empirically found to be 260 μIU/mL with mean OGT-stimulated insulin concentrations of 131.5 ± 24.8 μIU/mL for IS (mean ± SEM; min. 34.5, max. 257.0 μIU/mL; $n = 10$) and 675.2 ± 101.2 μIU/mL for ID (mean ± SEM; min. 398.9, max. 1403.0 μIU/mL; $n = 10$), respectively (Mann-Whitney test $p < 0.0001$). The two groups had comparable BCS values as an indicator of obesity (6.8 ± 0.4 vs. 7.1 ± 0.2; mean ± SEM; $n = 10$/group), comparable BW (in kg: 481.1 ± 29.0 vs. 408.8 ± 53.72; mean ± SEM, $n = 10$/group) and similar ages (in years: 14.2 ± 1.6 vs. 15.7 ± 1.7; mean ± SEM; $n = 10$/group). The OGT-triggered increase in insulin concentration was significantly greater in ID horses compared to IS horses (Fig. 1a; $p < 0.001$), but the increase in glucose concentration was only slightly higher in ID horses (Fig. 1b; $p = 0.98$).

An overview of the changes in the total metabolomic profiles of IS and ID horses during the OGT is given in Fig. 2. The PCA was used to downscale the dimensions of the data matrix resulting in PC1 and PC2 scores. The PCA scores plot showed a clear separation between the BASAL and the OGT-120/OGT-180 metabolic profiles

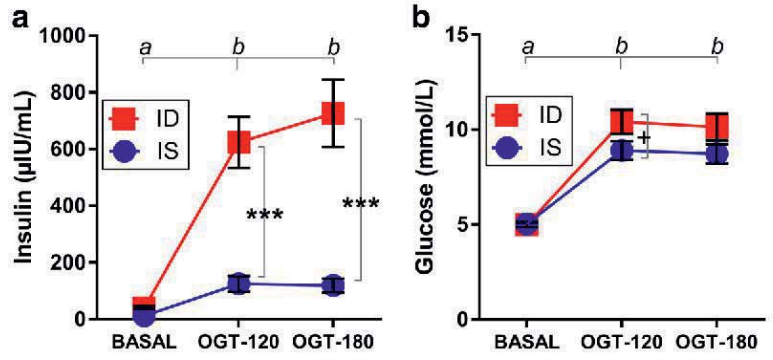

Fig. 1 Increase of (**a**) serum insulin (repeated measure two-way ANOVA (rmTWA) factor oral glucose testing (OGT) $p < 0.001$) and of (**b**) glucose (rmTWA factor OGT $p < 0.001$) concentration in insulin-sensitive (IS) and insulin-dysregulated (ID) horses during OGT. Measurements were carried out at three levels of OGT: immediately before glucose administration (BASAL), and 120 min (OGT-120) and 180 min (OGT-180) after administration. Means ± SEM are shown, $n = 10$, significant differences between IS and ID are indicated as ***($p < 0.001$), and a trend between IS and ID is indicated as $^{+}(0.05 < p < 0.1)$. a, b = different superscripts indicate significant differences between basal and challenged conditions as the main OGT effect in both ID and IS horses (Bonferroni post hoc test)

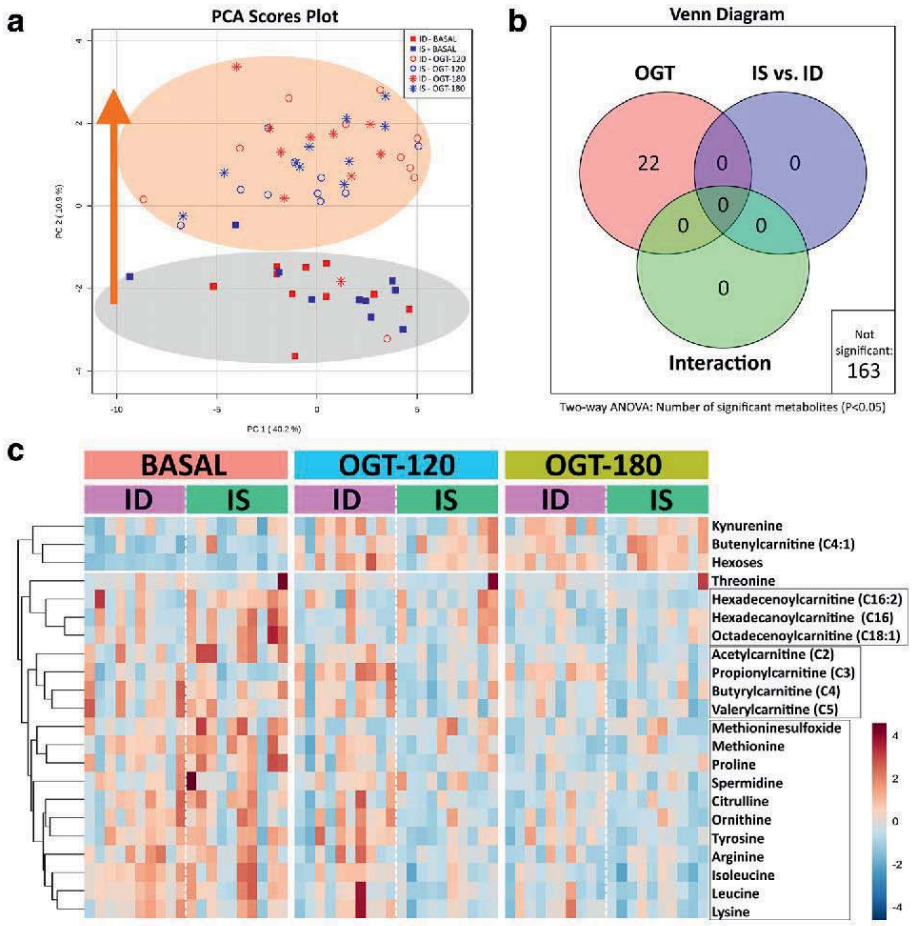

Fig. 2 **a** Principal component analysis scores plot showing metabolic profiles of insulin-sensitive (IS) and insulin-dysregulated (ID) horses sampled before glucose administration (BASAL), and 120 min (OGT-120) and 180 min (OGT-180) after administration. **b** Number of significant metabolites detected by repeated measure two-way ANOVA with false discovery rate correction, analyzing the effect of OGT, insulin sensitivity status (IS vs. ID) and respective interactions. **c** Heatmap illustrating relative concentrations of top 22 significant metabolites during OGT: BASAL, OGT-120 and OGT-180 for IS and ID horses

with a shift of all OGT-treated horses along the y-axis (PC2; Fig. 2a). Twenty-two metabolites were significantly affected by the OGT (Fig. 2b), which was the underlying cause of the shift observed in the PCA. The insulin sensitivity status did not affect the metabolite profile, and interaction between the two factors also remained non-significant (Fig. 2b). Accordingly, the remaining 163 metabolites studied did not show any significant differences by ANOVA (Fig. 2b). A heatmap was created by using the 22 metabolites identified by the ANOVA to visualize the metabolites responsible for the OGT effect (Fig. 2c). The first level of branches in the clustering tree separated between 3 metabolites having increasing concentrations and 19 metabolites having decreasing concentrations during the OGT. The former group consisted of kynurenine, butenylcarnitine and hexoses, while the latter group consisted of long-chain fatty acylcarnitines (C16-18), short-chain fatty acylcarnitines (C2-5), (modified) amino acids and biogenic amines. Hexose (Fig. 3a; $p < 0.001$) and kynurenine (Fig. 3b; $p = 0.002$) concentrations were increased at OGT-120 and OGT-180, whereas spermidine (Fig. 3c; $p = 0.006$) concentrations decreased during OGT in both IS and ID horses. Proteinogenic amino acids (Fig. 3d; $p = 0.024$), short-chain acylcarnitines (Fig. 3e; $p < 0.001$) and long-chain acylcarnitines (Fig. 3f; $p < 0.001$) also decreased during OGT.

