DOCTOR OF PHILOSOPHY

The influence of glucose availability on metabolism in skeletal muscle tissue and its role in the development of type 2 diabetes mellitus

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Award date:
2011

Awarding institution:
Bangor University

THE INFLUENCE OF GLUCOSE AVAILABILITY ON METABOLISM IN SKELETAL MUSCLE TISSUE AND ITS ROLE IN THE DEVELOPMENT OF TYPE 2 DIABETES MELLITUS

by

Francesco Sartor

Thesis submitted to the
University of Wales, Bangor
in fulfilment of the requirements of the degree of
DOCTOR OF PHILOSOPHY

2011

DEDICATION

To Helma, who makes my Italo-English idiolect comprehensible to other people, who supported me practically, morally and economically throughout my PhD, and without whom this work would not make any sense whatsoever.

" fantasy is a place where it rains"
Italo Calvino (Six Memos for the Next Millennium)

-That must be Wales-

SUMMARY

Humans, like many other animals, are congenitally attracted to sweet tasting food. In nature, sweet food is rather scarce, yet it plays a valuable role in the struggle for survival. Sweet food (sugars) has several precious characteristics such as high metabolic power (ATP produced/time), metabolic versatility, and it is preferred by vital tissues, such as brain. Moreover, like fat, sweet food is a good source of energy (energy dense). For this reason, sweet food taste and intake are positively rewarded by the human brain. This positive reward response must have evolved to ensure the required energy intake via efficient food selection. However, with the introduction of mass production of refined carbohydrate (CHO) and sugar this ancestral reward system is revealing to be potentially deleterious for our health. There is a great deal of evidence showing that high refined CHO and sugar consumption is associated with obesity, insulin resistance and type 2 diabetes mellitus (T2DM).

In this thesis we elucidate some aspects of the impact of exposure to high or low refined CHO/sugar intake on human physiology. In chapter 2, we present 2 studies. The first shows that overweight/obese (Ov/Ob) individuals perceive sweet (sucrose) solutions to be less sweet than lean (L) controls. However, the liking for sweet does not seem to differ between Ov/Ob and L individuals. Importantly, we have shown for the first time that implicit attitude towards sweet food/drinks is stronger among Ov/Ob than L people. The second study proves that 4 weeks of soft drink supplementation (2.1±0.2 g CHO per kg of body weight a day) can alter taste perception of sweet in lean, lightly active subjects. Moreover, explicit preference for sweet was increased in a subgroup of participants with initial low preference for sweet.

In chapter 3, we also present 2 studies. In the first study, we have adopted an in vitro approach to investigate the role of high glucose

availability on metabolism, glucose sensing, and insulin signalling in primary human myotubes established from needle muscle biopsies. We exposed the human myotubes to high D-glucose medium (15 mmol/L) and high insulin (10 μg/ml) and this led to an increased glycolytic activity and de novo lipogenesis, whereas oxidative capacity did not change. The expression of the glucose sensing protein MondoA was augmented by high glucose availability and so was the expression of thioredoxin-interacting protein (TXNIP), a protein linked to insulin resistance. Furthermore, exposure to a hyperglycemic condition diminished serine-threonine kinase Akt phosphorylation (thr 308) in response to insulin stimulation (100 nmol/L), revealing that high glucose availability can reduce insulin signalling response. In the second study we have analysed the effects of 4 weeks soft drink supplementation in lean, lightly active participants on metabolism and insulin sensitivity at whole body as well as cellular levels (muscle biopsies). This in vivo study shows various adverse health outcomes (i.e. increases in fat mass, and fasting glucose and insulin levels, and reduced basal fat oxidation) as a consequence of the soft drink consumption. Moreover, from the skeletal muscle analysis we found a strong trend towards an increase in MondoA protein expression and a decrease in glycogen synthase kinase -3β phosphorylation 1 h after glucose load. The disturbed insulin signalling that we found in vitro, due to exposure to high glucose, was confirmed in vivo with the syrup based soft drink intervention.

In chapter 4, we have adopted a two week low CHO and low energy regime in combination with a high intensity interval training (HIIT) to reduce exogenous and endogenous glucose availability with the aim to reduce the risk of T2DM in sedentary, obese individuals. The low CHO and low energy regime improved oral glucose insulin sensitivity, fat oxidation, and lipid profile in only two weeks. The addition of an energy-balanced HIIT was not sufficient to further enhance these parameters. However, HIIT improved cardiovascular fitness and preserved lean mass and circulating resistin levels.

In chapter 5, a case study of a diabetic patient is reported. The patient underwent 4 weeks of restricted CHO diet in combination with HIIT. This life style intervention has proven to be sufficient to reverse this patient's diabetic status. A randomized trial is required to verify these outstanding results.

In conclusion, the work presented in this thesis adds a large amount of original knowledge about the effects of refined CHO and sugar availability on a variety of physiological and also some psychological parameters. A stronger implicit drive to the consumption of sweet food/drinks was found in Ov/Ob individuals. One month of soft drink consumption is enough to change taste perception of sweet and increase preference for sweet in initial sweet dislikers. High sugar (glucose) availability and consequently high energy intake have deleterious effects on glucose regulation and metabolism at a whole body and a cellular level. Energy deficit and moderate CHO restriction (low exogenous glucose) seem to be effective in improving glucose regulation thereby reducing the risk of T2DM in obese individuals. HIIT (low endogenous glucose) per se does not further reduce the risk of T2DM, but does reduce the risk of cardiovascular diseases in obese, otherwise sedentary persons.

ACKNOWLEDGMENTS AND PUBLICATIONS

First of all, I would like to acknowledge my supervisor Dr. Hans-Peter Kubis for his valuable advice and feedback throughout my PhD. Moreover, I would like to thank Dr. Jeanette Thom for her supervision and helpful deadlines.

I am the principal investigator of all the chapters presented in this thesis. I have co-designed all the studies included in this thesis together with my supervisor/s. I have collected all the data myself, with the exception of study 1 presented in **Chapter 2**, where I have supervised the MSc student in charge of the data collection. I have performed the data analysis of all the studies of this thesis. I am grateful for the precious input of all the people who have been involved in my studies. In particular I would like to acknowledge Kevin Williams and Matthew Jackson for their technical support and friendship. Thanks to Dr. David Markland, Prof. Lucy Donaldson and MD. Verena Matschke, for his supervision of the IAT, her supervision of the taste tests, and taking needle muscle biopsies, respectively. Last but not least, my parents for their moral and economic support, my bestfriend Emanuele Vailati for his unreserved moral support, and the participants.

Publications:

Chapter 2: Francesco Sartor, Lucy F Donaldson, David A Markland, Helina Loveday, Matthew J Jackson and Hans-Peter Kubis. Taste perception and implicit attitude toward sweet related to Body Mass Index and soft drink supplementation *Appetite* (2011), DOI: 10.1016/j.appet.2011.05.107

Chapter 3: Francesco Sartor, Matthew J Jackson, Cesare Squillace, Anthony I Shepherd, Donald E Ayer, and Hans-Peter Kubis. Influence of high glucose availability on MondoA and TXNIP expression in

skeletal muscle of healthy, lean individuals in vivo and in vitro (submitted).

Chapter 4: Francesco Sartor, Helma M de Morree, Verena Matschke, Samuele M Marcora, Athanasios Milousis, Jeanette Thom, and Hans-Peter Kubis. High-intensity exercise and carbohydrate-reduced energy-restricted diet in obese individuals. *European Journal of Applied Physiology*. DOI: 10.1007/s00421-010-1571-y

Published abstracts

Chapter 3: Francesco Sartor, Matthew Jackson and Hans-Peter Kubis (2010). Influence of a four week energy drink intervention on glucose homeostasis in sedentary individuals. *Acta Physiologica* 198, Supp 677:P101.

Francesco Sartor, Matthew Jackson and Hans-Peter Kubis (2010). Influence of a four week energy drink intervention on glucose regulation in healthy sedentary individuals. *Medicine and Science in Sport and Exercise*, 42:5, Supp.

Chapter 4: Francesco Sartor, Helma M de Morree, Verena Matschke, Samuele M Marcora, Jeanette Thom, and Hans-Peter Kubis (2009). Combination of a low-carbohydrate diet with high intensity interval training – influence on insulin sensitivity, blood lipids and whole body metabolism in obese sedentary individuals. *Proceedings Physiological Society* 14, C13.

Francesco Sartor, Helma M de Morree, Samuele M Marcora, Verena Matschke, Jeanette Thom, and Hans-Peter Kubis (2009). Influence of a combined low-carbohydrate high intensity interval training intervention on insulin sensitivity, blood lipids and whole body metabolism in obese sedentary individuals. *Acta Physiologica* 195, Supp 669:O73

Chapter 5: Francesco Sartor, Athanasios Milousis, Matschke Verena, Marcora Samuele, Thom Jeanette, and Kubis Hans-Peter (2008). Reverting NIDDM by combining low-carbohydrate diet with high intensity interval training: A case report. *Journal of Sports Sciences* 26:1 S63

Other abstracts

Chapter 2: Francesco Sartor, Lucy F Donaldson, David A Markland, Matthew J Jackson and Hans-Peter Kubis. Behavioral and physiological changes due to 4 weeks soft drink consumption in healthy sedentary individuals (2010). *28th Annual Meeting of the Anglo Danish Dutch Diabetes Group,* Schæffergaarden, Denmark, 18-21 May.

Funding

David Edward Memorial PhD Scholarship (£ 42 000), 2006-09.
Chapter 4 and 5: North West Wales NHS Trust Grant (£ 5 000).
Hans-Peter Kubis, Francesco Sartor, Samuele Marcora, and Jeanette Thom. Low-carbohydrate diet combined with high intensity aerobic interval training in insulin resistant, overweight people, 2008.

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ABBREVIATIONS

ACC carboxylase	Acetyl-CoA
ACE inhibitor	Angiotensin-converting enzyme inhibitor
AKT kinase Akt	Serine-threonine
AMPK	AMP-activated protein kinase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUGC curve	Area under the glucose
BMI	Body mass index
Ca^{2+}	Calcium ion
CAMK	Ca2+/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid receptor
CHO	Carbohydrate
ChoRE	Carbohydrate response element
ChREBP	Carbohydrate response element binding protein
CK	Creatine kinase
CO_2	Carbon dioxide
CREB	cAMP response element-binding
CS	Citrate synthase
C2C12	Mouse myoblast cell line, C2C12
CV variation	Coefficient of
D	Diet
DE	Diet-exercise
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DXA	Dual energy X-ray absorptiometry
ECG	Electrocardiography
ECL	Electrochemilu minescence
ELISA	Enzyme-linked immunosorbent assay
FAS	Fatty acid synthase
FCS	Foetal calf serum
FUM	Fumarate hydratase
GABA acid	Gamma-aminobutyric
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GDP	Gross domestic product
GFAT	Glutamine fructose-6-phosphate amidotransferase
GIRE	Glucose insulin response element
gLMS	General labeled magnitude scale
GLUT	Glucose transporter
GS	Glycogen synthase
GSK-3 synthase kinase -3	Glycogen
G6P	Glucose-6-phosphate
HA1-ER	Tumorigenic human kidney-derived cell line

HDL	High-density lipoprotein
HFCS syrup	High fructose corn
HG	High-glucose
HIIT	High-intensity interval training
HK	Hexokinase
HOMA	Homeostasis model assessment
H_2O	Water
IAT test	Implicit association
Ig	Immunoglobulin
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
IL-10	Interleukin-10
INS line	Rat insulinoma cell
IP_3	Inositol trisphosphate
IRS substrate	Insulin receptor
ISI index	Insulin sensitivity
K^+	Potassium ion
kDa	Kilodalton
K562	Human erythromyeloblastoid leukemia cell line, K562
L	Lean
LDL lipoprotein	Low-density
L6	Rat myoblast cell line, L6
LXR	Liver X receptor
MAPK	Mitogen activated protein kinase
MHC	Myosin heavy chain
Mlx	Max-like protein x
mM	milliMolar
MUFA	monounsaturated fat
n	Number of participants
Na^+	Sodium ion
NAC	N-acetylcysteine
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NFATc1	Nuclear factor of activated T-cells, cytoplasmic 1
NG	Normal-glucose
NHS	National health service
OGIS	Oral glucose insulin sensitivity
OGTT test	Oral glucose tolerance
Ov/Ob	Overweight/Obese
P	Probability value
PBS	Phosphate buffered saline
PDK-1	Phosphoinositide-dependent protein kinase-1
PFK2-FBPase2	Phosphofructokinase-2-fructose bisphosphatase-2
PGC1α	Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PK	Pyruvate kinase
PKC	Protein kinase C

PP1	Protein phosphatase-1
PP2	Protein phosphatase-2
PUFA	Polyunsaturated fat
RER ratio	Respiratory exchange
RNA	Ribonucleic acid
(mRNA)	Messenger RNA
(rRNA)	Ribosomal RNA
(tRNA)	Transfer RNA
RNase	Ribonuclease
ROS species	Reactive oxygen
RQ	Respiratory quotient
RT-PCR	Reverse transcription polymerase chain reaction
RT-step	Reverse transcription step
RT	Room temperature
SAT	Saturated fat
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SEM	Standard error of the mean
SGLT	Sodium-glucose transporter
SMGM	Skeletal muscle growth medium
SREBP-1c	Sterol regulatory element binding protein-1c
SYBR-Green	Synergy Brands-Green
TCA	Tricarboxylic acid
TNF-α	Tumor necrosis factor-alpha
T2DM	Type 2 diabetes mellitus
TXNIP	Thioredoxin-interacting protein
VL	Vastus lateralis muscle
VCO_2	Carbon dioxide production
VO_2	Oxygen uptake
VO_{2max}	Maximal oxygen uptake
VO_{2peak}	Peak oxygen uptake
WHO	World health organization

CHAPTER I

GENERAL INTRODUCTION

Humans display an innate attraction to sweet tasting substances. This passion and the practically unlimited availability of sweet tasting products in the current industrialised epoch expose humans to health threatening problems. At first we will give a brief account of the historical relationship between human passion for sweet and the consequent sugar production and consumption. The aim of this succinct first paragraph is mainly to underline the duration and scale of this "human-sweet" relationship, and therefore to substantiate the exigency of advancing our understanding of the causes and the effects of high refined-carbohydrate/sugar availability on human metabolism and health. Our investigation into the causes of sweet food/drinks consumption will bring us to study human taste perception, food preference/selection and their association with the reward system in the brain. Although we will not investigate human reward directly, its introduction is necessary to understand behaviours that may lead to overnutrition, weight gain and obesity. We will also focus our attention on automatic associations, which are likely to explain subconscious behaviours related to sweet food preference. We will then describe the metabolism of refined-carbohydrate/sugar (i.e. glucose) in normal conditions, introducing the complex pathways regulating glucose uptake and disposal. Since the skeletal muscle tissue is of major importance for glucose disposal, our analysis will focus mostly on this tissue. In particular, we will elucidate the role of newly discovered glucose sensing proteins in the skeletal muscle, which will be of central importance in this thesis. Whereupon, we will draw our attention to the effects of high refined-carbohydrate/sugar consumption and its negative consequences on health (e.g. insulin resistance). We will enumerate the literature published during the last decade, which shows a clear association between refined-carbohydrate/sugar consumption and obesity and insulin resistance. This available literature gives credit to the necessity to study the influence of carbohydrate availability on metabolism in skeletal muscle tissue and its role in the development of type 2 diabetes mellitus. Finally, we will present the possible benefits of reducing carbohydrate/sugar

availability on people's health. Low carbohydrate/sugar availability will be achieved by combining a reduced-carbohydrate diet regime (exogenous glucose) with high intensity exercise (endogenous glucose).