A volcano plot comprised of fold change and t-test statistics was analyzed using the results of BASAL samples to identify the metabolites with the greatest discriminating potential between IS and ID horses (Fig. 4a). This plot shows in its upper left corner the metabolites of interest (MOI), those that have the highest fold change and the lowest p value of the t-test comparing IS vs. ID. Two metabolites were clearly identified: trans-4-hydroxyproline (FC = 1.59, $p = 0.003$) and methionine sulfoxide (FC = 2.09, $p = 0.013$), with higher concentrations in

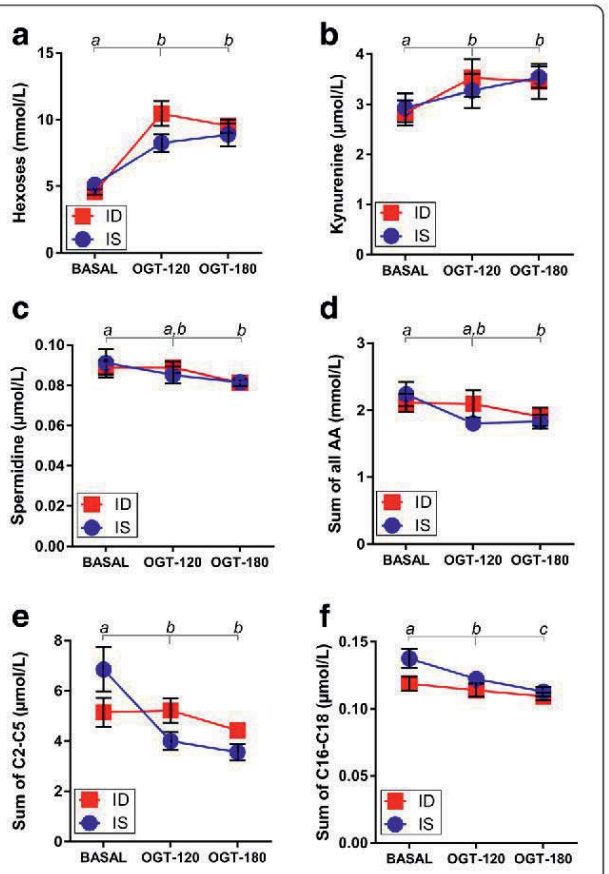

Fig. 3 Metabolites of importance which were significantly affected by OGT in insulin-sensitive (IS) and insulin-dysregulated (ID) horses. Concentration of (**a**) hexoses (repeated measure two-way ANOVA (rmTWA), factor OGT $p < 0.001$) and (**b**) kynurenine (rmTWA, factor OGT $p < 0.01$) increased, while (**c**) spermidine (rmTWA, factor OGT $p < 0.01$), (**d**) sum of all proteinogenic amino acids (rmTWA, factor OGT $p < 0.05$) (**e**) sum of short-chain (C2, C3, C4 and C5) acylcarnitines (rmTWA, factor OGT $p < 0.001$) and (**f**) sum of long-chain (C16, C16:1, C16:2, C:18, C18:1, C:18:2) acylcarnitines (rmTWA, factor OGT $p < 0.001$) decreased. Insulin status had no significant effect. Means ± SEM are shown, $n = 10$; a, b, c = different superscripts indicate significant differences between basal and challenged conditions as the main OGT effect in both ID and IS horses (Bonferroni post hoc test)

IS horses compared to ID horses. Further assessment of the dynamic changes of these MOI during the OGT revealed that both trans-4-hydroxyproline (Fig. 4b) and methionine sulfoxide (Fig. 4c) concentrations decreased significantly in the IS group, but not in the ID group. Analyzing the effect of insulin sensitivity status by Bonferroni post hoc test revealed that these metabolites had significant differences only under BASAL conditions, with lower concentrations in the ID group (Fig. 4b: trans-4-hydroxyproline $p = 0.040$; Fig. 4c: methionine sulfoxide $p = 0.010$).

Discussion

This study used a targeted and quantitative metabolomics analysis to describe novel metabolic profiles associated with equine insulin dysregulation in two approaches. (1) Basal concentrations before OGT challenge are suitable for identifying metabolites which can predict the metabolic characteristics of horses associated with insulin sensitivity or dysregulation. The dynamic responses after oral glucose load seen at OGT-120 and OGT-180 enables the identification of pathways which are causally affected by the oral glucose load as a single challenge in both IS and ID horses. The ID individuals have a higher pancreatic insulin secretion in response to a certain glucose load than IS individuals [1, 2, 6]. These dynamic changes of glucose and insulin were – as expected – clearly visible at 120 (OGT-120) and 180 (OGT-180) min after oral glucose load in the present study, confirming the classification into two groups (IS and ID) by the clustering algorithm. The threshold used here is considered independent of the cut-offs used clinically based on insulin concentrations during OGT and was declared as a differentiation criterion applicable to the animal cohort used in the current study to establish two experimental groups with a physiological and a pathophysiological response, respectively. Therefore, it should be emphasized that the metabolites identified here cannot be extrapolated for clinical diagnostic use; our findings provide rather a basis for hypotheses generation concerning insulin dysregulation mechanisms and potential candidates for future biomarker development. Moreover, the OGT induced a severe insulin increase with a wide dynamic range, reflecting that insulin sensitivity is not a dichotomous state of being either IS or ID, but rather that ID exists in different intensities. The typical condition of hyperinsulinemia was intensive in several ID horses, but this was not accompanied by a correspondingly strong hyperglycemia. This equine-specific pathophysiological phenomenon requires future clarification regarding its underlying mechanisms.

The corresponding metabolic profiles were studied by using the AbsoluteIDQ p180 Kit (Biocrates Life Science AG). This targeted metabolomics assay of kit format was designed by the developer to quantify a defined set of metabolites reflecting specific metabolic disorders, such as obesity, insulin resistance, proinflammation, dysregulation of glucose and lipid metabolism, and mitochondrial dysfunction. Although this assay was developed for human purposes, it is a species-independent analytical tool to accurately detect small molecules in various biological samples and, accordingly, we were able to detect a wide range of circulating acylcarnitines, amino acids, biogenic amines, glycerophospho- and sphingolipids, and hexoses in horses. The assay kit applied was considered suitable for acquiring phenotypic patterns of equine metabolism affected by insulin dysregulation because the metabolic syndrome of horses has been demonstrated to exhibit some common symptoms with

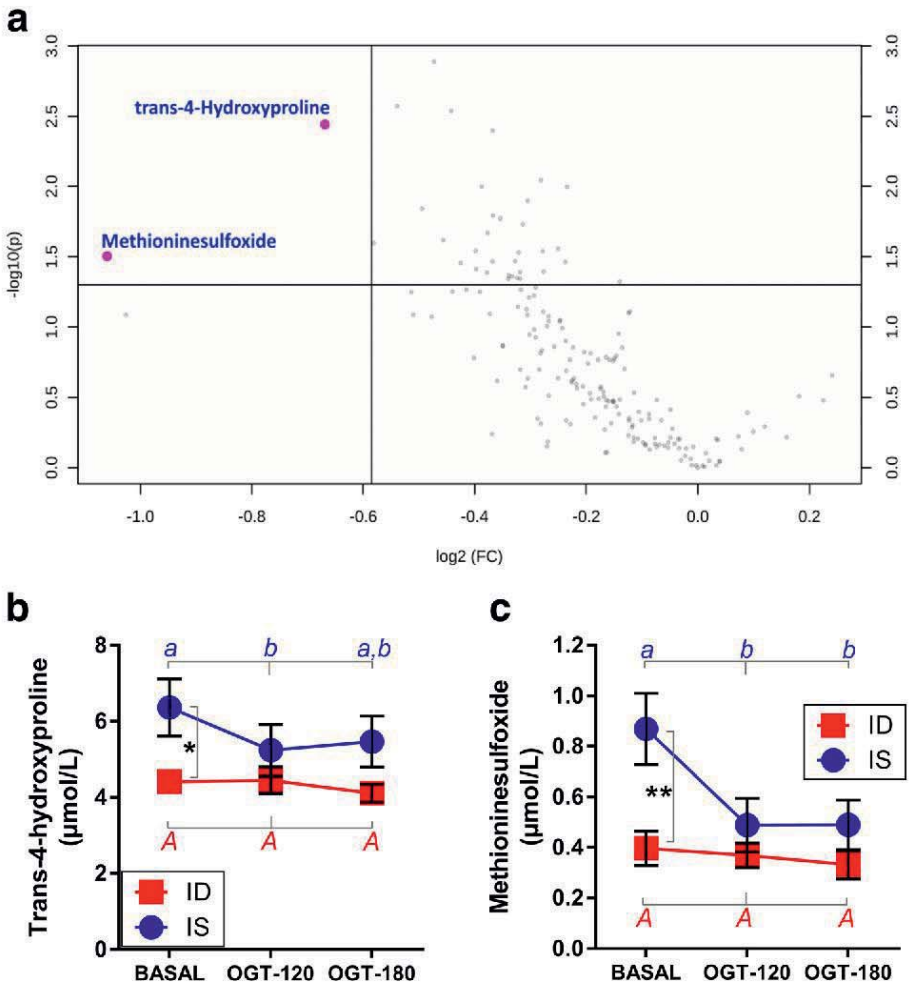

Fig. 4 **a** Volcano plot highlighting metabolites of importance (trans-4-hydroxyproline $p = 0.0036$; methinonine sulfoxide $p = 0.0134$) which were different between insulin-sensitive (IS) and insulin-dysregulated (ID) horses under unchallenged conditions (only BASAL) (**b**) Trans-4-hydroxyproline (repeated measure two-way ANOVA (rmTWA), factor insulin sensitivity status $p < 0.05$, factor OGT $p = 0.06$) and (**c**) methionine sulfoxide (rmTWA, factor insulin sensitivity status $p < 0.05$, factor OGT $p = 0.06$) concentrations shown in serum of IS and ID horses at three levels of OGT: before glucose administration (BASAL), and 120 min (OGT-120) and 180 min (OGT-180) after administration (means ± SEM, $n = 10$). Significant differences between IS and ID are indicated as ** ($p < 0.01$), * ($p < 0.05$). a, b = different superscripts indicate significant differences ($p < 0.05$) between basal and challenged conditions in IS horses, while no OGT effect was observed (as indicated by A) in ID horses (Bonferroni post hoc test)

the human metabolic syndrome: adiposity, chronic inflammation and mitochondrial dysfunction [1, 2].

Metabolic response to oral glucose challenge

Metabolomic profiles changed overall after the OGT in a similar manner, irrespective of insulin sensitivity status and the time after challenge. Twenty-two metabolites were significantly affected by the OGT. The most obvious pattern seen was the rise of the hexose concentration at OGT-120 and OGT-180 in both IS and ID horses. Since circulating hexoses in adult horses consist mostly of glucose, this increase was expected after the OGT and was in accordance with the result of the serum glucose measurement.

Kynurenine concentrations were also increased due to the OGT in both IS and ID horses. Kynurenine is a known proinflammatory marker originating from tryptophan metabolism, which is increased in human patients with diabetic retinopathy [10]. Kynurenine is generated by indoleamine 2,3-deoxygenase, a rate-limiting enzyme, which is activated by inflammatory signals and reactive oxygen species (ROS) related to obesity and insulin resistance [10, 11]. In general, most of the ID horses are reported to be obese [1, 2]; however, the IS and ID horses in this study had comparable BCS values (as an indicator of obesity) and BW. Adipose tissue in humans was identified as a major source of indoleamine 2,3-deoxygenase activity and inflammatory cytokines [10]. Furthermore, acute short-term oral glucose load led to an

inflammatory profile even in nondiabetic humans [12]. Although inflammatory cytokine levels were not determined in the horses of this study, the elevated kynurenine concentrations probably indicate a proinflammatory effect of short-term high glucose load by OGT. Therefore, the acute oral glucose load by OGT appeared to affect the molecular health of the horses and was also reflected by decreased spermidine concentrations in both IS and ID horses. Spermidine is a polycationic biogenic amine which is produced by various tissues. One of its predominant molecular functions is to inhibit nonenzymatic glycation of proteins and nucleic acids [13]. The strong reduction in spermidine concentrations indicated its utilization in the prevention of glycation-derived damage of molecules. To summarize, OGT with 1.0 g glucose/kg BW in horses, even as a short-term challenge, can potentially trigger a shift towards a proinflammatory metabolic phenotype.

Administering glucose in high amounts by OGT necessarily induced a shift in substrate availability for energy-gaining pathways. Horses are known to have a well-developed short-chain fatty acid (SCFA) metabolism, with large amounts of butyrate absorbed from the colon [14]. Thus, their energy metabolism depends largely on SCFA, while glucose must be produced by gluconeogenesis from gluconeogenic amino acids, at least when fed on a diet rich in crude fiber and low in grain. During OGT, gluconeogenic needs decreased and the excess of glucose could be used for ATP generation, while utilization of long-chain fatty acids was diminished, a physiological effect called the Randle cycle [15]. Increasing insulin levels due to the OGT probably accounted for short-term protein anabolism. This was reflected in the metabolomics approach in IS and ID horses by a decrease of all proteinogenic amino acids. A reduced utilization of SCFA was reflected by a decrease of short-chain acylcarnitines, mitochondrial degradation products derived mainly from SCFA metabolism. Moreover, enhanced insulin-mediated anti-lipolysis was reflected by reduced concentrations of long-chain acylcarnitines, mitochondrial degradation products of long-chain fatty acids.

Novel metabolites and pathways associated with insulin resistance

No clear differences between IS and ID horses (if present) could be detected by ANOVA, PCA or the heatmap because of the dominance of the OGT effect on the total metabolome. Physiologically, this means that the response to glucose was equally intensive in both IS and ID horses, superposing any potential effects of insulin dysregulation. However, the metabolomics approach was also performed to identify the basal differences of metabolic profiles as a function of insulin sensitivity status.