A brief historical introduction on sugar production and consumption in the westernised countries

Undoubtedly human beings have a strong attraction towards sweet tasting food and drinks. We can trace this attraction to sweet back to a Palaeolithic painting (about 15,000 years old), found in a cave near Valencia in Spain, in which a man is pictured robbing honey from a beehive (Edson, 1958; Pfaffmann, 1978) (**Fig. 1.1**). Honey is the oldest known sweetener in history. It is easily recognisable by its sweet taste and syrupy texture. Honey is chemically composed mainly of fructose (38.2%), glucose (31.3%), and other di- or trisaccharides (10.2%) (Bogdanov *et al.*, 2008). Honey was the most important sweetener used in Western Europe until the 16th century (Ballinger, 1978).

Although Asian Indians already produced cane sugar, or better molasses, from sugar cane plants (not later than 400 B.C.), the migration of sugarcane growing and sugar (molasses) extraction took 2,000 years to be brought from India to the Atlantic coasts of Africa (Ballinger, 1978). Sugarcane was introduced in Madeira, an island 520 km west of the Moroccan Atlantic coast, immediately after its Portuguese colonisation in 1420. The first recorded cane sugar shipment to England was in 1456 (Ballinger, 1978). Sucrose and cellulose are the two main chemical constituents of sugarcane (Chen & Chou, 1993). Sucrose, a white small crystal, is a disaccharide composed of glucose and fructose.

Until 1500, sugar in Western Europe was generally regarded as a medicine (*Saccharum officinarum*) or as an article of luxury (Ballinger, 1978). The Portuguese first (~1526), and later the British, Dutch and

Danish began to produce cane sugar systematically in their various colonies, consequently they began to provide Europe with sugar on the regular basis. In fact, "sugar was the first agricultural product to be shipped from America to Europe in commercial quantities" (Ballinger, 1978).

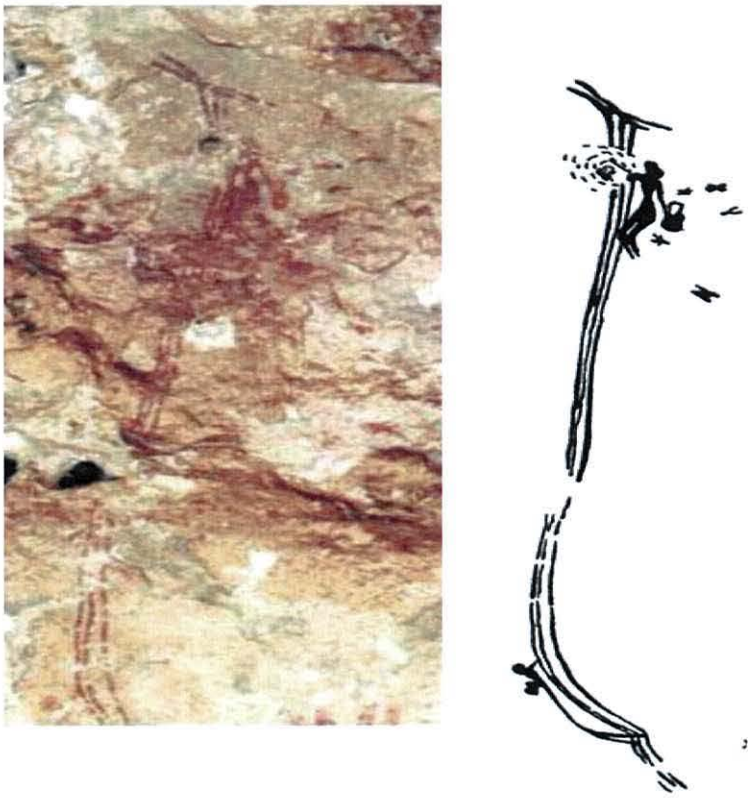


Figure 1.1 Palaeolithic cave painting from "las cuevas de la araña" (the spider cave) in Bicorp, Valencia, Spain.

It was mainly during the Napoleonic Wars that, because of the British continental blockade, alternatives to cane sugar were developed. In 1747 the German chemist Andreas Marggraf extracted sugar (sucrose) from beet. But it was not until 1799 that the first factory for the production of sugar from beets was established, in France (Steward & Bernstein, 1942). Soon, beet sugar industries appeared in Germany, Italy, The Netherlands, and Russia. In the United States the beet sugar production started only in the 1870s. In 1899, about 82,000 tons of beet sugar were produced in the United States, this represented just 3.4% of U.S. sugar consumption that year (Ballinger, 1978).

In 1811, a Russian scientist, Gottlieb Sigismund Kirchhof, reported his findings that when dry starch is heated together with some water and acids it acquires some of the properties of vegetable gums (sweet tasting syrup). In 1855, 11 million pounds of dextrose (D-glucose) were produced from starch in France and in 1874 about 44 million pounds were produced in Germany (Ballinger, 1978). Corn syrup became a significant industry in the late 1920s. In 1927, the total consumption of syrup and dextrose made up about 11% of U.S. sugar consumption (Ballinger, 1978). However, corn syrup use increased mainly during World War II, because of the shortage of cane sugar and beet sugar. During the fifties and sixties, corn syrup prices became constantly lower than sucrose prices, and this led to an increasing usage of corn syrup in food industries (**Fig. 1.2**).

Nowadays, corn syrup is leading the market of added sugars in food and beverages, as shown in **Figure 1.3**. There are three types of corn syrups: high fructose corn syrup 42 (HFCS 42; 42% fructose and 52% glucose), high fructose corn syrup 55 (HFCS 55; 55% fructose, 42% glucose, and 3% polysaccharide), and glucose syrup, which is 100% glucose. The majority of U.K. starch, which is derived from North American maize, is used for glucose syrup production (Bowler *et al.*, 1985). Conversely, in the United States the vast majority of starch is used to produce HFCS 42 or HFCS 55 (ERS, 2004).

It should not be neglected that sugar is not the only source of CHO. Complex CHO, such as starch, is of primary importance in the human diet. With the advent of industrial revolution in the 17th century the milling of cereals changed, removing almost all the indigestible materials and increasing yield and palatability (Miller & Colagiuri, 1994). Thus, with the industrialisation, per capita sugar and refined starch availability increased drastically. For these reasons it is sensible to look for associations between dietary refined starch/sugar

availability and the increasing incidence of obesity, insulin resistance and type 2 diabetes mellitus.

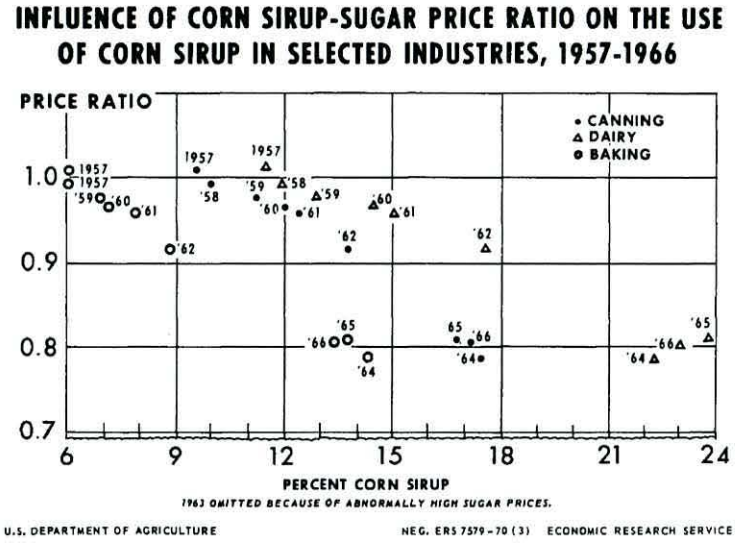


Figure 1.2 Decrease in corn syrup price, increase in corn syrup consumption, 1957-1966 (from USDA).

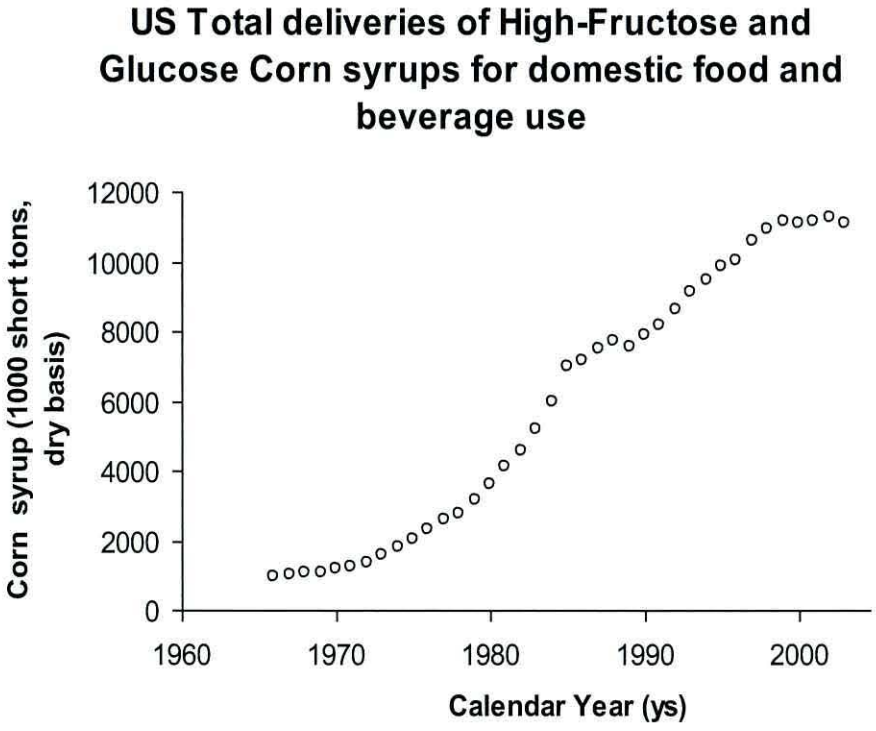


Figure 1.3 *Sugar and Sweetener Situation and Outlook Yearbook*, 2004 Economic Research Service USDA.

Mechanisms regulating sugar intake

Taste

The sense of taste originates on the lingual surface, but also on the palate, epiglottis, larynx, pharynx and oesophagus (Pfaffmann, 1978). The majority of the molecules that can be tasted are detected by taste cells clustered in taste buds (Buck, 2000). "Taste buds are comprised of modified epithelial elements clustered together in a barrel-shaped aggregate opening to the oral surface via a small pore at the top" (Pfaffmann, 1978). Frank and Pfaffmann (1969) observed that taste cells have a degree of chemical specificity. However, taste buds responsive to sweet, salty, bitter, sour, and savoury are found in all regions of the tongue (Buck, 2000). Two different mechanisms are believed to transduce sweet taste. In the first, sweet tastants bind to G protein, which increases cAMP, thereby activating cAMP-dependent kinase that in turn inactivates K^+ channels via phosphorylation, depolarising the taste cell. In the second, G protein seems to increase IP_3 concentration which induces Ca^{2+} release (Buck, 2000). The taste information collected in the tongue is transported to the brain via the chorda tympani and the glossopharyngeal nerves. These afferents have a first synapse in the gustatory area of the nucleus of the solitary tract, located in the brain stem, and a second synapse in the ventral posterior medial nucleus of the thalamus; finally the signals reach the gustatory cortex (anterior insula-frontal operculum) placed in the subcentral area (Brodmann area 43) (Buck, 2000).

The gustatory system has three dimensions: quality (sweet, salty, bitter, sour and savoury), intensity, and affect (pleasant/unpleasant) (Small *et al.*, 2003). These three dimensions are not orthogonal, but they influence each other (Pfaffmann, 1980). The pleasantness of a substance varies by changing its subjective intensity following an inverted U-shaped curve (Wundt curve) (Pfaffmann, 1980; Veldhuizen *et al.*, 2006). Taste intensity activates mainly the amygdala, whereas affective valence (pleasantness/unpleasantness) has been located in the

anterior cingulated cortex and caudolateral orbitofrontal cortex (Small *et al.*, 2003). With appropriate training, humans can rate taste intensity irrespective of positive or negative valence (e.g. sweet/pleasant; bitter/unpleasant) (Small *et al.*, 2001). However, it is extremely hard to isolate pleasantness from intensity (Veldhuizen *et al.*, 2006), because both clearly contribute to palatability (hedonic reward) and therefore to food selection (explicit preference).

Reward

Food seeking and food consumption are the major activities of most animals (Rozin, 1976). Consuming sufficient food allows animals to maintain adequate energy stores, which are essential for their survival (Saper *et al.*, 2002). Energy is required to ensure physiological functions, maintain a constant body temperature, and produce work (e.g. movement). Mammalians have evolved a potent reward system, which drives feeding behaviour (Saper *et al.*, 2002). As L. Tiger (1992) puts it, "making essential behaviours pleasurable increases the likelihood the species will survive". Undoubtedly, sweet food is highly palatable (Dum *et al.*, 1983; Bergmann *et al.*, 1985), and this property makes it a potent reinforcer (Silveira *et al.*, 2010). The fact that humans, and other animals such as rats, find sweet food palatable could be attributable to efficient food selection. In fact, sweet food has several precious properties. Sugars have high metabolic power and capacity (ATP produced/time) (Margaria, 1967; Heck *et al.*, 2003), and metabolic versatility (**Fig. 1.4**). Moreover sugars are preferred by vital organs, such as the brain (Maher *et al.*, 1994). Citing N.R. Carlson: "because the brain controls eating, it seems reasonable that hunger might be triggered by a decrease in the brain's primary fuel" (1991). Furthermore, along with fat, sugar is a good source of energy (high energy density) (Bowman *et al.*, 2004). All these properties of sugars explain why animals' brains potently reward sweet food tasting and ingestion (Sclafani, 2004). Indeed, in rats the reward produced by

sweet taste overtakes the reward produced by cocaine (Lenoir *et al.*, 2007).

Reward is a complex response, and indeed it is composed of three components: a hedonic or "liking" component, a motivational or "wanting" component, and a learning component (Berridge & Robinson, 2003). K.C. Berridge (1996) states that wanting corresponds to the concept of appetite or craving and liking corresponds to the concept of palatability. The lateral hypothalamic area plays a key role in appetite regulation, particularly via melanin concentrating hormone and orexin. Both melanin concentrating hormone and orexin have three targets: the brain stem motor system involved in chewing, licking, and swallowing; the autonomic or visceral nervous system involved in salivation and esophageal, and gastric motility etc.; and the ascending arousal system. Orexin seems to be regulated by glycaemia (blood glucose levels); indeed, hypoglycaemia is known to increase orexin gene expression. However, only melanin concentrating hormone seems to have projections to an important area of the brain involved with reward, the nucleus accumbens (Saper *et al.*, 2002).