Therefore, a volcano plot comprising fold change and t-test statistics was analyzed using the results of BASAL samples to identify the metabolites with the greatest discriminating potential between IS and ID horses. Trans-4-hydroxyproline and methionine sulfoxide attracted attention, with higher concentrations in IS horses compared to ID horses. During the OGT, both MOI, trans-4-hydroxyproline and methionine sulfoxide, decreased significantly in the IS group, but not in the ID group. However, the concentration of these metabolites was only different between IS and ID under BASAL conditions, with lower concentrations in the ID group. It is suggested that both MOI belong to the oxidant–antioxidant system of an organism to cope with oxidative stress directly (methionine sulfoxide) or indirectly (trans-4-hydroxyproline; synthesis needs vitamin C, an antioxidant) [11, 16, 17]. Oxidative stress is defined as oxidant–antioxidant disequilibrium due to either increased ROS production, a decrease in the capacity of antioxidant system to defend the organisms against ROS or both. The ROS promote cell damage and can modify endocrine signaling pathways, such as the insulin response cascade leading to insulin resistance [18], one of the potential underlying processes for insulin dysregulation. Disturbances of the oxidant–antioxidant equilibrium have been well-studied in horses suffering from various diseases, but decent markers are still not sufficiently characterized [19, 20]. It is especially not clear yet whether a disequilibrium of the oxidant–antioxidant system is causally relevant for the development of insulin dysregulation in horses. Equine insulin dysregulation and disturbances in the oxidant–antioxidant system are currently understood poorly. Banse et al. [21] suggested that obesity-associated hyperinsulinemia was connected with oxidative stress in skeletal muscle, but they did not find any evidence for oxidative damage to skeletal muscle in obese hyperinsulinemic horses. However, protein carbonyls – products of protein oxidation by ROS – were significantly reduced in the skeletal muscle of obese hyperinsulinemic horses [21]. Consistent with these findings, both MOI found in this study decreased with increasing glucose and insulin concentrations due to the OGT in serum, with a more pronounced decrease in IS horses. Accepting the former suggestion that acute oral glucose load causally promoted a shift into a more inflammatory status even in healthy horses, a decrease in plasma markers of oxidative stress might reflect an increasing disequilibrium in tissue oxidant–antioxidant balance in IS horses; this condition is intensified in ID horses.

Conclusion

It can be concluded as a working hypothesis that higher concentrations of trans-4-hydroxyproline and methionine sulfoxide under basal conditions are a positive sign

for health and insulin sensitivity, indicating that healthy horses can remove oxidatively modified amino acids from cells more effectively to avoid cell damage. The lower basal values of these MOI and their decrease during OGT, as well as the concurrent increase of the proinflammatory marker kynurenine are assessed as negative signs and indicate that oxidative stress is involved in the pathway to insulin dysregulation of horses. However, the underlying mechanisms by which oxidized amino acids were produced and modulated in equine tissues is widely unknown so far. Furthermore, because of the small sample size ($n = 10$ per group) and the lack of a clinical diagnosis, the potential significance of trans-4-hydroxyproline and methionine sulfoxide as biomarkers for equine insulin dysregulation has to be tested in larger populations under clinical settings. However, the high diversity of the experimental horse population presented regarding breed, age and BW suggests a universal pathophysiological potential for equine insulin dysregulation. Further research based on these implications should focus on defining criteria for the early diagnosis of insulin dysregulation in horses.

Additional file

Additional file 1: Online Resource 1. List of metabolites measured by the Absolute-IDQ p180 Kit. Name and abbreviation of metabolites measured in the targeted metabolomics analysis approach by using the Absolute-IDQ p180 Kit of Biocrates Life Sciences AG (Innsbruck, Austria). (DOCX 17 kb)

Abbreviations

ANOVA: Analysis of variance; BCS: Body condition score; BW: Body weight; FIA-MS/MS: Flow-injection analysis and tandem mass spectrometry; ID: Insulin-dysregulated; IS: Insulin-sensitive; LC-MS/MS: Liquid chromatography and tandem mass spectrometry; MOI: Metabolite of interest; OGT: Oral glucose test; rmTWA: Repeated measures two way analysis of variance; ROS: Reactive oxygen species; SCFA: Short-chain fatty acids; SEM: Standard error of the mean

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TW, KF and KH designed the study; ÁK and KH analyzed and interpreted the data; ÁK, TW, KF and KH drafted and revised the data; ÁK, TW, KF and KH approved the final version of the manuscript and agreed to be accountable for all aspects of the study.

Ethics approval and consent to participate

Samples from ID horses and ponies were collected during routine diagnostic procedures in the Clinic for Horses, Hannover, and owners gave written informed consent for the study.

Consent for publication

Owners gave written informed consent for publication by means of signing our official client acceptance form.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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6 GENERAL DISCUSSION

A combination of investigation into the analyses of blood samples with analyses of molecular levels enables the correlation and assessment of activation of several signaling pathways involved in insulin signaling. In the present study, the OGT and the CGIT resulted in sufficient stimulation of metabolic processes, reflected via changes in endocrine and metabolic profiles assessed by blood analyses for insulin, glucose, TRG, NEFA, cortisol and fructosamine. Furthermore, molecular examination of tissue biopsies revealed a stimulation-provoked phosphorylation of the main targets in insulin signaling. This PhD project is the first study investigating the insulin signaling cascade in equids under basal and stimulated conditions on a molecular protein level in various equine tissues in combination with detailed analyses of the metabolic condition. The results of the present study indicate that IV-administered exogenous insulin could stimulate the insulin signaling cascade in various tissues. However, highly individual variations in molecular responses were detected between horses. Further research is needed to evaluate the physiological peculiarities of equine insulin signaling and to assess if and how this signaling is modulated in horses with HI and ID.

Stimulation of healthy warmblood horses by OGT and CGIT in the present study indicated profound changes in the metabolic and endocrine profile of the horses. In the OGT procedure, enteral-absorbed glucose provoked a rapid increase in the insulin response with prolonged HI. However, individual insulin responses were highly variable even in these healthy warmblood horses, supporting the idea that IS is not a dichotomous state of either IS or ID but rather that IS exists in different intensities. Analyses of the insulin concentration of horses and ponies with unknown insulin status support this idea. The range of post OGT insulin concentrations ranged from 34.5 to 1403.0 µIU/mL. Therefore, subsequent research is needed to investigate the insulin concentrations during OGT as prognostic factors in EMS horses. We expected that higher insulin concentrations were negatively correlated with long-term outcome and survival.

Insulin dynamics in response to orally applied glucose and enteral absorption were both monophasic and biphasic during OGT performed in the present study. This is in

accordance with previous studies investigating the pancreatic secretion pattern of insulin in response to oral glucose challenge (Smith et al. 2016) or IV glucose stimulation (Hoffman et al. 2003). Insulin secretion in response to oral glucose is generally described as much more variable compared to the reproducible pattern of insulin secretion in response to IV glucose application in humans (Wilcox 2005).