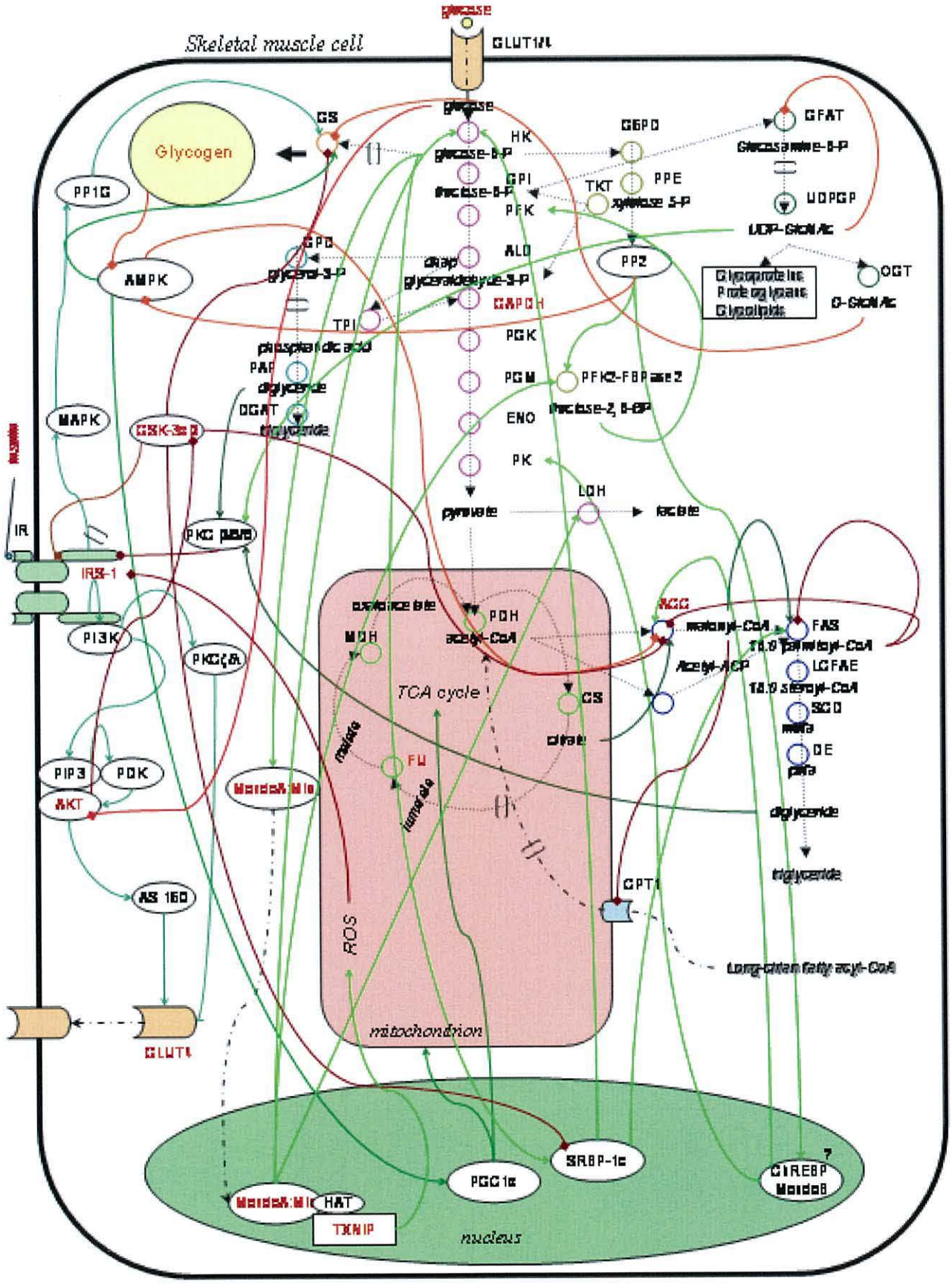


Figure 1.4 Principal cellular pathways associated with glucose desposal and metabolism in the skeletal muscle cell.

The nucleus accumbens has projections to the motor system which may influence feeding behaviour (Saper *et al.*, 2002). It sends signals via spiny neuron projections that release γ-aminobutyric acid (GABA) and opioid neurotransmitters to the ventral pallidum which is thought to be linked specifically to the hedonic or "liking" reward (Smith & Berridge, 2005). Opioid neurotransmitters seem to be closely associated with hedonic reward. In fact, enhancement of taste hedonic impact is especially mediated by opioid circuits localised in the posterior ventral pallidum (Smith & Berridge, 2005). The injection of opioid agonists in the nucleus accumbens shell increases intake of palatable sweet food (Zhang & Kelley, 2002), while opioid antagonists have the opposite effect on palatable food intake (Kelley *et al.*, 1996). When rats are maintained on a diet of intermittent sugar access they show changes in the opioid receptors, and this pattern has also been shown to generate addiction (Avena *et al.*, 2008).

GABA and dopamine are hypothesised to be more related to "wanting" reward (Berridge & Robinson, 1998). A range of foods can release dopamine in the nucleus accumbens, including sugar (Bassareo & Di Chiara, 1997; Hajnal *et al.*, 2004; Liang *et al.*, 2006). Moreover, repeated sugar consumption increases dopamine turnover in the nucleus accumbens (Hajnal & Norgren, 2002). Dopamine function seems to be related more to novelty, attention, or anticipation rather than to the immediate hedonic reward (Saper *et al.*, 2002).

Preference for sweet

People may have two different attitudes towards an object (e.g., sweet item) at the same time, explicit and implicit (Greenwald & Banaji, 1995; Wilson *et al.*, 2000).

- *Explicit preference for sweet*

Explicit preference requires thinking consciously about two or more items under analysis (introspection) and selecting one item rather than another. Taste is the most important factor in the process of food selection (Glanz *et al.*, 1998). Liking (hedonic reward response) is the basis for selection, even though there are also other motives (Birch, 1999). "As a general rule, molecules that serve as energy, such as sugars, are perceived as sweet by humans and tend to be accepted by both humans and animals" (Pfaffmann, 1975). Food preference has genetic as well as environmental factors. It is often the case that the acceptance and rejection in response to certain flavours are congenital and are evident even before experiencing the consequences of their ingestion (Galef, 1996). In fact, newborn babies show strong appetitive responses to sweet tasting substances and equally strong negative responses to both sour- and bitter-tasting substances (Rosenstein & Oster, 1988). This innate attraction to the sweet taste of sugar is true also for many other species (Vigorito & Sclafani, 1988; Ackerman *et al.*, 1992). Genetic predispositions include predisposition to reject novel tastes (neophobia) and to prefer familiar tastes (Birch, 1999). However, the environment also plays an important role in food preference. For instance, humans can learn to prefer food because of its context and consequences (Birch, 1999). If a particular food produces nausea and vomiting it will be identified as bad, and therefore it will not be selected again.

- *Implicit preference for sweet*

Implicit or automatic preference does not require a conscious thought (no introspection); individuals are usually not aware of their implicit preference about something (Friese *et al.*, 2006). The implicit preference shows the automatic or unconscious liking of one item over another. A measure of implicit preference, the Implicit Association Test (IAT), was introduced by Greenwald, McGhee and Schwartz (1998). Implicit attitudes are unconsciously held positive or negative evaluations of an object and reflect automatic associations in memory

between concepts (Greenwald *et al.*, 2002). The assumption behind the IAT is straightforward, if two concepts are highly associated (e.g. healthy food-positive), then they are easier to associate via IAT's sorting tasks than if the two concepts are poorly associated (e.g. unhealthy food-positive) (Greenwald & Nosek, 2001).

Implicit preference has been assessed by IAT to study food choices (Maison *et al.*, 2001; Roefs & Jansen, 2002). Several investigations have studied the implicit preference towards sweet food/drinks and fruit. The studies have found that students of both genders had a more positive association with fruits than with snack bars (Karpinski & Hilton, 2001; Perugini, 2005; Richetin *et al.*, 2007). Maison *et al.* (2001) showed a similar implicit preference for juices over sodas. Additionally, when people's ability to control their behaviour is reduced, sweets consumption is primarily predicted by automatic sweets attitudes. In this condition of "mental weakness", high levels of dietary restraint standards corresponded to high sweets consumption (Hofmann *et al.*, 2007). Recently, implicit preference was analysed for high-sweet-fat food versus high-salted-fat food in women suffering from premenstrual dysphoric disorder (PMDD), who are known to have increased carbohydrate (CHO) intake in the luteal phase (Yen *et al.*, 2010), revealing that these women have a higher implicit preference for high-sweet-fat food than controls (Yen *et al.*, 2010). However, to date nothing is known about the implicit preference for sweet food in normal weight versus obese individuals.

Changes in the mechanisms regulating sugar intake

Since our taste determines our food choices, changes in taste could resolve in food choice changes. Traditionally it was thought that taste was predetermined and stable over time (Bartoshuk *et al.*, 1994). However, this view has been disproved by investigations that reported experience-induced changes in glucose sensitivity (Eylam & Kennedy, 1998) and monosodium glutamate sensitivity (Kobayashi & Kennedy,

2002). The fact that taste is a plastic system is of paramount importance when investigating its relationship with food choice.

Changes in taste could determine alterations in hedonic and motivational reward, leading to a change in food preference. It is well known that an artificial alteration of reward alters food intake. For example, delta-9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive substance found in cannabis, increases the intake of sweet food (Koch & Matthews, 2001). Δ^9-THC seems to enhance sugar palatability, as observed by an increased orofacial "liking" elicited by taste of sucrose (Jarrett *et al.*, 2005). Additionally, microinjections of anandamide, an endogenous cannabinoid neurotransmitter, into the nucleus accumbens shell amplify the hedonic impact of sweetness, especially in the dorsal hotspot of the medial shell of the nucleus accumbens (Mahler *et al.*, 2007). Importantly, the cannabinoid CB_1 receptor system in the brain (hypothalamus and nucleus accumbens) as well as in the peripheral organs (pancreas, adipose tissue, skeletal muscle, and liver) plays a role in the regulation of food intake. Centrally this happens by hedonic and homeostatic energy regulation, and peripherally it reduces energy expenditure and increases lipogenesis (Matias *et al.*, 2008). This experimental evidence suggests that changes in reward can change food intake (i.e. food selection/preference).

There is some evidence that changes in taste lead to food preference changes. Patients with otitis media have a damaged trigeminal system which induces taste alterations; they have a significantly higher intake of sweets and are more likely to be overweight (Bartoshuk *et al.*, 2006). Moreover, on the anterior tongue loss of taste shows damage to the chorda tympani nerve affecting preference for and intake of sweets (Bartoshuk *et al.*, 2006). Taste also seems to be affected by environmental factors like intake of certain micronutrients. For example, taste thresholds for salt are lower in patients with adrenal cortical insufficiency, who experience urinary sodium loss (Henkin *et*

al., 1963); and higher salt consumption leads to lower taste intensity scores (Bertino *et al.*, 1982). There is the possibility of a parallelism between salt and sugar. Indeed, individuals who have a lower sensitivity for sweetness have a higher intake of added sugar (Duffy *et al.*, 2003). There is also some evidence that exposure to sweet food/drinks can alter preference for sweet. For example, a short term exposure study showed that children's preference for the same sweet orangeade was augmented after 8 days of sweet orangeade exposure (Liem & de Graaf, 2004). This change in preference was probably determined by changes in taste. Acceptance of sweetness is innate in human beings because of evolutionary reasons (need of calories). Nowadays, the superabundance of sweet food and drinks encourages individuals to chronically consume sweet food and drinks (overnutrition). The constant exposure to sweetness might therefore amplify its acceptance (i.e. increased preference), which eventually becomes morbid acceptance, or better, craving. However, there is little literature on the effects of chronic exposure to refined CHO, such as in soft drinks, on taste perception and food acceptance.

Obesity, taste, reward, and preference

Obesity is a pathological condition in which excess fat has accumulated in the body (Martin, 2010). Obesity is present when a person has a body mass index (BMI) of 30 or over (NIH, 2000; Martin, 2010). Although the aetiology of obesity is multifactorial, the excessive fat deposition is often caused by over-nutrition (i.e., energy intake greater than energy expenditure) (Bray & Gray, 1988). Obesity is after smoking, the most preventable cause of death in the USA (McGinnis & Foege, 1998; Allison *et al.*, 1999). The incidence of obesity has constantly increased over the past 4 decades (Flegal *et al.*, 2010). In 2000, direct and indirect costs related to obesity amounted to $117 billion, 1.2% of the American gross domestic product (GDP) (USDHHS, 2001; Yach *et al.*, 2006).

Over the past few years, many scientists have tried to identify taste perception differences in the obese population, reaching discordant results (Donaldson *et al.*, 2009). These contrasting results may be due to the different methodologies used (Bartoshuk *et al.*, 2006). This does not allow us to understand if and how obese individuals perceive taste for sweet differently from the lean. On the one hand, the early studies carried out by J. Grinker show that obese individuals are not more sensitive to sweet taste and they do not have a lower detection threshold for sweetness (Grinker & Hirsch, 1972; Grinker, 1978). Moreover, Grinker shows that obese people perceive changes in sweet intensity in the same way as lean individuals (Grinker & Hirsch, 1972). On the other hand, Bartosuk *et al.* (2006) show that obese persons perceive sweet as being less intense than normal weight controls. Furthermore, perceived sweet pleasantness seems to be either the same or lower in obese people compared with normal weight individuals (Frijters & Rasmussen-Conrad, 1982; Felsted *et al.*, 2007).

It appears that sensitivity to reward and overeating are strongly associated (Davis *et al.*, 2004). Obese individuals have an enhanced sensitivity in the brain areas associated with the sensory processing of food (e.g. sensory areas related to lips, tongue and mouth) (Wang *et al.*, 2002). Moreover, in women food craving and BMI seem to be associated with reward sensitivity (Franken & Muris, 2005). Conversely, some authors report obesity to be associated with hypo-dopaminergic functioning (Wang *et al.*, 2004). For instance, as obese women have lower alcohol and marijuana use than age-matched lean counterparts (Kleiner *et al.*, 2004; Warren *et al.*, 2005), overeating may compete with those pharmacological agents (Davis *et al.*, 2007). However, it seems that this phenomenon is present mainly in heavily obese individuals (BMI>40) (Davis *et al.*, 2007). Interestingly, obese persons with binge eating disorder may be influenced by a hyper-reactivity to the hedonic properties of food (Davis *et al.*, 2009a).

The preference for sweet in the obese population is also an unclear matter. For Grinker (1978) taste preference for sweet is equivalent or lower in obese than in normal weight individuals, but Bartoshuk *et al.* (2006) report that obese people have an increased liking for sweet. It also seems that obese people have a different implicit food preference from their non-obese counterparts (Drewnowski *et al.*, 1992; Capaldi, 1996). To date it is not clear whether obese individuals have a stronger explicit and implicit attitude towards sweet food and drinks than normal weight controls.