The OGT performed in the present study are considered to be indirect methods to assess IS in horses. The OGT tests the capacity of endogenous insulin to shift absorbed glucose into peripheral IS target tissues. In contrast to the OGT, the CGIT is a direct method to assess IS in horses and tests the capacity of the exogenous insulin to shift the injected glucose into IS tissues. This was assessed by analyses of blood metabolites and indirectly by detecting the extent of phosphorylation of signaling components, such as insulin receptors, in the healthy horse. It was assessed that phosphorylation of insulin signaling molecules represent activation of the cascade. Therefore, it could be expected that the activation of the cascade failed in IR. However, it has been shown that ID can occur independently of IR (De Laat et al. 2016). The metabolomics approach revealed that even basally, oxidative stress in ID horses occurred with HI, which can lead to IR, at least in humans and rodents (Vinayagamoorthi et al. 2008, Zeyda and Stulnig 2009, Oxenkrug 2013).

Interestingly, IV and oral tests in ponies did not reveal similar results regarding the insulinemic state (De Laat et al. 2016). Results of the present study underline these recently reported findings. Four horses responded via oral glucose challenge in an OGT with markedly higher insulin concentrations in response to the oral glucose challenge compared to the remaining eight horses, and these differences could not be detected in CGIT. These findings highlight and support the hypothesis proposed recently that equine ID may be linked to gastrointestinal factors (De Laat et al. 2016). Differences between animals may also be explained by variations in gastric emptying rate, intestinal glucose absorption, hepatic extraction or urinary glucose spilling if the renal threshold of 9 to 10 mmol/L is exceeded due to hyperglycemia provoked by absorption of high amounts of glucose from the GIT (Carlson 1996). Due to the study design without the collection of urine during the OGT and CGIT procedure, we cannot assess the urinary glucose losses and potential impairments of study results by urine

glucose spilling. The renal threshold can be exceeded during standard-dosed OGT as well as during initial hyperglycemia in CGIT as shown by the glucose concentrations in the first and third study part. Tóth et al. (2009) optimized the FSIGTT protocol to reduce urinary glucose spilling and suggested 100 mg/kg BW glucose as the optimal dose for implementation of FSIGTT. Whether reduction of the glucose applied during our modified CGIT is possible remains unknown so far but should be investigated in further studies. Dosing glucose for the implementation of oral glucose challenge tests is a frequently discussed issue (Manfredi 2016, The Equine Endocrinology Group 2016, De Laat and Sillence 2017). A potential lowering of the glucose dosage during OGT may be equally sufficient for stimulation due to supra-physiological stimulation with 1 g/kg BW. This is especially relevant, as metabolome analyses revealed signs of pro-inflammation during OGT. Interestingly, both IS and ID individuals responded with decrease spermidine and increased kynurenine concentrations. Spermidine inhibits nonenzymatic glycation of proteins and nucleic acids (Gugliucci and Menini 2003) and, therefore, decreased concentrations during OGT reflect its utilization in the prevention of glycation-derived damage to molecules. Moreover, spermidine reduced inflammation and seemed to be involved in the regulation of lipid metabolism and cell growth (Minois 2014). By contrast, increased kynurenine concentrations were linked directly to inflammation. Kynurenine is synthesized by the enzyme tryptophan dioxygenase in response to immune activation (Opitz et al. 2011). Interestingly, Wang et al. (2010) showed that kynurenine has properties which dilate blood vessels during inflammation. Therefore, hyperglycemia and HI during OGT may lead to vascular or endothelial dysfunction, a condition already linked to the development of endocrinopathic laminitis (Morgan et al. 2016). Whether short-term stimulation and pro-inflammatory shift during OGT has already compromised patients remains unknown, but lowering glucose concentration during OGT and, therefore, reducing hyperglycemia and HI might reduce any side effects. However, this hypothesis needs to be confirmed by dose-comparison studies with a special focus on the diagnostic value and accuracy to preserve diagnostic significance and evaluate the optimal dosing of glucose.

Interestingly, we were not able to identify the horses with higher insulin response in OGT by their phenotype. There was neither a correlation between BCS and insulin response during OGT, nor insulin clearance in CGIT. Moreover, differentiation of horses and ponies into IS and ID individuals based on their insulin response in OGT in the third research project did not lead to different average BCS values between groups. Individuals with high insulin responses had comparable BCS values to those with lower insulin responses, highlighting that high BCS is not necessarily indicative of ID nor does low BCS preclude ID. Taking these findings together, the first hypothesis had to be refuted. It was not possible to show body condition-related changes in endocrine and metabolic responses to oral glucose and intravenous glucose and insulin stimulation either in healthy horses or in ID horses and ponies.

Application of glucose via nasogastric tubing in the OGT procedure induced an initial increase in serum cortisol concentrations, whereas IV application of glucose and insulin did not result in increased cortisol concentrations during CGIT. Therefore, the initiation of metabolic processes affecting glucose homeostasis via exogenous glucose application or even combined glucose and insulin application may not necessarily be reasonable for the activation of stress responses rather than application via nasogastric tubing, and the procedure of tubing itself may be the cause of cortisol increase.

A combined injection of insulin and glucose provoked an initial increase in insulin and glucose concentrations, followed by an even decline. The CGIT was performed with porcine zinc-insulin that is licensed as Caninsulin® (Caninsulin® - 40 I.E./ml, MSD, Unterschleißheim, Germany) in Germany for the treatment of diabetes in cats and dogs. This type of insulin is a formulation of 30 % amorphous zinc-insulin and 70 % crystalline zinc-insulin. However, performance of CGIT with porcine zinc-insulin is for off-label use, due to the fact that the commercially available porcine zinc-insulin formulation in Germany is not licensed for IV usage. The human recombinant insulin used in the original CGIT protocol developed by Eiler et al. (2005) has pharmacological differences compared to the porcine zinc-insulin formulation used in the present study. Usage of porcine zinc-insulin resulted in a prolonged elimination phase and slower return of glucose levels to baseline concentrations in the present study compared to

the original literature describing the glucose and insulin kinetics following CGIT with human recombinant insulin.

The pharmacokinetics of porcine zinc-insulin has been examined in dogs after subcutaneous application in multiple studies (Graham et al. 1997, Fleeman et al. 2009). Graham et al. (1997) observed a two-step response in blood insulin concentrations after subcutaneous injection. The first peak of insulin concentration is thought to be due to the absorption of the amorphous component, while the second peak is thought to be due to the absorption of the crystalline component. The first peak occurred after about three hours and the second after about nine hours. The human recombinant regular insulin, a fast-acting insulin analog, is modified by the replacement of one amino acid, provoking either faster disaggregation of the former hexamer structure following injection and, therefore, increased absorption or modified receptor binding by the altered tertiary structure (Owen and Roberts 2004). However, impaired absorption can be neglected due to IV injection. If faster disaggregation of hexamer structures impairs pharmacological properties after IV injection remains unknown. Interestingly, none of the study horses developed clinical signs of marked hypoglycemia in response to CGIT performed with porcine-zinc insulin. Severe clinical hypoglycemia is reported to be a complication during the CGIT procedure, which requires an immediate injection of glucose to prevent hypoglycemic shock (Bröjer et al. 2013). This is the case when insulin regulation and sensitivity are unaffected and IS tissues respond with marked glucose uptake, especially when performed in horses with questionable IR. Whether the usage of porcine zinc-insulin is advantageous compared to human recombinant insulin for the implementation of CGIT under clinical conditions or not remains unknown. Nevertheless, reliable reference ranges have to be established to allow usage in clinical settings and safe identification of individuals suffering from IR. Due to the close structural homology between equine and porcine insulin (in amino acid 9 of the A chain), we speculate that porcine zinc-insulin, used in the present study for the implementation of CGIT and IV stimulation, acts more comparably to the endogenous equine insulin in contrast to human recombinant insulin, which differs in amino acid 9 on the A chain and amino acid 30 on the B chain (Ho et al. 2008, Kuuranne et al. 2008, Ho et al. 2011).