Leptin and Resistin

Leptin

Leptin is a hormone produced mainly by the adipose tissue (Stanley *et al.*, 2005). Circulating leptin levels are linked to energy stores and acute energy balance (Stanley *et al.*, 2005). Indeed, plasma leptin levels correlate well with fat mass (Maffei *et al.*, 1995). Leptin is known to reduce appetite (i.e. exogenous leptin administration) (Ahima *et al.*, 1996) and food consumption (Halaas *et al.*, 1995). It crosses the blood brain barrier by a saturable system independent of insulin (Banks *et al.*, 1996). In the brain, leptin receptors are mainly located in the hypothalamus, the arcuate nucleus, the ventromedial and dorsomedial hypothalamus, the lateral hypothalamic area and the medial preoptic area (Fei *et al.*, 1997; Elmquist *et al.*, 1998; Hakansson *et al.*, 1998). Leptin inhibits the activity of orexigenic neurons (Stanley *et al.*, 2005). Mutations of the gene *ob*, which expresses leptin, are very rare but lead to hyperphagia (abnormally high appetite and overeating) and obesity (Pelleymounter *et al.*, 1995). The majority of the obese population has a raised plasma leptin (Maffei *et al.*, 1995) and these chronic high leptin levels seem to induce central resistance to leptin (Halaas *et al.*, 1997). Therefore, leptin resistance appears to be a consequence of obesity, and it might also contribute to the aetiology of obesity (Stanley *et al.*, 2005). Interestingly, leptin seems to affect taste

perception. A study in mice has shown that leptin selectively inhibits sweet taste response through activation of outward K^+ currents and that in diabetic *db/db* mice taste cells are not affected by leptin levels (Kawai *et al.*, 2000). However, not much is known about the relationship between leptin levels and sugar preference and consumption in humans.

Resistin

Like leptin, resistin is also a hormone produced by adipose tissue. Its name comes from the fact that resistin was initially thought to induce insulin resistance (Steppan *et al.*, 2001). Animal studies showed that knockout mice have an increase in glucose tolerance with high fat-diet (Sul, 2004). Recently, the role of resistin in insulin resistance has become uncertain (Filkova *et al.*, 2009). It seems that resistin may be involved in inflammation, endocrine and tumor diseases (Filkova *et al.*, 2009). It has been observed in pregnant women that resistin levels correlates positively with high fat intake (Jansson *et al.*, 2008). However this needs confirmation.

Cytokines and macronutrients

Several cytokines, such as TNFα, IL-10, and IL-6 are expressed in adipose tissue (Yudkin *et al.*, 2000; Juge-Aubry *et al.*, 2005). In particular circulating IL-6 has been found to increase with adiposity in healthy men and women (Mohamed-Ali *et al.*, 1997). Augmented or reduced adiposity as a consequence of an increased or reduced energy intake from CHO, might determine increases or decreases in cytokines, which could cause a change in macronutrient intake. Studies on cytokine-induced sickness behaviour conducted in animals have shown that increased levels of cytokines decrease food intake and alter diet composition (Dantzer, 2001). In particular, IL-1β infusion, at the same time as decreasing food intake, increased CHO consumption and reduced protein intake, while fat ingestion remained unaltered

(Dantzer, 2001). Further evidence is needed in humans to confirm the link between cytokines and macronutrient intake.

Endocrine regulation of glucose

For a number of reasons which we have just introduced, absorbable CHO is a very important and common macronutrient in most habitual diets of human beings. This and the following paragraphs will serve to introduce the CHO digestion, absorption, disposal and regulation. The majority of the CHO ingested (dietary fibres excluded) is converted into glucose (80%). Blood glucose levels (glycaemia) are tightly regulated (ranging from 4 to 7 mM) by a hormonal network. Insulin and glucagon are the main actors in this regulatory system. Insulin is synthesised in and secreted by the β-cells of the pancreatic islets of Langerhans. Insulin secretion is stimulated by high intracellular glucose availability in the β-cells. In physiological conditions this transient intracellular high glucose availability is determined by postprandial hyperglycaemia. Postprandial hyperglycaemia is caused by absorbable CHO ingestion, digestion, and absorption. In the small intestine, glucose is absorbed by active transport; coupling to the symport of Na^+ (SGLT1), and by facilitated diffusion via GLUT2 (Kellett & Brot-Laroche, 2005). Once the absorbed glucose reaches the β-cells, raising their intracellular glucose availability, insulin secretion is induced. Increased glycaemia leads to an increase in blood insulin levels (hyperinsulinaemia). Higher circulating insulin levels allow the rapid removal of glucose from the blood stream by stimulating glucose uptake into peripheral tissues (i.e. skeletal muscle, adipose tissue and liver). The postprandial hyperglycaemia is temporary and thanks to the insulin response it is normalised within two to three hours in healthy people (Woerle *et al.*, 2003). Insulin also enhances energy storage by increasing glycogen synthesis and fatty acid synthesis. Conversely, glucagon is produced and secreted by the α-cells of the islets of Langerhans when blood glucose levels are low (hypoglycaemia).

Glucagon acts mainly on the liver where it stimulates the hepatocytes to release glucose via glycogenolysis and gluconeogenesis.

Uptake and metabolism of glucose in skeletal muscle cells

Insulin signalling

Skeletal muscle is the principal tissue involved in glucose disposal (Katz *et al.*, 1983).
At resting (overnight fasting) glycaemic levels (5 mM), glucose constantly enters the skeletal muscle cells via facilitated diffusion (GLUT1) (Mueckler, 1990). However, during postprandial hyperglycaemia muscle glucose uptake increases drastically via insulin-dependent active transport (GLUT4) (Klip *et al.*, 1990). Circulating insulin binds to specific recognition sites on skeletal muscle cells (Burant *et al.*, 1986). These specific recognition sites for insulin were localised for the first time in rat liver in 1971 (Freychet *et al.*, 1971). Insulin receptors have two extracellular α-subunits and two transmembrane β-subunits connected by disulfate bonds (Burant *et al.*, 1986).

Insulin binds to the α-subunit inducing tyrosine autophosphorylation of the β-subunit (Hubbard *et al.*, 1994). This phosphorylation induces a subsequent phosphorylation of the insulin-receptor substrate (IRS) proteins (Gual *et al.*, 2005). There are four isoforms of IRSs (IRS-1, IRS2, IRS-3, and IRS-4) (White, 1998). It has been shown that IRS-1 is involved in cell growth (Tamemoto *et al.*, 1994) as well as glucose uptake (Araki *et al.*, 1994). IRS-1 phosphorylation can have positive or negative regulatory functions (Gual *et al.*, 2005). For instance, phosphorylation of IRS-1 on the tyrosine residues stimulates insulin responses, but on serine residues it can either enhance or terminate the insulin effects (Gual *et al.*, 2005). The phosphorylation of tyrosine 612 and tyrosine 632 located on IRS-1 activates phosphatidylinositol-3-kinase (PI3K) (Gual *et al.*, 2005). PI3K consists of two subunits; the

regulatory subunits p85 interacts with the IRS-1 docking site, and the catalytic subunit p110 phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2), thereby generating phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Saltiel & Pessin, 2002).

A PH domain within serine-threonine protein kinase Akt, also referred to as PKB or Rac, is a target of PIP3 (Corvera & Czech, 1998). The binding of PIP3 to Akt generates a conformational change required for its phosphorylation by 3-phosphoinositide-dependent protein kinase (PDK-1) (Corvera & Czech, 1998). PDK-1is in turn activated by PIP3. Activated Akt phosphorylates its 160 kDa substrate (AS160), which induces GLUT4 translocation from intracellular vesicles to the plasma membrane (Sano *et al.*, 2003). GLUT4 translocation to the plasma membrane increases glucose uptake. PDK-1 activates atypical protein kinase C λ and ζ (PKC λ / ζ), which also promotes GLUT4 translocation (Bandyopadhyay *et al.*, 2002; Farese *et al.*, 2007). At the same time, Akt inhibits glycogen synthase kinase-3 (GSK-3) by phosphorylation (Cross *et al.*, 1995). The inhibition of GSK-3 releases glycogen synthase (GS) from inhibition. In this way, a large part of the augmented influx of glucose, due to increased GLUT4 presence on the plasma membrane, is deviated towards glycogen synthesis. Moreover, glycogen synthesis is promoted not only by insulin via GSK-3 inhibition. Insulin-dependent mitogen activated protein kinase (MAPK/90 kDa) activation, and consequently protein phosphatase-1 (PP-1) phosphorylation, also directly activates GS enzymatic function (Srivastava & Pandey, 1998). The salient points of the insulin signalling pathways are represented schematically in **Figure 1.4**.

Glucose sensing

Ingested refined CHO and sugar are digested to glucose and absorbed by the small intestine, inducing a rise in glycaemia. The skeletal muscle tissue is responsible for the major part of the glucose disposal by insulin-dependent glucose uptake (DeFronzo, 1997). The increased

influx of glucose in the skeletal muscle cells induces an increase in glycolysis, glycogen synthesis, pentose shunt, hexosamine pathway, tricarboxylic acid (TCA) cycle (also known as citric acid cycle or Krebs cycle), and *de novo* lipogenesis (**Fig. 1.4**).

Emerging evidence shows that glucose and glucose metabolites are sensed within the muscle cells by specific molecules promoting regulatory gene expression (Desvergne *et al.*, 2006). Once inside the muscle cell, glucose is promptly converted into glucose-6-Phosphate (G6P) by the enzymatic action of hexokinase (HK) (**Fig. 1.4**). In the hepatic and adipose tissues, G6P seems to be a key signal metabolite for inducing up regulation of fatty acid synthase (FAS), L-pyruvate kinase (L-PK), and S14 (Ferre, 1999; Desvergne *et al.*, 2006). Diaz Guerra *et al.* (1993) described a regulatory region of the *L-PK* gene, which interacts with glucose. This region has similarities with the regions found on *FAS* and *S14* genes (Desvergne *et al.*, 2006), these are called glucose/insulin response elements (GIREs) or carbohydrate response elements (ChoREs). Yamashita *et al.* (2001) purified a protein binding to the ChoRE of the *L-PK* gene (ChREBP, also known as MondoB) of liver nuclear extracts prepared from rats, but they did not detect CHREBP/MondoB in skeletal muscle. ChREBP/MondoB is activated by G6P (Davies *et al.*, 2008), and apparently by dephosphorylation mediated by PP2A induced in turn by xylulose-5-phosphate in high glucose conditions; even though its regulation via phosphorylation has been questioned (Tsatsos & Towle, 2006) it translocates to the nucleus (Kawaguchi *et al.*, 2001) and promotes lipogenesis by activating acetyl-CoA carboxylase (ACC) and L-PK (Ma *et al.*, 2005).

MondoA, a paralog of ChREBP/MondoB, is highly expressed in the adult human skeletal muscle tissue (Billin *et al.*, 2000). MondoA is known to translocate from the outer mitochondrial membrane to the nucleus (Sans *et al.*, 2006). Similarly to ChREBP/MondoB, G6P makes MondoA accumulate inside the nucleus (Peterson *et al.*, 2010). HK

inhibition reduces G6P-dependent MondoA translocation into the nucleus (Stoltzman *et al.*, 2008). MondoA is a heterodimerisation partner for max-like protein x (Mlx) (Billin *et al.*, 2000), which is a common partner for several transcription factors (Billin *et al.*, 1999). The MondoA:Mlx complex activates the transcription of lactate dehydrogenase-A (LDH-A), HK and phosphofructokinase 2-fructose bisphosphatase 2 (PFK2-FBPase2) genes (Sans *et al.*, 2006). Thus, the MondoA:Mlx complex is a necessary and sufficient regulator of glucose flux (Sans *et al.*, 2006). MondoA also activates thioredoxin-interacting protein (TXNIP) (Stoltzman *et al.*, 2008). TXNIP binds to and inhibits the thioredoxin-NADPH-dependent reduction of protein disulfides (Nishiyama *et al.*, 1999). TXNIP's negative regulation of the thiol-reducing system makes cells more susceptible to oxidative stress by reactive oxygen species (ROS) (Nordberg & Arner, 2001). Moreover, TXNIP reduces glucose uptake acting as a negative feedback (Parikh *et al.*, 2007).

There are other glucose sensors present in the skeletal muscle tissue. One is the sterol regulatory element binding-protein 1c (SREBP-1c) (Guillet-Deniau *et al.*, 2004). SREPB-1c up-regulates the lipogenic flux in response to high glucose availability. In fact, SREBP-1c activates HK, FAS, ATP-citrate lyase (Guillet-Deniau *et al.*, 2002) and ACC (Guillet-Deniau *et al.*, 2004). Another glucose sensor is liver X receptor (LXR), an important regulator of the lipid and cholesterol metabolism in liver and in skeletal muscle tissue (Muscat *et al.*, 2002). LXRβ seems to be the main LXR subtype in the skeletal muscle (Hessvik *et al.*, 2010).

Considering that intracellular high glucose availability has been linked to insulin resistance (Tomas *et al.*, 2002), and is a feature of tumor cells (Pedersen, 2007), it is not surprising that glucose metabolites, such as G6P, are involved in the tight regulation of the main metabolic and insulin signalling pathways of the skeletal muscle cells. It is also reasonable to think that glucose metabolites can be involved in the

disruption of the "metabolic harmony" of the muscle cell (Herman & Kahn, 2006).

Glucose storage

A meal rich in refined CHOs and sugar produces a temporary overflow of glucose, which is disposed by the muscle tissue via insulin-dependent glucose uptake. In response to this augmented inflow of glucose, the muscle cell increases its glycolytic activity, thus increasing in parallel glycogen synthesis, glycolysis, and the final oxidation of glucose into CO_2 and H_2O. The increased production of CO_2 as a consequence of increased glucose oxidation can be measured indirectly by measuring the respiratory exchange ratio (RER). In acid-base balance, the CO_2 produced during metabolism is equivalent to the respiratory output of CO_2 (VCO_2). Therefore, the ratio between VCO_2 and oxygen uptake VO_2 in this condition can be referred to as the respiratory quotient (RQ). Indeed, following a meal containing CHO (e.g. 920 kcal of CHO) RER increases from 0.84, a typical fasting value, to 1.05 an hour after CHO ingestion; meaning that virtually only glucose is being oxidised (Saltzman & Salzano, 1971).

Within 6 hours following glucose ingestion 96% of glucose is disposed by the peripheral tissues (Woerle *et al.*, 2003), mainly in the muscle tissue (90%) (DeFronzo *et al.*, 1985). Of the total glucose disposed, 67% enters glycolysis, whereas 33% is stored as glycogen (Woerle *et al.*, 2003). After glucose ingestion and disposal both oxidative and nonoxidative glycolysis are increased; oxidative glycolysis accounts for 65% of the total glucose catabolised (Woerle *et al.*, 2003). Two factors regulate net whole body glycogen retention: the size of the CHO load and the level of initial glycogen stores (Fery *et al.*, 2003). The level of glycogen stores in turn depends on the diet, the duration of fasting, and physical activity (Fery *et al.*, 2003). Moreover, net CHO accumulation after a meal depends on the balance between glycogen synthesis and endogenous glycogen mobilisation, which, although

reduced, persists during the postprandial period (Barrett *et al.*, 1994; Fery *et al.*, 2003; Woerle *et al.*, 2003).

Glycogen is a branched polymer of glucose (starch) and is present in virtually all organisms, from unicellular organisms to humans (Roach, 2002). Glycogen is located in the cytoplasm, possibly in the vicinity of membranes (Cardell *et al.*, 1985). It is a large molecule because it contains glucose and water (Flatt, 1995). G6P allosterically activates glycogen synthesis (Villar-Palasi & Guinovart, 1997) and deactivates HK activity (Gregoriou *et al.*, 1983). It seems that glycogen does not only serve as glucose storage but it regulates glucose flux as well (Jensen *et al.*, 2006). In fact, high glycogen concentration reduces glycogen synthase activity (Danforth, 1965) and glucose uptake in skeletal muscle (James *et al.*, 1985; Jensen *et al.*, 1997; Derave *et al.*, 2000; Aslesen *et al.*, 2001). Glycogen storage has a limited capacity, and a continuous high glucose availability might result in decreasing glycogen synthase function, and increasing glucose metabolite concentration, lipogenesis, and insulin resistance.