Basal NEFA concentrations, determined prior to OGT and CGIT, showed high inter-individual variation. Due to overnight starvation prior to testing, lipolysis in the AT was promoted in study horses and resulted in NEFA release and high serum NEFA concentrations. This was reproducible and additionally occurred after fasting prior to biopsy sampling. Basal NEFA concentrations were comparable to concentrations measured prior to OGT and CGIT. Bertin et al. (2016) observed fat mobilization within four hours of fasting in horses and speculated that the initiation of fat mobilization indicates quick exhaustion of liver glycogen stores. Previous studies in humans investigated the effect of acute high serum NEFA concentrations and described increased hepatic glucose output, reduced peripheral IS and modified glucose stimulated insulin secretion (Amery et al. 2000). Moreover, starvation has been shown to cause IR in humans (Newman and Brodows 1983). By contrast, chronic elevation of NEFA is associated with decreased glucose-stimulated insulin secretion and reduced insulin synthesis in humans (Amery and Nattrass 2000). Therefore, fasting prior to assessment of IS is discussed controversially. Knowles et al. (2017) showed that OST results differ depending on the fasting status and reported significantly higher insulin responses in OST after fasting compared to non-fasting conditions in horses. In accordance with this, Bertin et al. (2016) concluded that the longer the horses were fasted, the more likely they were to be classified as IR. However, over-night fasting is currently recommended for in-feed OGT (The Equine Endocrinology Group 2016) and a three hour fast is recommended prior to the implementation of OST (Bertin et al. 2016). The optimal fasting time for the implementation of CGIT is discussed controversially and ranged from 14 hours (Bröjer et al. 2013) to feeding low non-structural carbohydrate roughage overnight (Morgan et al. 2015). We decided to fast all horses for 14 hours to create similar conditions for all dynamic diagnostic tests and stimulation procedures. Interestingly, fasting prior to glucose challenge tests for diagnostic purposes on IR in humans is recommended for eight hours (Horowitz et al. 1993). In addition to disagreement between studies for optimal fasting time prior to testing, differences in the testing principles and their physiological modes of action should be considered. Moreover, species-specific characteristics regarding digestion and fermentation impede direct transference.

The NEFA concentrations under the challenge of OGT and CGIT decreased to comparable minimal concentrations with similar dynamics in OGT and CGIT in all individuals. This is in accordance with previous studies reporting reduced NEFA concentrations during EHC in horses (Suagee et al. 2011, Urschel et al. 2014a). The reduction of plasma NEFA concentration is due to the inhibitory effects of insulin on the lipolytic enzyme HSL, resulting in reduced release of NEFA from the AT (Meek et al. 1999). Although the extent of phosphorylated HSL did not decrease significantly in all AT after the IV injection of insulin, the results of the present study suggest similar conditions in horses. Moreover, anti-lipolysis was reflected by reduced concentrations of mitochondrial degradation products of LCFA in IS and ID horses and ponies during OGT. The results of the first study part indicate a saturation of the suppression of lipolysis by insulin already observed with intermediate insulin concentrations provoked by the oral challenge in OGT. In accordance with these findings, EHC studies indicated that insulin concentrations, similar to postprandial insulin concentrations, were sufficient for maximal anti-lipolytic effects in horses (Urschel et al. 2014a). Additionally, the anti-lipolytic effect seemed to be independent of the insulin's origin, indicated by the equal efficacy of exogenous porcine zinc-insulin and endogenous equine insulin.

Insulin stimulates fatty acid synthesis in the AT and LT through increased glucose uptake and formation and storage of TRG. The enzyme responsible, FAS, is increased by the activation and phosphorylation of acetyl-CoA carboxylase (Saltiel and Kahn 2001). The total serum TRG concentrations did not differ during the stimulations in the present study, though tissue TRG content was not measured in the biopsy samples. In contrast to lipogenesis, insulin inhibits lipolysis in the AT profoundly throughout the inhibition of HSL (Lonnroth and Smith 1986). Anti-lipolytic effects are mediated by dephosphorylation and, thereby, deactivation of HSL, suppressing TRG breakdown (Wilcox 2005). Interestingly, the extent of the phosphorylated HSL did not differ significantly between basal unstimulated and stimulated hyperinsulinemic conditions. However, concomitant analyses of blood samples collected during the stimulation process and the biopsy collection indicated a significant decrease of serum NEFA concentrations in response to IV stimulation comparable to the NEFA decrease in CGIT described above. A possible explanation for the absent change in the

phosphorylation extent of HSL may be a time-consuming adaptive process in the AT regulation induced by insulin. Biopsies were collected in the early elimination phase of glucose, indicating high plasma clearance by either insulin-mediated tissue uptake or even urinary glucose spilling. Therefore, the collection of biopsies and investigation of insulin's influence on lipid metabolism at later time points with NEFA concentrations reaching a nadir with minimal concentrations might have resulted in a more profound change in HSL phosphorylation on a molecular level.

Morrison et al. (2017) examined the AT morphology and protein expression of HSL in lean and obese horses. The adipocyte area was generally significantly greater in obese horses compared to lean horses. Interestingly, expression of HSL was significantly lower for RPAT in obese animals compared to NUAT, SCAT, epicardial AT and omental AT. These results confirm that equine obesity is associated with changes in the expression of lipolytic proteins in certain depots, which may indicate functional differences between regional ATs (Morrison et al. 2017). However, it was not possible in the present study to detect the basal difference in the total extent of HSL or of phosphorylated HSL, nor in response to stimulation, reflected by changes in the extent of phosphorylation between lean and obese horses.

Despite morphological differences between various ATs (Frayn 2000), visceral AT has been shown to express different metabolic characteristics compared to SCAT, with higher metabolic activity (Giorgino et al. 2005). In a study performed on human AT, variations in insulin action on different AT were observed (Montague and O'rahilly 2000). Bolinder et al. (1983) reported lower sensitivity to the anti-lipolytic effects of insulin in human visceral AT compared to SCAT (Bolinder et al. 1983). Comparison of protein expression among variable equine tissues may be interesting to draw conclusions of differences in metabolic activities. Regional adiposity is common, especially in horses and ponies with ID or IR, and variable protein expression in theses specific AT depots may provide further information about the pathophysiological link between ID or IR and regional adiposity. Further *in vitro* examination of the tissues biopsies collected under the influence of different agents, such as insulin or β-adrenergic simulators, may have provided additional information on the metabolic activity of the different equine tissues. Proof of activation or deactivation indicated by

phosphorylation or dephosphorylation of selected enzymes and resulting changes in metabolites would have been an additional advantage. However, immunofluorescent staining performed in RPAT, NUAT and SCAT of the horses in the present study illustrated heterogeneous distribution of InsR-β without differences in fluorescent intensity between the three AT investigated. This suggests that potential metabolic differences between equine AT might not be directly related to the extent of InsR-β in theses tissues.

The FAS, as a counterpart enzyme to HSL, promotes lipogenesis on insulin stimulation. The total content of FAS did not differ between basal and stimulated conditions in any tissues investigated. However, we were not able to investigate the phosphorylation status of FAS, which may have provided interesting additional information on the anabolic influence of lipid metabolism by short-time stimulation with insulin and glucose. Moreover, there was no correlation of the FAS content and BCS.

Biopsies were collected during the elimination phase of glucose by provoked insulin-mediated uptake in insulin-dependent tissues to detect the maximal activation of insulin signaling induced by IV injection of insulin and glucose in the present study. Tissue-specific variations in acute response of insulin signaling were observed. Interestingly, the increased extent of InsR-β was only detected in LT, whereas further downstream targets, such as PKB/AKT, showed a marked increase in the extent of phosphorylation in all tissues investigated. Western blot analysis of InsR-β in LT and MT produced reliable and specific bands for quantification by densitometry, whereas the antibody (phosphorylated insulin receptor-β (Try1162/1163), Santa Cruz Biotechnology Inc.) used for the detection of phosphorylated InsR-β did not provide satisfying bands usable for reliable quantification by densitometry in the AT samples. Therefore, failure to detect an increased extent of phosphorylated InsR-β in AT may also be due to either a very low abundance, equine AT-specific isoforms, or methodological problems. However, immunofluorescent staining in NUAT, SCAT and RPAT indicated the expression of InsR-β and satisfactory detection.

The InsR-β did not respond with an increased extent of phosphorylation in the MT following IV insulin injection in the present study. However, all tissues expressed an

enhanced AMPK-α phosphorylation. The AMPK is a key player for the maintenance of cellular energy homeostasis (Jeon 2016). The AMPK-α is not involved directly in the insulin signaling due to its catabolic role in tissues which are inverse to the anabolic role of insulin (Weikel et al. 2016). Nevertheless, it was decided to investigate this protein, because it is linked to cellular glucose uptake. It was shown that AMPK-α can modulate an alternative pathway to translocate GLUT4 into plasma membranes under exercise conditions (Murgia et al. 2009, Richter and Hargreaves 2013). Furthermore, Duehlmeier et al. (2010) reported insulin-dependent GLUT4 translocation in equines, as has been described in rodents and farm animals (Duehlmeier et al. 2005) but highlighted that insulin-stimulated GLUT4 activation in ponies is lower than that reported for pigs and cows under the same experimental conditions.

The horses in the present study were kept under similar feeding and management conditions and were not exercised during the study. Thus, exercise-induced activation of AMPK-α is unlikely. Moreover, previous studies did not report any significant effect of age and feeding state on AMPK phosphorylation (Sivinski et al. 2015) and no effect of age on AMPK phosphorylation has been detected in our study. Despite these facts, the equine MT and AT appeared to respond to insulin simultaneously with an activation of AMPK-α, which should result in an increase of glucose uptake by these tissues. The analyses of plasma glucose concentrations in corresponding blood samples during biopsy collection indicated decreased glucose concentrations. Thus, the glucose dynamics suggest that the glucose clearance observed may not be solely achieved by LT, in which it was possible to detect an increased extent of phosphorylated InsR-β, rather than AT and MT being involved in blood glucose clearance by AMPK-α-mediated glucose uptake. These data suggest that the insulin-glucose homeostasis in horses can be partially different to humans and rodents. Based on the usage of a nonspecific antibody directed against AMPK-α, it was not possible to differentiate between predominant α1 or α2 activation. However, AMPK can be activated by a lack of energy and hypoxia (Musi et al. 2001, Jeon 2016). Interestingly there is growing evidence, that ROS may regulate AMPK activity (Cardaci et al. 2012, Jeon and Hay 2015). This is of special interest, as two MOI associated with oxidative stress were found by analyses of metabolic profiles in IS and ID horses and ponies. All horses

showed similar metabolic responses during OGT, whereas analyses of basal samples revealed differences. The IS individuals had higher trans-4-hydroxyproline and methionine sulfoxide concentrations compared to ID individuals. Interestingly, both metabolites decreased during OGT in IS horses and ponies, whereas their concentrations in ID horses and ponies were not influenced. Methionine sulfoxide and trans-4-hydroxyproline are associated with the oxidant-antioxidant system and are involved either directly (methionine sulfoxide) or indirectly (trans-4-hydroxyproline) in oxidative stress (Padh 1991, Levine et al. 1996, Stadtman and Levine 2003). Previous research showed that oxidative stress can modify endocrine signaling and result in IR (Vinayagamoorthi et al. 2008). Nevertheless, studies performed in obese hyperinsulinemic horses did not find oxidative damage in skeletal MT (Banse et al. 2015). Thus, the direct link between equine ID and impaired oxidant-antioxidant systems is still missing. However, the results of the present study support the hypothesis of impaired oxidative-antioxidative balance in horses suffering from severe ID.

Despite the potential influence of ROS, AMPK is of special interest regarding IR and ID due to the fact that this protein is one of the important proteins for potential medical interventions. Metformin (dimethylbiguanide) is an oral biguanide that has been used in human medicine for the treatment of diabetes type II as an insulin-sensitizing drug (Nathan et al. 2006). It is an AMPK agonist (Zhou et al. 2001) and has been used in several studies and clinical settings for the treatment of horses with disturbed insulin regulation (Durham et al. 2008, Rendle et al. 2013). Despite low oral bioavailability in horses (Tinworth et al. 2010), the low cost compared to levothyroxine for the treatment of obese and IR horses and ponies is one explanation for its frequent usage in equine medicine (Durham 2012). Metformin is often described as an anti-hyperglycemic drug, although its mechanisms and spectrum of actions are incompletely understood. However, our findings on potential equine-specific insulin-glucose homeostasis partially mediated by AMPK may provide potential implications for metformin usage in horses and needs further investigations.

The increased extent of phosphorylation of PKB/AKT observed in all tissues investigated is in accordance with previous studies reporting increased activation of

PKB/AKT in response to increased insulin infusion rates in horses (Mccutcheon et al. 2006, Urschel et al. 2014b). Moreover, Loos et al. (2017) reported increased PKB/AKT phosphorylation in horses provoked by the feeding of a high protein diet. By transmission of the insulin signal, dephosphorylation and, thereby, inactivation of GSK-3, PKB/AKT is essential for glucose regulation by glucagon synthesis (Cross et al. 1995). The basal extent of GSK-3β did not differ between basal and stimulated conditions in the present LT samples. Phosphorylation of GSK-3α and GSK-3β did not change after two hours of HI in previous studies (Tiley et al. 2008). However, further tissues and the phosphorylation status of GSK-3β were not investigated in the present study.