Insulin resistance and type 2 diabetes mellitus in skeletal muscle

Insulin resistance refers to the impairment of insulin action in insulin-target tissues (i.e. skeletal muscle, adipose tissue and hepatic tissue) (DeFronzo, 1997). Lack of insulin action can be divided into decreased sensitivity and decreased responsiveness (Kahn, 1978). Because of the diminished response, a higher concentration of insulin (hyperinsulinaemia) is required to maintain normal circulating glucose levels (Martin, 2010). When hyperinsulinaemia can no longer overcome the defect in insulin action, resulting in hyperglycaemia, type 2 diabetes mellitus (T2DM) develops (Reaven, 1988). T2DM, also called non-insulin dependent or maturity-onset diabetes mellitus, is a metabolic disease, which usually occurs after the age of 40, but can also develop in younger people (Martin, 2010). It is important to underline that insulin resistance is a precursor state of T2DM (Martin

et al., 1992). Indeed, insulin resitance manifests long before hyperglycaemia appears (DeFronzo, 1988).

Genetic as well as environmental factors are known to be implicated in the pathophysiology of insulin resistance (Diamond, 2003). Some environmental factors seem to be more important than others, for instance lifestyle related factors (e.g. early life events, physical inactivity and diet) (Steyn *et al.*, 2004). Clearly, the global diabetes epidemic is brought on by genetic susceptibility, sedentary lifestyle, and over-nutrition (Zimmet *et al.*, 2001).

The pathophysiology of T2DM involves impaired insulin action and insulin secretion (DeFronzo, 1988). Wayer *et al.* (1999) indicated that insulin action and insulin secretion progressively deteriorate with the transition from normal glucose tolerance to impaired glucose tolerance accompagning augmented body weight. Moreover, they showed that the progression from impaired glucose tolerance to T2DM was determined by a further diminution of insulin action and secretion, a further increase in body weight, and an increase in basal endogenous glucose output (Wayer *et al* 1999). Glucose uptake is dramatically reduced in insulin resistant skeletal muscle (Abdul-Ghani & DeFronzo, 2010). A decreased insulin-dependent glucose uptake is ascribable to impaired insulin signalling, including impaired glucose transport and glucose phosphorylation, and to impaired glucose oxidation and glycogen synthesis (Abdul-Ghani & DeFronzo, 2010).

High glucose availability and insulin resistance

Continuous high energy food intake and a lack of energy expenditure (sedentarism) challenge the glycaemic regulation (Manson *et al.*, 1992; Salmeron *et al.*, 1997). Because plasma glucose regulation becomes weaker, hyperglycaemic periods are prolonged. High glucose availability or its metabolite concentration (e.g. G6P) in the sarcoplasm influence several signalling pathways leading to metabolic

adaptations in skeletal muscle which result in reduced insulin sensitivity and reduced oxidative capacity.

Glucose uptake measured by glucose clamp in hyperglycaemic conditions has been found to be only slightly reduced in diabetic patients (DeFronzo *et al.*, 1982). Insulin stimulated glucose uptake in diabetic patients during hyperglycaemic clamp is 10-fold higher than in the absence of insulin (Kelley & Mandarino, 1990), thus intracellular glucose is expected to be high. Indeed, high intracellular glucose availability (6.3 ± 2.2 mmol/L) does occur in type 2 diabetic patients during hyperglycaemia (~13 mmol/L) and hyperinsulinaemia (240 $pmol \cdot min^{-1} \cdot m^2$) (Bonadonna *et al.*, 1996). This demonstrates that intracellular high glucose availability is present even in insulin resistant cells in a hyperglycaemic and hyperinsulinaemic environment. Considering that in healthy humans only 23% of total plasma glucose is endogenous (Woerle *et al.*, 2003), it is reasonable to think that high CHO diet combined with low energy expenditure, caused by inactivity, encourages prolonged postprandial periods of hyperglycaemia in otherwise normal glucose tolerant individuals. This could clearly contribute to the development of a diabetic phenotype in skeletal muscle with an abundance of fast glycolytic fibres with low oxidative capacity as well as an impaired response of insulin signalling pathways.

Chronic hyperglycaemia is directly linked to insulin resistance and therefore T2DM (Rossetti *et al.*, 1987). High glucose availability has been broadly established as a cause of insulin resistance in skeletal muscle (Fell *et al.*, 1982; Richter *et al.*, 1988a; Richter *et al.*, 1988b; Hansen *et al.*, 1992; Davidson *et al.*, 1994; Gulve *et al.*, 1994; Kawanaka *et al.*, 1999; Kawanaka *et al.*, 2001; Oku *et al.*, 2001; Han *et al.*, 2003). There are several mechanisms by which hyperglycaemia induces insulin resistance (Kawanaka *et al.*, 2001). High glucose-induced UDP-N-acetylhexosamine accumulation (Marshall *et al.*, 1991b, 1991a; Robinson *et al.*, 1993; Baron *et al.*, 1995; Virkamaki *et*

al., 1997), protein kinase C activation (Pillay *et al.*, 1996; Filippis *et al.*, 1997; Laybutt *et al.*, 1999) and increased glycogen content (Fell *et al.*, 1982; Jensen *et al.*, 1997; Kawanaka *et al.*, 1999) have all been shown to induce insulin resistance.

Glycogen

Insulin resistance is characterised by impaired insulin-stimulated glycogen synthesis (Abdul-Ghani & DeFronzo, 2010). Impaired glycogen synthesis was also documented in normal glucose tolerant offspring of T2DM patients (Abdul-Ghani & DeFronzo, 2010). However, in healthy humans, glycogen stores are far from being full under normal *ad libitum* CHO diets. When these stores are saturated via a massive CHO overfeeding, glucose oxidation and *de novo* lipogenesis dispose the glucose in excess (Acheson *et al.*, 1988). However, higher glycogen content is observed in healthy lean and obese subjects with high CHO/sugar diets (Gollnick *et al.*, 1972; Flatt, 1996). Conversely, He and Kelley (2004) did not find higher muscle glycogen content in obese people compared to lean, but they did find that glycogen was lower in type I muscle fibre than in type IIa and IIx/d fibres. It has also been shown that obese people have less type I muscle fibres and more type IIa and IId/x fibres (Wade *et al.*, 1990); indirectly proving that obese individuals have a higher glycogen storage within the skeletal muscle tissue. As previously mentioned, an increase in glycogen content reduces GS activity and this has been associated with insulin resistance (Jensen *et al.*, 2006).

Free glucose

Free glucose itself can cause insulin resistance. As previously mentioned, a high glycogen concentration in the muscles causes a reduction in glycogen synthase activity and glucose uptake, and additionally the reduced glycogen synthase leads to increased levels of G6P, glycolytic flux, and accumulation of non-phosphorylated glucose

(Jensen *et al.*, 2006). Accumulation of non-phosphorylated glucose is present in T2DM patients (Bonadonna *et al.*, 1996). High free glucose *per se* seems to have a direct effect on insulin-mediated glucose uptake by deactivation of Akt (Kurowski *et al.*, 1999; Oku *et al.*, 2001; Tomas *et al.*, 2002).

Glycogen-synthase kinase 3

GSK-3, which inhibits GS function, is elevated in skeletal muscle of T2DM patients (Nikoulina *et al.*, 2000). In vitro experiments on adipocytes, and ovary and embryonic kidney cell lines indicate that GSK-3 can attenuate insulin-stimulated phosphorylation of tyrosine residues in IR and IRS-1 by phosphorylating serine residues of these proteins (Eldar-Finkelman & Krebs, 1997; Greene & Garofalo, 2002; Liberman & Eldar-Finkelman, 2005). Recently, GSK-3 was proven to induce IRS-1 degradation as a consequence of high glucose availability in primary hepatocytes (Leng *et al.*, 2010). Chronic (4-day) treatment of human myotubes with GSK-3 inhibitors (CT98014, CHIR98023, and LiCl) increased GS activity, both basal and insulin-stimulated glucose uptake, and ~3.5 folds IRS-1 amounts, but, it did not change Akt phosphorylation (Nikoulina *et al.*, 2000). GSK-3 also deactivates ACC activity by phosphorylation and adipocyte determination- and differentiation-dependent factor 1 (ADD1)/SREBP-1c transcriptional activity in adipocytes and hepatocytes (Kim *et al.*, 2004; Terrand *et al.*, 2009). GSK-3 seems to be up-regulated also by glucosamine (Singh & Crook, 2000). Therefore, GSK-3 is a key signalling molecule in high glucose availability-induced insulin resistance. Indeed, GSK-3 inhibitors are considered as possible anti-diabetic drugs (Martinez *et al.*, 2002).

Gene expression regulation

Aas *et al.* (2004) showed that in cultivated human skeletal muscle cells high glucose availability can reduce insulin stimulated glucose uptake

and glycogen synthesis even after a short time of exposure, e.g. two days. This suggests that effects on metabolic gene expression and insulin signalling might happen very early in the development of diabetes. It has been suggested that in skeletal muscle high glucose availability can influence several transcription or co-transcription factors, such as MondoA, SREBP-1c, LXR, and peroxisome proliferator-activated receptor coactivator 1 alpha (PGC1α), which are known to decrease oxidative capacity and at the same time increase glycolytic capacity of the muscle cell (Guillet-Deniau *et al.*, 2004; Sans *et al.*, 2006; Mitro *et al.*, 2007; Hanke *et al.*, 2008).

Of the 198 genes regulated by glucose in muscle cells, 152 (~75%) require MondoA for their transcription (Stoltzman *et al.*, 2008). Following accumulation of MondoA:Mlx complex in the nucleus, glucose is necessary to allow this complex to occupy the target promoters and to recruit histone H3 acetyltransferase, which triggers MondoA:Mlx-induced gene expression (Peterson *et al.*, 2010). Arrestin domain-containing protein 4 (ARRDC4) and TXNIP are the two most highly MondoA dependent genes (Stoltzman *et al.*, 2008). ARRDC4 and TXNIP are paralogs and although not much is known about the first, the latter is known to be involved in ROS formation by the mitochondria, possibly causing mitochondrial dysfunction (Parikh *et al.*, 2007; Saxena *et al.*, 2010). Importantly, TXNIP expression is regulated by glucose ChoRE, and MondoA:Mlx complex is its transcription factor (Stoltzman *et al.*, 2008). TXNIP is an important negative regulator of glucose uptake in skeletal muscle cells (Parikh *et al.*, 2007). MondoA is clearly very important in the regulation of intracellular glucose homeostasis, and continuous high glucose supplies might disturb such tight regulation. However, to date there are no studies which investigate the role of MondoA in high glucose availability in human skeletal muscle cells in vitro and in vivo.

Hyperglycaemia-induced ROS overproduction by the mitochondria seems also to inhibit glyceraldehyde-3-phosphate dehydrogenase

(GAPDH), a key enzyme of glycolysis (Bouche *et al.*, 2004). GAPDH controls gene expression, DNA replication and repair, and tRNA export (Sirover, 1997). It seems that its inhibition might lead to PKC and hexosamine pathway activation (Du *et al.*, 2003).

The oxidative capacity of skeletal muscle in insulin resistant and diabetic individuals is severely impaired (Kelley & Mandarino, 1990; Kelley *et al.*, 1999). Muscles from obese and diabetic individuals possess a markedly reduced mitochondrial volume density even if corrected for muscle fibre type (Kelley *et al.*, 2002). Moreover, their muscle have a decreased mitochondrial adenosine triphospate (ATP) synthesis, lower oxidative fibre type I to glycolytic type II ratio, reduced glycogen synthesis, and a reduced expression of the important co-factor of mitochondrial gene expression, PGC1α (Mootha *et al.*, 2003; Patti *et al.*, 2003; Lowell & Shulman, 2005; Franks & Loos, 2006).

It is reasonable to think that high glucose availability would decrease mitochondrial oxidative phosphorylation and would promote slow-to-fast fibre type conversion by reducing PGC1α activation (Mootha *et al.*, 2004). This is expected not only in diabetic subjects but presumably also in sedentary individuals consuming high levels of sugars, for example present in soft-drinks. In fact, diabetic patients do have a lower PGC1α expression (Mootha *et al.*, 2003; Patti *et al.*, 2003) and they also show a higher density of fast muscle fibre type than healthy subjects (Oberbach *et al.*, 2006).

High refined carbohydrate/sugar diets and the incidence of obesity, insulin resistance, and type 2 diabetes mellitus

In the last decade, many investigations and reviews have found an association between refined starch and/or soft drink consumption and obesity (Ludwig *et al.*, 2001; Ebbeling *et al.*, 2002; Schulz *et al.*, 2002; St-Onge *et al.*, 2003; Berkey *et al.*, 2004; Bray *et al.*, 2004; Gross *et*

al., 2004; Schulze *et al.*, 2004; James & Kerr, 2005; Bes-Rastrollo *et al.*, 2006; Malik *et al.*, 2006; Dhingra *et al.*, 2007; Vartanian *et al.*, 2007; Palmer *et al.*, 2008; Bleich *et al.*, 2009; Fung *et al.*, 2009; Nissinen *et al.*, 2009; Olsen & Heitmann, 2009; Hu & Malik, 2010). However, there is also some literature, that tends to discredit the association between carbohydrate/soft drink consumption and obesity (Drewnowski & Bellisle, 2007; Gaesser, 2007; Forshee *et al.*, 2008; Gibson, 2008; Gomez-Martinez *et al.*, 2009; van Baak & Astrup, 2009; Mattes *et al.*, 2010). A recent systematic review has highlighted that many of the publications which weakened the carbohydrate/soft drink consumption-obesity association are funded by food industries (Vartanian *et al.*, 2007). Generally, these industry funded studies weaken the association between soft drink intake and weight gain by adjusting for total energy intake. Since this association is in part mediated by the increase in total energy intake, this adjustment reduces its statistical significance (Hu & Malik, 2010).

Many investigations show a link between refined carbohydrate/soft drink consumption and insulin resistance/type 2 diabetes mellitus (Liu *et al.*, 2000; Liu, 2002; Gross *et al.*, 2004; Schulze *et al.*, 2004; Vartanian *et al.*, 2007; Yoshida *et al.*, 2007; Palmer *et al.*, 2008; Bleich *et al.*, 2009; Mohan *et al.*, 2009; Stanhope *et al.*, 2009; Hu & Malik, 2010). Other studies observed that substituting refined CHO with "lente" (slow-release) CHO, rich in dietary fibres and with a low glycaemic-index, decreases insulin resistance and risk of type 2 diabetes mellitus (Fung *et al.*, 2002; Hodge *et al.*, 2004; Oh *et al.*, 2005; de Munter *et al.*, 2007; Krishnan *et al.*, 2007; Jenkins *et al.*, 2008; Mellen *et al.*, 2008).

As a matter of fact, refined carbohydrate/soft drink consumption has increased over the past four decades (ERS, 2004). From 1988-1994 to 1999-2004 the percentage of adults drinking soft drinks in the U.S. has increased from 58% to 63%, with the per capita consumption of soft drinks increased from 157 ± 5 kcal/d to 203 ± 5 kcal/d over the same

period of time (Bleich *et al.*, 2009). This increase in dietary refined CHO availability and simple sugar availability has produced a large rise in exogenous glucose availability (Foster-Powell & Miller, 1995).

Low carbohydrate/glucose and energy availablilty to reduce the risk of type 2 diabetes mellitus

Hypoglycaemic diet and insulin resistance

Low carbohydrate (low-CHO) diets have been shown to reduce plasma insulin and glucose concentrations in overweight/obese people with insulin resistance (Foster *et al.*, 2003; Dansinger *et al.*, 2005). In particular, several studies have demonstrated low-CHO diets to be very effective in reducing fasting glucose and insulin even in diabetic patients (Garg *et al.*, 1988; Parillo *et al.*, 1992; Low *et al.*, 1996; Samaha *et al.*, 2003; Boden *et al.*, 2005; Yancy *et al.*, 2005). In several cases, low-CHO diets have been reported to be more effective than low-fat diets in reducing hyperglycaemia and hyperinsulinaemia (Garg *et al.*, 1988; Parillo *et al.*, 1992; Low *et al.*, 1996; Samaha *et al.*, 2003; Dansinger *et al.*, 2005). Moreover, low-CHO diets have been demonstrated to be a very effective method to lose weight for obese and overweight people (Brehm *et al.*, 2003; Foster *et al.*, 2003; Boden *et al.*, 2005; Dansinger *et al.*, 2005; Westman *et al.*, 2006; Ebbeling *et al.*, 2007).

It is important to underline the fact that modest and substantial increases in body weight are associated with an increased risk of T2DM (Wannamethee & Shaper, 1999). The well established relationship between being overweight and having diabetes appears in its precise significance when considering that almost 65% of the adult population in the United States is overweight (Hill *et al.*, 2003) and similar trends have been found in the United Kingdom (Avenell *et al.*, 2004).

One of the major side effects of a low-CHO diet might be an increased ketosis (Kekwick & Pawan, 1957; Benoit *et al.*, 1965; Krehl *et al.*, 1967; Worthington & Taylor, 1974; Lewis *et al.*, 1977; Larosa *et al.*, 1980; Wing, 1995). On the one hand, higher ketosis encourages appetite suppression (Astrup *et al.*, 2004), but on the other hand it may induce gout. A daily ingestion of around 150-200 g of CHO is known to avoid any possible risk of starvation ketosis (de Munter *et al.*, 2007). Low-CHO diets have been indicated to positively influence lipid levels (Brehm *et al.*, 2003; Samaha *et al.*, 2003; Stern *et al.*, 2004; Yancy *et al.*, 2004) and improve some cardiovascular risk factors (Astrup *et al.*, 2004). Finally, several studies, on either obese or obese diabetic individuals, have shown that two weeks of low-CHO diet is sufficient to reduce plasma insulin and glucose levels (Lewis *et al.*, 1977; Parillo *et al.*, 1992; Boden *et al.*, 2005).

Exercise-induced glycogen depletion and insulin resistance

Although low-CHO diets can reduce plasma glucose levels in hyperglycaemic individuals, they cannot bring glycaemic levels back to the normal physiological range (Low *et al.*, 1996; Brehm *et al.*, 2003; Samaha *et al.*, 2003; Boden *et al.*, 2005; Dansinger *et al.*, 2005; Yancy *et al.*, 2005). Fell *et al.* (1982) have demonstrated in rats that glucose uptake was significantly higher (60-80%) at the same insulin concentration, when muscle glycogen was kept low, than when glycogen was raised by CHO feeding. However, low-CHO diet *per se* does not seem to decrease skeletal muscle glycogen sufficiently to enhance the glucose uptake. Miller *et al.* (1984) reported that 5 weeks of low-CHO diet leads to a greater glycogen depletion in the liver than in the skeletal muscle of male rats. Simi *et al.* (1991) confirmed the effect of low-CHO diet on liver glycogen depletion, but they did not find any statistical difference in muscle glycogen concentrations between 12 weeks of low-CHO diet and 12 weeks of high-CHO diet in sedentary rats. These results seem to be in agreement with the notion that muscle glycogen turnover is reduced (Hultman & Nilsson, 1975).

By employing exercise-induced glycogen depletion, muscle glycogen storage should be diminished. This can be replenished from plasma glucose and could further reduce hyperglycaemia. Muscle glycogen depletion is greater (≈ 70%) with high intensity aerobic exercise (90% of maximal oxygen uptake (VO_{2max})) than with low intensity aerobic exercise (≈ 40% at 60% VO_{2max}) (Gollnick *et al.*, 1974; Vollestad & Blom, 1985). Although glycogen is not easily depleted in insulin resistant muscles (Del Prato *et al.*, 1993), glycogen synthesis is reduced (Roden & Shulman, 1999). Therefore, exercise-induced glycogen depletion should keep glycogen levels low. In particular, high intensity intermittent exercise has been shown to be very effective to deplete muscle glycogen (MacDougall *et al.*, 1977; Vollestad & Blom, 1985). When deep glycogen depletion is required, the exercise intensity must be higher than 75% of VO_{2max} (Edgerton & Roy, 2006). Moreover, a diet providing less than 200 g/day of carbohydrates is shown to permit little resynthesis of muscle glycogen (Bergstrom *et al.*, 1967). Kjær *et al.* (1990) have reported that high intensity exercise is a good method to increase glucose clearance in both healthy and diabetic people. Glucose clearance is the measure of the tissue's activity to remove glucose (Radziuk & Lickley, 1985). Several studies have found improved insulin sensitivity following high intensity aerobic exercise in insulin resistant and non-insulin resistant people (Ronnemaa *et al.*, 1986; Kjaer *et al.*, 1990; Mourier *et al.*, 1997; Loimaala *et al.*, 2003; Dela *et al.*, 2004; DiPietro *et al.*, 2006).

Combination of exercise and diet

Several studies have shown that a combination of controlled diet and exercise leads to better glucose regulation in obese and diabetic people (Saltin *et al.*, 1979; Kaplan *et al.*, 1987; Eriksson & Lindgarde, 1991; Pan *et al.*, 1997; Rice *et al.*, 1999; Roberts & Barnard, 2005). None of the previous studies though, combined exercise-induced glycogen degradation with a low-CHO diet. Racette *et al.* (1995) tested the

effect of low-CHO and low-fat diets combined with aerobic exercise at 65% of maximal oxygen consumption (which is known not to degradate glycogen) in obese, non-insulin resistant women. They found that seven weeks diet plus exercise promotes a greater weight loss, in particular in the low-CHO/Ex group (11 kg loss vs. 6 kg in the low-fat/Ex group). Low-CHO/Ex seemed also to be the most effective in reducing fat mass. Fasting glucose and insulin were already in a normal physiological range from baseline (5.0 ± 0.3 mmol/L and 83 ± 24 pmol/L, respectively). However, a trend (P = 0.09) for a greater decrease in plasma glucose in the low-CHO groups was found. Fasting insulin decreased in both conditions.

Cellular responses to low-CHO diet and exercise-induced glycogen depletion

PGC1α increases the expression of mitochondrial oxidative phosphorylation genes (Mootha *et al.*, 2004) and promotes slow-to-fast fibre type conversion (Lin *et al.*, 2002; Handschin *et al.*, 2007). Hanke *et al.* (2008), by using muscle cell cultures from rabbits showed that the expression of PGC1α is increased with low cellular glucose availability (glucose free medium). The reduction in PGC1α might be due to a lack of activation by AMP-activated kinase (AMPK) and Calmodulin IV kinase (CaMK IV) (Zong *et al.*, 2002; Jager *et al.*, 2007). Lack of contractile muscle activity reduces AMPK and CaMK IV activation by low cytoplasmic Ca^{2+} concentration (Wu *et al.*, 2002). On the contrary, the higher the intensity of exercise, the greater the AMPK activation (Chen *et al.*, 2003). AMPK has been demonstrated to be inhibited by high glycogen (as a consequence of high glucose availability) (Jørgensen *et al.*, 2007) and by protein phosphatase 2A (PP2A) activation (triggered by glucose via the pentose phosphate pathway (Gimeno-Alcaniz & Sanz, 2003) and by ceramide via fatty acid synthesis) (Lin *et al.*, 2002). Taken together, these indications show that high glucose availability combined with physical inactivity might lead to AMPK and CaMK IV inactivation which seems to cause

mitochondrial dysfunction. Moreover, AMPK inhibits the activity of the first enzyme of the fatty acid synthesis, ACC by phosphorylation (Munday *et al.*, 1988). Convesely, high-intensity exercise and low-CHO low-energy diet activate CAMK and AMPK, which effectively stimulate mitochondrial biogenesis and GLUT4 expression (Ojuka, 2004).

Aims of the thesis

The general aim of this thesis is to advance our understanding of the factors causing the large human consumption of refined CHO/sugar and the effects of the consequently high exogenous glucose availability on metabolism and insulin function in the skeletal muscle tissue. Another aim of this thesis is to elucidate the effectiveness of low glucose availability as a lifestyle intervention to reduce the risk of T2DM in obese, sedentary individuals.

Research questions

Chapter two: Do overweight/obese individuals have a different taste perception and implicit attitude towards sweet food/drinks than lean individuals? How does chronic soft drink usage change taste perception and explicit/implicit attitude towards sweet food/drinks?

Chapter three: How does chronic glucose availability influence insulin signalling and cell metabolism in human primary skeletal muscle cells? How does periodic high glucose availability (soft drink supplementation) influence insulin signalling and whole body and cell metabolism *in vivo*?

Chapter four: After ascertaining that high glucose availability has several deleterious effects on one's health, what are the effects of a short period of low glucose availability on obese, sedentary individuals?

Chapter five: Is the lifestyle intervention adopted in **Chapter four** applicable to T2DM patients?

CHAPTER II

TASTE PERCEPTION AND IMPLICIT ATTITUDE TOWARD SWEET RELATED TO BODY MASS INDEX AND SOFT DRINK SUPPLEMENTATION

Abstract

These studies examined the differences in sweet taste perception and implicit attitude toward sweet between lean and overweight/obese adults; and tested the effects soft drink consumption has on sweet taste, explicit preference and implicit attitude toward sweet in lean participants. In study 1, 34 adults (20 females, 14 males; 22 lean, 11 overweight/obese; 23 ± 3 yrs) were assessed for sweet taste intensity and pleasantness. Implicit attitude toward sweet was assessed by implicit association test (IAT). In study 2, 12 lean, lightly active adults (7 females and 5 males; 26 ± 6 yrs) underwent one month soft drink supplementation (≈ 760 mL/day). This increased their daily carbohydrate intake by 2.1 ± 0.2 g/kg body weight. Sweet taste perception, explicit preference and implicit attitudes to sweet were assessed. Overweight/obese participants perceived sweet and salty tastes as less intense (-23% and -19%, respectively) and reported higher IAT scores for sweet than lean controls (2.1-fold). The supplementation changed sweet intensity and pleasantness ratings and it increased explicit preference (2.3-fold) for sweet in a subgroup of initial sucrose-dislikers. In conclusion, overweight/obese individuals are more implicitly attracted to sweet; and their altered taste perception may be determined by the environment. One month of soft drink supplementation changed sweet taste perception of lean participants.

Introduction

In westernized countries the consumption of soft drinks has increased consistently over the last three decades (Nielsen & Popkin, 2004; Vereecken *et al.*, 2005; Duffey & Popkin, 2007; Barquera *et al.*, 2008). Indeed, the total estimated delivery of caloric high-fructose corn syrup and glucose syrup for domestic food and beverages has increased 11.6 times from 1966 to 2003 in the U.S. (ERS, 2004). Additionally, soft drinks provide the largest amount (47%) of added sugars in the diet of US Americans (Guthrie & Morton, 2000). The strong palatability of sweet taste (Lenoir *et al.*, 2007) along with the high energy density and the low cost of soft drinks (Drewnowski & Bellisle, 2007) might explain their escalating popularity.

Considering the low physical activity levels in westernized countries (Booth *et al.*, 2000; Hayes *et al.*, 2005), high and chronic soft drink consumption has a detrimental impact on public health. In fact, there is epidemiologic and experimental evidence that high consumption of soft drinks is associated with weight gain and obesity (Malik *et al.*, 2006). Consuming one or more soft drinks per day is associated with increased odds of developing metabolic syndrome (Dhingra *et al.*, 2007). Furthermore, a high consumption of soft drinks has been associated with an increased risk for development of type 2 diabetes (Schulze *et al.*, 2004; Montonen *et al.*, 2007; Palmer *et al.*, 2008).

Taste is the most important factor influencing food choice (Glanz *et al.*, 1998). Sweetness is a basic drive of food selection (Booth *et al.*, 1987; Birch, 1999), and there is a positive correlation between percent of calorie intake from sweet and sweet preference (Mattes & Mela, 1986). Several investigations have tried to elucidate the relationship between sweet taste perception and obesity (Donaldson *et al.*, 2009), yet there is still some divergence on the nature of this relationship (Bartoshuk *et al.*, 2006). Taste is a plastic system (Heath *et al.*, 2006). Indeed, hedonic ratings for high-fat food were reduced by 12 weeks of reduced-fat diet, when the sensory exposure to fat was restricted

(Mattes, 1993). Accordingly, it is possible that a regular consumption of soft drinks might move the taste threshold for sweet to a higher level and potentially alter perception of both intensity and pleasantness. These effects could eventually enhance regular soft drink consumers' preference for sweet tasting food/drinks. There is some existing evidence that a short term period (8 days) of sweet orangeade exposure can increase children's preference for this orangeade (Liem & de Graaf, 2004). However, this effect was not seen in young adults (Liem & de Graaf, 2004). This discrepancy was probably due to the fact that in that study children liked the sweet orangeade more than the adults who tended to reduce their orangeade consumption throughout the study (Liem & de Graaf, 2004). We hypothesized that if a leading soft drink on the market, preferred by adults, is chosen and a longer time of exposure is applied, the preference for sweet could be increased in adults as well.

Implicit or non-conscious preferences or attitudes might also play a key role in the high consumption of soft drinks particularly among obese individuals. Implicit attitudes are unconsciously held positive or negative evaluations of an object and reflect automatic associations in memory between concepts (Greenwald *et al.*, 2002). The implicit association test (IAT) is frequently used to assess implicit attitudes. The IAT is a computerized method for indirectly assessing attitudes toward an object by measuring the strengths of associations among concepts through the speed with which individuals respond to the presentation of stimuli associated with the concepts.
The assumption behind the IAT is straightforward, if two concepts are highly associated in memory (e.g. sweet food and favorable attributes), then they are easier to associate via IAT's sorting tasks, than if the two concepts are weakly associated (e.g. sweet food and unfavorable attributes) (Greenwald & Nosek, 2001). Thus response latencies are faster for strongly associated concepts. It seems that obese people have different implicit food preferences from their non-obese counterparts (Drewnowski *et al.*, 1992; Capaldi, 1996). To the authors' knowledge

it is not yet known whether implicit attitude toward sweet differs in obese people. Furthermore, if soft drink-induced taste alteration changes explicit preferences (e.g. conscious food choices), chronic soft drink consumption might eventually lead to an implicit preference for sweet tastes.