In addition to the regulation of glucose homeostasis and lipid metabolism, stimulation of protein synthesis is one of the basal insulin effects (Hemmings and Restuccia 2012). All the tissues examined in the present study showed a marked expression of phosphorylated mTOR under stimulation, compared to basal conditions. These results underline the similarities of horses and other species and are in accordance with previous studies investigating mTOR in equine MT (Urschel et al. 2011, 2014b). Feeding resulted in increased mTOR signaling and the authors suggest that dietary amino acids appear to be the main mediators of this effect on muscle protein synthesis (Urschel et al. 2011). However, equine skeletal muscle showed increased activation of both upstream and downstream factors in mTOR signaling under insulin infusion, with a concomitant drop in amino acid concentrations (Urschel et al. 2014b). Decreased proteinogenic amino acids during OGT in the present study further supports this hypothesis and reflects protein anabolism. However, based on a combination of the results, it was suggested that activation of mTOR was mediated mainly by insulin.

The results presented in this PhD project indicate that it was possible to provoke changes in phosphorylation of key components involved in insulin signaling in equine tissues by an IV injection of insulin. Interestingly, it was not possible to detect differences in insulin signaling between lean and obese horses. However, equine-specific variations in the regulation of glucose homeostasis have been observed and ought to be the subject of further studies. Moreover, endocrine and metabolic changes have been detected based on blood analyses during IV and oral challenges. To test

whether these endocrine and metabolic changes differ in IS and ID equids, OGTs were performed on horses presented for EMS diagnostics to the Clinic for Horses. The targeted metabolomics approach was used in this PhD project to study the OGT-induced influences on the metabolic profile. The AbsoluteIDQ p180 Kit (Biocratis Life Science AG, Innsbruck, Austria) was originally designed to quantify a defined set of metabolites reflecting specific metabolic disorders in humans, such as obesity, IR, pro-inflammation, dysregulation of glucose and lipid metabolism, and mitochondrial dysfunction. However, due to significant similarities between the human MetS and EMS, this assay was considered to be suitable to investigate the metabolic profile during OGT and to study conspicuous features of equids affected by ID. Moreover, the assay kit has been used in several studies investigating metabolic conditions or changes in animal experiments related to IR or impaired lipid metabolism (Huber et al. 2016, Kenez et al. 2016). The kit covers a wide range of circulating acylcarnitines, amino acids, biogenic amines, glycerophospho- and sphingolipids, and hexoses and allows more in-depth analysis of metabolic pathways than analyses of selected metabolites and hormones performed previously at the beginning of this PhD project.

Interestingly, all horses responded in a similar manner during the OGT procedure and the insulin status did not affect their metabolic profile. Thus, the third hypothesis was refuted. However, it was possible to detect 22 metabolites which were significantly affected by oral glucose challenge during OGT. As mentioned previously, increased kynurenine reflects pro-inflammatory conditions during OGT. Furthermore, kynurenine is known to be associated with obesity and IR in humans (Oxenkrug 2013). Research in human medicine indicated that acute short-term oral glucose challenge can promote inflammatory profiles even in IS individuals (Sesti et al. 2014). Several studies investigated inflammatory cytokines in horses affected by EMS and suffering from obesity, HI, IR or laminitis and found increased inflammatory markers (Vick et al. 2007, Tadros et al. 2013, Wray et al. 2013, De Laat et al. 2014, Burns et al. 2015, Suagee et al. 2015). However, it was not possible to provide analyses of cytokine profiles in the study horses during OGT and CGIT or in horses and ponies with unknown insulin status during OGT. Analyses with the targeted metabolomics approach enabled the assessment of a wide range of endogenous metabolites reflecting complex

physiological processes. Consequently, results provided interesting information to support the findings observed and hypotheses stated in previous study parts and generates multiple further hypothesis for following research projects. One of these central questions is whether lower basal trans-4-hydroxproline and methionine sulfoxide concentrations are consistently indicative of ID and how oxidative stress is linked to ID or IR in equids.

7 MAJOR FINDINGS AND CONCLUSION

In this PhD project, physiological and pathophysiological variations in IS, glucose homeostasis and lipid metabolism in horses were investigated. Oral and IV challenges provoked marked endocrine and metabolic responses in both lean and obese horses. These responses were detectable based on clinical investigations by analyses of blood samples, and on molecular levels in *in vivo*-stimulated tissues collected via biopsies. However, no body condition-related differences could be observed in dynamic metabolic and endocrine responses. Furthermore, IV challenge with insulin and glucose resulted in tissue-specific variations in the acute response of insulin signaling. The results of this study revealed not only important similarities between horses and other species in certain aspects of insulin-mediated signaling, but also provided data to suggest that equine insulin and glucose homeostasis is partially different to humans and rodents. Therefore, the adoption and assumption of physiological conditions and measures for treatment derived from other species is complicated. Moreover, the cross-talk between insulin actions and specific ATs and other tissues, such as MT and LT, has not only been investigated on a molecular level, but also by a targeted metabolomics approach. Consequently, we were able to describe shifts in the metabolic profile of horses under oral glucose challenge and have identified hints of pro-inflammatory conditions. These findings require critical evaluation of diagnostic tests and refinement of diagnostic procedures. Finally, two metabolites associated with impaired oxidative-antioxidative balance have been identified in horses and ponies with severe ID, potentially providing a basis for further examinations of pathophysiological mechanisms and diagnostic interventions. The results of the present PhD project highlight the current difficulties in the accurate assessment of impaired insulin regulation in horses. Variable metabolic responses to insulin stimulation cannot be allocated to variations in body condition, either on clinically recognizable parameters or on the extent of protein phosphorylation. Testing horses and ponies for IR and ID should be differentiated and specific clinical conditions may require either one or the other. Moreover, the importance of IR in terms of ID should be investigated in further studies. Notwithstanding, the lack of knowledge about

pathophysiological variations in insulin signaling in horses and ponies with IR, assessment of postprandial HI under standardized conditions provoked by OGT allows the identification of the clinical features of ID.

This PhD project provides another piece in the puzzle to unravel pathophysiological and physiological variations in IS, glucose homeostasis and lipid metabolism in horses. Equine-specific insulin signaling, the marginal relationship of IS to obesity and strong associations of high plasma insulin concentration to cellular oxidative stress depicted not only a special physiological condition but also indicate novel pathophysiological conditions underlying EMS.

8 FUTURE PERSPECTIVES

Having shown that insulin signaling in various equine tissues can be investigated by procedures described in this PhD project, further studies can be based on this experimental setting. Hyperinsulinemic and hyperglycemic conditions provoked by IV stimulation provoked a tissue-specific variation in insulin signaling in healthy horses. Equine-specific regulation of glucose homeostasis by AMPK should be studied further to support and confirm our hypothesis.

Furthermore, studies should investigate insulin signaling in horses with altered insulin regulation under IV and oral stimulation. This may provide valuable information for the characterization of the pathomechanisms leading to IR and ID. Moreover, it is of great interest to find out whether horses suffering from ID and IR will differ in the expression and activation of key proteins of the insulin signaling cascade in IS tissue among each other or in comparison to healthy horses. This may help to unravel and differentiate IR and ID and provide information for potential drug-based approaches for the treatment of metabolic pathologies such as EMS.

This research project used a targeted metabolomics approach successfully for the investigation of metabolic profiles in horses. Multiple hypotheses can be generated from our results and have already provided bases and ideas for future research projects investigating EMS and ID in horses. Further studies are needed to investigate the impaired oxidative-antioxidative balance in ID and IR horses and the metabolites trans-4-hydroxproline and methionine sulfoxide should be tested in larger populations and various clinical settings for their relevance and potential usage in diagnostic purposes in terms of ID and IR.

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