Moreover, eating behavior changes, possibly induced by taste alterations, might have some links to increased adipokines and/or cytokines with augmented adiposity induced by the soft drink supplementation. Leptin levels vary directly according to the amount of body fat mass (Considine *et al.*, 1996). Leptin is an adipokine that is involved in long term regulation of appetite (Friedman & Halaas, 1998). Leptin diminishes the perception of food reward and enhances the response to satiety signals generated during food consumption (Farooqi *et al.*, 2007). Although, the role of leptin in satiety has been extensively investigated and is well estabilished; leptin modulates many other systems as well (Harris, 2000). A study in mice has shown that leptin selectively inhibits sweet taste responses through activation of outward K^+ currents, and that in diabetic *db/db* mice, taste cells are not affected by leptin levels (Kawai *et al.*, 2000). It is possible that changes in taste sensitivity and concomitant food preference are influenced by altered leptin levels. For this reason we also investigated the possible involvement of leptin on sugar consumption.

Studies on cytokine-induced sickness behavior conducted on animals have shown that increased levels of cytokines decrease food intake and alter diet composition (Dantzer, 2001). In particular, IL-1β infusion, at the same time as decreasing food intake, increased carbohydrate consumption and reduced protein intake, while fat ingestion remained unaltered (Dantzer, 2001). Several cytokines such as TNFα, IL-10 and IL-6 are expressed in the adipose tissue (Yudkin *et al.*, 2000; Juge-Aubry *et al.*, 2005), in particular IL-6 has been found to increase with adiposity in healthy men and women (Mohamed-Ali *et al.*, 1997). Augmented adiposity as a consequence of an increased energy intake

from carbohydrates (e.g. soft drink consumption), might determine increases in cytokines which could cause a change in macronutrient intake.

Thus we conducted two studies. The purpose of study 1 was to test the hypothesis that there are differences in sweet-salty taste perception and/or implicit attitude toward sweet between lean and overweight/obese young adults. The aim of study 2 was to test the hypotheses that one month of consumption of a commercially available soft drink would alter taste perception (intensity and pleasantness) of sweet, and explicit preferences and implicit attitudes to sweet in healthy, lightly active participants. Secondarily, study 2 aimed to observe any correlation between leptin levels and sugar consumption, and between cytokine levels and changes in macronutrient intake.

Methods

Study 1

- *Participants and Study design*

Thirty-four young healthy adults, fourteen males and twenty-one females (mean age 22.8 ± SD 2.5 yrs; BMI 24.7 ± 4.7) were recruited via university e-mail announcements within the Bangor University population. The participants completed a medical questionnaire to verify that they did not have any chronic diseases. Sweet and salty taste and IAT toward sweet were tested twice. The thirty-four participants were split into two groups according to their BMI. The lean (L) group had a BMI ≥18 and <25, the overweight/obese (Ov/Ob) group had a BMI ≥25. Consequently, twenty-two participants (seven males and fifteen females) were allocated in the L group, eleven participants (seven males and four females) in the Ov/Ob group (**Table 2.1**), and one subject was excluded because of a BMI less than 18.

- *Taste test*

Eleven concentrations of sucrose (0, -0.5, -0.75, -1, -1.25, -1.5, -1.75, -2, -2.25, -2.5, -2.75 log [sucrose] mol/L) and seven concentrations of sodium chloride (-1, -1.25, -1.5, -1.75, -2, -2.25, -2.5 log [NaCl] mol/L) were prepared with demineralised water. These concentrations were used to determine perception across threshold and into the suprathreshold concentration range, rather than at a single suprathreshold concentration. Generalized Labeled Magnitude Scales (gLMS) (Green *et al.*, 1996) of intensity (150 mm) and pleasantness (± 86 mm) were adopted to measure the perceptions of intensity and pleasant/unpleasantness of the sucrose or sodium chloride solutions by the participants. The low (barely detectable) and the high (the strongest imaginable sensation of any kind) anchor points of the intensity scale and the low and high anchor points of the pleasantness scale (anchors:

most unpleasant imaginable, most pleasant imaginable, midpoint 0=neutral) were established prior to the beginning of the test. The use of the gLMS was carefully explained to all participants prior to testing by means of standard information, but the participants received no prior training. All solutions (5 ml each) were presented at room temperature. The various concentrations were presented in a random order and they were labeled with undetectable code names. Moreover, the operator who provided the solutions did not know their concentrations. Intensity and pleasantness were assessed using a standard 'sip-and-spit' procedure. The participants were asked to sip the solution and wash their mouths with it for about 5 s, discharge the solution and rate how strong and how pleasant the taste was using the gLMS (Mattes, 2009).

The participants were informed whether the solutions were meant to be sweet or salty, but not about their concentration. Between each taste they rinsed their mouths with dematerialized water for about 20 s. The order of the taste tests (sucrose intensity and pleasantness, sodium chloride strength and pleasantness) was randomized.

All participants were asked to refrain from alcohol for 24 hours prior to the test days and from caffeine consumption on test days. They were asked to be well hydrated before undergoing this test. Before the test the participants were asked to complete the Spielberger State and Trait questionnaire (1983) as anxiety is known to affect taste perception (Heath *et al.*, 2006).

- *Implicit Association Test*

Inquisit 3.0 (2008) which measures response latencies to keyboard presses with millisecond accuracy was used to generate the test and collect the data. The IAT was presented in seven blocks, five of which were practice trials to acquaint participants with the stimulus materials

and categorization rules. The target category exemplars comprised images of sweet (e.g. chocolate, cola drinks) and non-sweet (e.g. savoury foods, water) foods and drinks. Practice blocks comprised 20 trials each. The critical test blocks were the fourth (20 trials) and fifth (40 trials), labeled compatible blocks, where the sweet exemplars were paired with positive attribute words (e.g. 'pleasure') on one response key and the non-sweet exemplars with the negative attribute words (e.g. 'tragic') on another response key, and the sixth and seventh blocks (20 and 40 trials respectively), labeled incompatible blocks, in which these pairings were reversed. There were eight images and attribute words in each category. Exemplar and attribute stimuli were presented randomly without replacement within blocks, independently for each subject. Order of presentation of compatible and incompatible blocks was counterbalanced across participants. Response latencies were recorded for the test block trials and an IAT score was computed from the mean difference between performance on the compatible and incompatible blocks using the *D*-score algorithm for IAT data (Greenwald *et al.*, 2003).

Study 2

- *Participants and study design*

A pre-test post-test, within participants design was used to test our research hypotheses. An initial screening for lifestyle and soft drink consumption was executed via qualitative questionnaires. Questionnaires were administrated by the head researcher. The physical activity and soft drink consumption scores used in this study were defined as follows: 1 = physically inactive (little or no exercise), 2 = lightly active (light exercise or sports 1-3 days a week), 3 = moderately active (moderate exercise or sports 3-5 days a week) and 4 = very active (hard exercise or sports 6-7 days a week). Soft drink consumption scores: 1 = more than 4 pints a week, 2 = 1 to 2 pints a week, 3 = less than 1 pint (or can) a week and 4 = none. Out of 213

people screened, 32 were considered eligible. Healthy people with low physical activity, no more than 1-3 days of light exercise a week, and consuming less than one pint of soft drink and or fruit juices per week were considered eligible to take part.

Twelve participants, seven females and five males (age: 26 ± 6 yrs, height: 1.73 ± 0.09 m, weight: 65.5 ± 9.6 kg, BMI 21.7 ± 1.5, physical activity scores: 2.00 ± 0.60, soft drink scores: 3.42 ± 0.51) took part in this study. Participants were informed that upon completion of testing they would receive a £100 monetary compensation. Before and after the intervention period, participants attended our laboratories for two testing sessions. Although all the participants included in this study were physically lightly active, they were asked to refrain from heavy exercise for 24 h prior to all tests. Moreover, the subjects were asked to keep their normal physical activity constant throughout the duration of the intervention. Unless otherwise mentioned, all the tests described in study 2 were conducted before or 36 hours after the last soft drink supplementation.

- *Diet diaries*

Diet diaries were recorded in order to assess energy intake and macronutrient changes due to the intervention. Participants were introduced to diet diaries via standard instructions (Gibson, 1993) and were informed about the importance of the accuracy and precision of their reports for this study. Then, participants were asked to keep a seven day diet diary for a week before the intervention started. The adherence of their records to the standards required was checked at the pre-test sessions and further instructions about the standards required were given. During the supplementation month participants were asked to keep a fourteen day diet diary. The fourteen days were randomly chosen over the intervention period of four weeks. Energy balance was estimated from measurements of changes in body composition by using Elia *et al.*'s method (2003).

- *Taste test, IAT and preference test*

Taste and IAT were carried out on the participants of study 2 as described in study 1. Since no differences in salty taste perception were expected to occur with the intervention, intensity and pleasantness scores of salty taste were measured as a control. Additionally, a sucrose preference test was conducted [adapted from (Liem & Mennella, 2002)]. Five different sucrose solution concentrations (0, -0.5, -0.75, -1 and -1.25 log [sucrose] mol/L) were presented in ten random pairs. After tasting the two sucrose solutions the participants had to indicate the one they preferred. The number of times in which the participants preferred the highest concentration was calculated as a percentage of the total number of presentations. The median preference score at baseline was used to split the group into "sucrose-likers" (preferred the most concentrated solution > median 55%) and "sucrose dislikers" (preferred the most concentrated solution < median- scores). The preferences for sweetness by the "sucrose-likers" and the "sucrose dislikers" were compared using a Fisher's exact test.

A pilot study conducted on eight participants showed that the soft drink used in this study (Lucozade Energy, orange and apple flavors) had intensity and pleasantness scores in the range of the sucrose solutions used in the taste tests (Orange: 2.95 ± 0.98 cm, 0.77 ± 0.51 cm; Apple: 3.76 ± 1.52 cm, 0.61 ± 2.71 cm, respectively).

- *Analytical procedures*

An overnight fasting venous blood sample (4 ml) was collected into heparinized vacutainers from the antecubital vein of each subject. The plasma sample aliquots were then stored at -40°C for later analysis. Cytokines (IL-10, IL-6 and TNF-α) and adipokines (leptin and resistin) were also analyzed from fasting plasma samples by ELISA (Bender MedSystems GmbH, Vienna, Austria and BioVendor, Laboratoní

Medicína, Modrice, Czech Republic; respectively). Out of range values were excluded from statistical analysis.

- *Study intervention*

The participants underwent a four week soft drink (Lucozade Energy, GlaxoSmithKline plc, UK) supplementation on top of their habitual diet. Soft drink supplementation was carried out on the basis of a carbohydrate intake ≈ 2.0 g/kg body weight per day. Post-test analysis determined a carbohydrate intake of 2.1 ± 0.2 g/kg body weight per day. This corresponded, on average, to 760 mL energy drink per person per day. Experimenters provided the participants with the energy drinks (on average 2 times a week). Participants were told to keep all the other aspects of their habitual diet constant throughout the duration of the intervention. Participants' urine was collected throughout the intervention period without prior notice on random days. The participants were told that their urine samples were tested for energy drink specific markers in order to check the compliance of the participants to the supplementation. This deception was implemented to improve adherence to the protocol. Participants were debriefed at the end of the study. In addition, empty drink bottles were collected.

The experimental protocols of these two studies were approved by the School of Sport, Health and Exercise Sciences Research Ethics Committee (Bangor University) in accordance with the Declaration of Helsinki. Participants were asked to sign the informed consent prior to taking part in the study. Taste tests, explicit and implicit tests were performed by the same investigator in all cases – investigator and participants were blind to the hypotheses of the studies.

Statistical Analysis

The statistical analysis was performed using SPSS 11.5 standard version. Unless otherwise specified, all data are reported as means ± standard deviations. For study 1 the differences between the L group

and Ov/Ob group were analysed via two-way between-between ANOVA, where group and gender were the between participants factors. In case of the taste tests, three way repeated measures ANOVA were used with concentration as the within participants factor. The outcomes of study 2 were analysed with Student's paired-samples t-tests. Taste curves were analysed by three-way repeated measures ANOVAs (time and concentration as within participants factors and gender as between participants factor). Preference test and IAT scores (for split groups) in study 2 were analysed via two-way repeated measures ANOVAs (time as within participants factor and subgroups as between participants factor). A within-participants correlation coefficient was computed for the correlations between IAT and preference scores, leptin levels and sugar intakes and leptin levels and preference scores using the method described by Bland and Altman (1995). This method adjusts for repeated observations within participants (baseline and post intervention) by using multiple regression with 'subject' treated as a categorical factor using dummy variables. Appropriate post-hoc test with Bonferroni correction were used. The significance level was set at 0.05 (two-tailed). The significance level for trends was set at 0.10.

Results

Study 1

- *Taste tests in Overweight-Obese and lean participants*

The overweight-obese participants tasted the sweet sucrose solutions as being 23% less intense than the lean participants. In the sucrose taste test there was a significant increase in intensity scores with increasing concentrations, as expected (main effect of concentration, $F(3,94) = 95.3$, $P<0.001$, partial $\eta^2 = 0.767$). The Ov/Ob group showed significantly lower sucrose intensity scores across the range of concentrations compared to the L group (main effect of group, $F(1,29) = 7.01$, $P<0.050$, partial $\eta^2 = 0.195$) (**Fig. 2.1A**). We found a tendency toward a significant interaction between group and gender in the sucrose intensity scores (group × gender interaction, $F(1,29) = 3.35$, $P<0.100$, partial $\eta^2 = 0.104$). No 3-way interaction or other 2-way interactions were found for sucrose taste intensity.

Pleasantness scores were not affected by sucrose concentration; no significant main effect of concentration was found in the sucrose pleasantness test ($F(2,71) = 1.55$, $P=0.214$, partial $\eta^2 = 0.051$). No differences were seen between the Ov/Ob group and the L group (main effect of group, $F(1,29) = 1.92$, $P=0.176$, partial $\eta^2 = 0.062$), however, males rated the sucrose solutions 5-fold more pleasant than females (main effect of gender $F(1,29) = 5.65$, $P<0.050$, partial $\eta^2 = 0.163$) (**Fig. 2.1B**). A trend for a group × gender interaction was also found in the sucrose pleasantness scores ($F(1,29) = 15.7$, $P<0.100$, partial $\eta^2 = 0.115$). No 3-way interaction or other 2-way interactions were found for sucrose taste pleasantness.

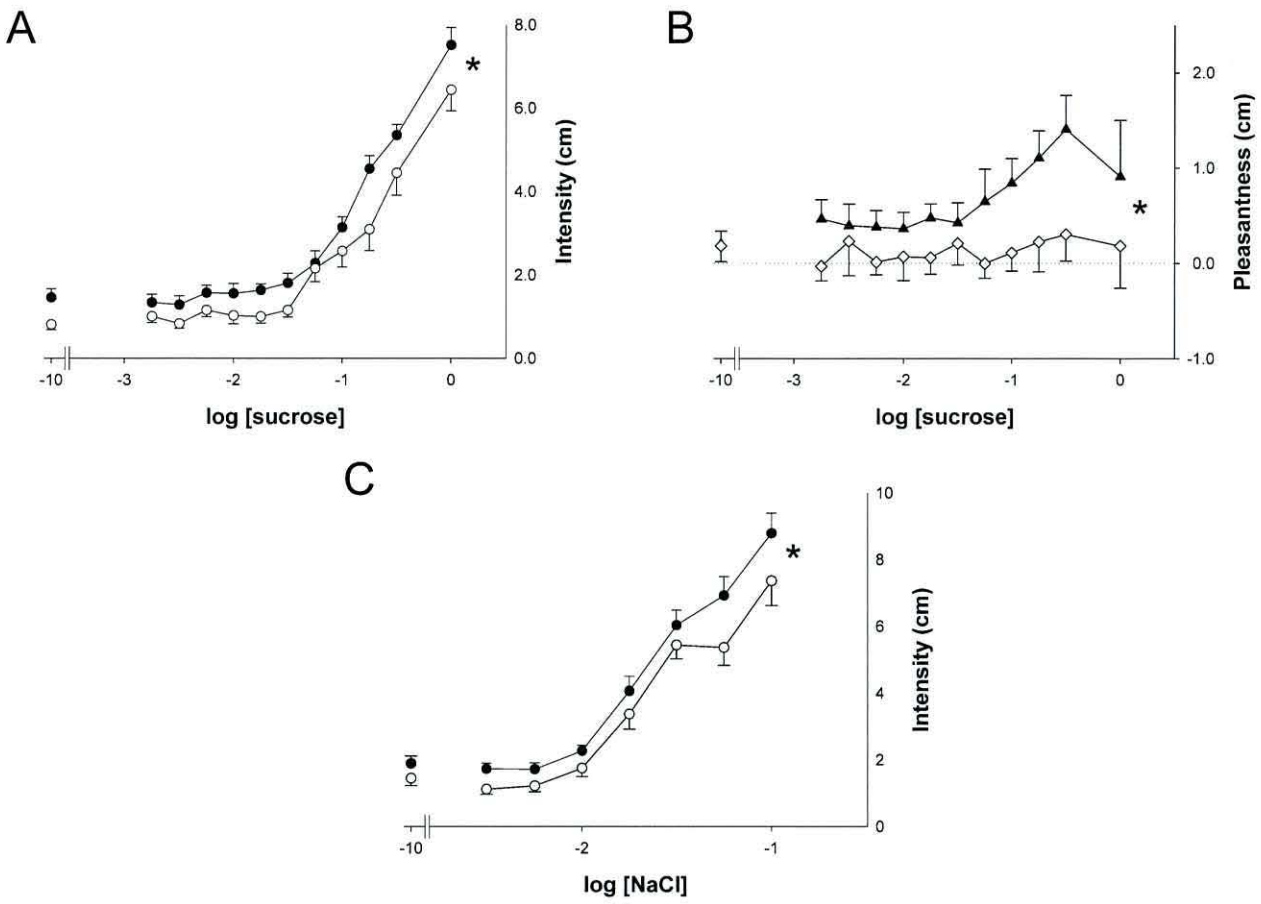


Figure 2.1 A) Sucrose intensity scores for lean (closed circles, n = 22) and overweight/obese (open circles, n = 11) young adults (study 1). * $P<0.05$ main effect of group. B) Sucrose pleasantness score differences between males (closed triangles, n = 14) and females (open diamonds, n =19). * $P<0.05$ main effect of gender. C) Salt intensity score differences between lean (closed circles) and overweight/obese (open circles) young adults. * $P<0.050$ main effect of group. Data points are expressed as mean and SEM. Intensity scale equivalents (A,C): barely detectable = 0 cm; weak = 1.1 cm; moderate = 2.7 cm; strong = 5.5 cm; very strong = 8 cm; strongest imaginable sensation of any kind = 15 cm. Pleasantness scale equivalents (B): neutral = 0 cm (dotted line); slightly pleasant 0.5 cm; moderately pleasant = 1.9 cm; pleasant 3 cm; very pleasant = 4.5 cm; most pleasant imaginable = 8.6 cm; slightly unpleasant = -0.5 cm; moderately unpleasant = -1.9 cm; unpleasant = -3; very unpleasant -4.5 cm; most unpleasant imaginable = -8.6.

Salt taste test revealed a significant increase in intensity scores with increasing concentrations (main effect of concentration $F(2,84) = 77.6$, $P<0.001$, partial $\eta^2 = 0.728$) and the Ov/Ob group rated all sodium chloride solutions as less intense (-19%) than the L group (main effect of group, $F(1,29 = 4.12$, $P<0.050$, partial $\eta^2 = 0.124$), (**Fig. 2.1C**). Salt pleasantness tests showed only a significant main effect of concentration ($F(2,81) = 39.4$, $P<0.001$, partial $\eta^2 = 0.576$). Higher concentrations were rated significantly less pleasant than lower concentrations. No other effects were found for salt taste intensity and pleasantness.

- *Implicit attitude changes in Overweight-Obese participants*

The overweight-obese participants had a 2.1-fold stronger automatic attraction to sweet than the lean counterparts. The computer based IAT indicated that the Ov/Ob group had significantly higher scores than the L group ($F(1,29) = 6.48$, $P<0.050$, partial $\eta^2 = 0.183$) (**Table 2.1**), indicating a stronger implicit attitude to sweet in overweight/obese participants. A significant Pearson correlation between IAT scores and BMI was observed ($n = 33$, $r = 0.36$, $P<0.050$). No main effect of gender or group × gender interaction was found.

Study 2

- *Eating behavior and energy intake*

Energy and macronutrient intakes gathered from subject's diet diaries are reported in **Table 2.2**. During the soft drink intervention participants changed their diet composition by increasing the CHO by 12% and decreasing fat and protein by 10% and 2% respectively. The amount of alcohol consumed before and during the intervention showed a similar reduction, it was on average 22 kcal/d less during the intervention.

Table 2.1 Subject's characteristics (study 1) and BMI and IAT differences between lean and Overweight/Obese subjects.

	L (22)	L ♂ (7)	L ♀ (15)	Ov/Ob (11)	Ov/Ob ♂ (7)	Ov/Ob ♀ (4)
Age (yrs)	23.1 ± 2.9	23.1 ± 2.8	23.1 ± 3.0	22.2 ± 1.6	22.8 ± 1.2	21.0 ± 1.6
Height (m)	1.70 ± 0.08	1.79 ± 0.06[‡]	1.66 ± 0.06[‡]	1.72 ± 0.08	1.76 ± 0.06[‡]	1.61 ± 0.01[‡]
Weight (kg)	64.3 ± 8.6[†]	70.7 ± 7.8	61.3 ± 7.4	89.0 ± 8.7[†]	88.9 ± 11.1	89.3 ± 8.7
BMI (kg/m^2)	22.2 ± 2.0[†§]	21.9 ± 1.9[‡§]	22.3 ± 2.1[‡§]	30. 2± 3.6[†§]	28.6 ± 3.5[‡§]	33.1 ± 0.5[‡§]
IAT scores	0.40 ± 0.57*	0.24 ± 0.58	0.48 ± 0.57	0.85 ± 0.30*	0.82 ± 0.35	0.90 ± 0.23

BMI = body mass index, IAT = implicit association test, Ov/Ob = overweight/obese group, L = lean group* Significant main effect of Group (P<0.050). † Significant main effect of Group (P<0.001). ‡ Significant main effect of Gender (P<0.050). § Significant Group × Gender interaction (P<0.050).

Table 2.2 Average energy and macronutrient intake (means ± standard deviations) at baseline and during the 4 weeks of soft drink supplementation (study 2)

	Baseline	During the intervention	t-value (df = 11)	P level	partial η^2
Energy intake (kcal/d)	2383 ± 624	2413 ± 542	-0.169	0.869	0.003
Protein (g/d)	81.5 ± 21.2	70.6 ± 22.1	2.891	0.015*	0.432
CHO (g/d)	265 ± 69	339 ± 68	-3.276	0.007**	0.494
Sugar (g/d)	95.0 ± 40.4	179.5 ± 34.1	-9.698	<0.001***	0.895
MUFA (g/d)	40.5 ± 15.0	32.7 ± 16.9	1.980	0.073[#]	0.263
PUFA (g/d)	12.4 ± 7.5	8.1 ± 5.0	2.742	0.019*	0.406
SFA (g/d)	41.6 ± 18.1	30.8 ± 16.4	2.251	0.046*	0.315
Dietary fiber (g/d)	19.5 ± 5.6	17.8 ± 6.0	1.578	0.143	0.184

CHO = carbohydrate, MUFA = monounsaturated fat, PUFA = polyunsaturated fat, SFA = saturated fat [#] $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n = 12$

The supplementation was intended to increase the daily energy intake by about 600 kcal/day. Our diet diary data showed a non-significant increase in energy intake of only about 30 kcal a day. Estimation of energy balance established by body composition changes (see study 2, **Chapter 3**) indicates that an increase of circa 1 kg of fat mass with no changes in lean mass in one month corresponds to about 345 kcal energy surplus a day.

- *Perception of sucrose and salt intensity and pleasantness*

The soft drink intervention altered both intensity and pleasantness taste perceptions of sweet (**Fig. 2.2A**). Temporal and long-standing anxiety as measured by state and trait Spielberger inventory were within the normal range prior to the intervention, and did not significantly change with the soft drink supplementation (State: 30.4 ± 9.8 vs. 30.9 ± 6.6, $t(11) = -0.22$, $P=0.831$; Trait: 32.3 ± 7.3 vs. 35.6 ± 11.6, $t(11) = -1.44$, $P=0.177$). It is unlikely therefore that anxiety level contributed to any change in taste perception. Analysis of variance of the sucrose intensity tests showed a significant time × sucrose concentration interaction ($F(11,110) = 3.11$, $P<0.001$, partial $\eta^2 = 0.237$), meaning that the intervention altered the perception of taste intensity for sucrose. Sucrose intensity scores significantly increased with increasing concentration (main effect of concentration, $F(3,31) = 83.8$, $P<0.001$ partial, $\eta^2 = 0.893$). No significant main effect of time was found ($F(1,10) = 0.07$, $P=0.799$, partial $\eta^2 = 0.007$). Post-hoc Bonferroni tests for simple main effects of time (sucrose intensity before vs. sucrose intensity after intervention) revealed a significant difference only for -2 10mM (-2log[M]) and 178mM (0.75log[M]) concentrations ($t(11) = 3.77$, $P<0.010$, and $t(11) = 2.97$, $P<0.050$).

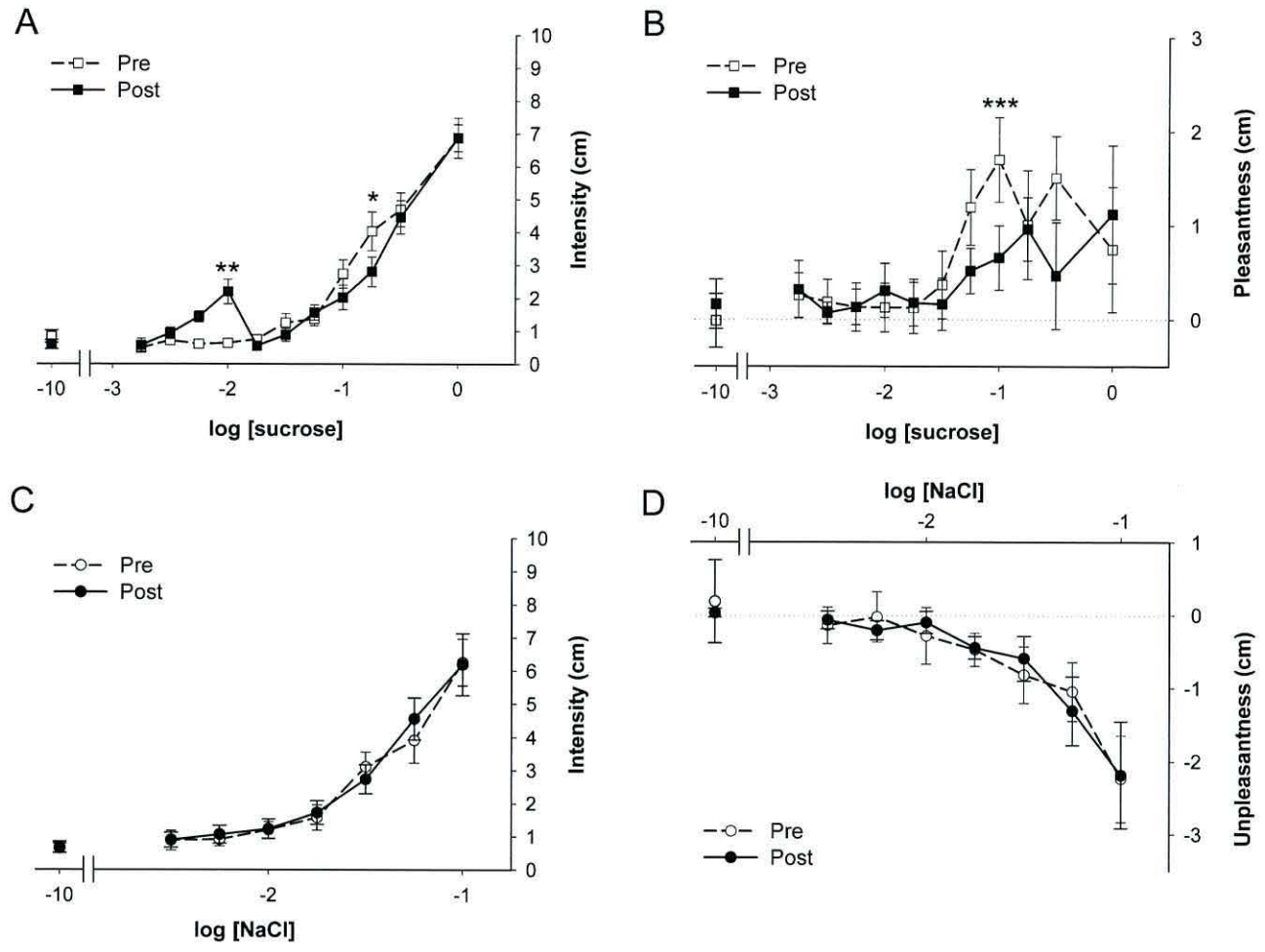


Figure 2.2 Changes in taste perception induced by 4 weeks of soft drink consumption (study 2). Baseline and post intervention sucrose scores are represented as squares (A, B) and sodium chloride scores as circles (C, D). Pre intervention scores are represented with open symbols and post intervention scores with closed symbols. Data points are expressed as mean and SEM. * $P<0.050$, ** $P<0.010$, *** $P<0.001$. For intensity and pleasantness equivalents see legend of **figure 2.1**. $n = 12$.

A significant time × sucrose concentration interaction ($F(11,110) = 1.91$, $P<0.050$, partial $\eta^2 = 0.148$) was found for pleasantness. Follow-up Bonferroni corrected t-tests showed a significant decrease in pleasantness with soft drink intervention only for 100mM (-1log[M] ($t(11) = 5.58$, $P<0.001$) (**Fig. 2.2B**). Whereas there was no significant main effect of sucrose pleasantness concentration ($F(2,23) = 2.42$, $P=0.105$, partial $\eta^2 = 0.195$). No significant main effect of time was

found for sucrose pleasantness ($F(1,10) = 0.35$, $P=0.567$, partial $\eta^2 = 0.034$). Gender did not contribute to differences in sucrose taste perception; no gender interactions or main effects were detected for sucrose intensity or sucrose pleasantness.

No effect of the soft drink supplementation was detected in the salt taste tests. There was no significant time × concentration interaction for salt intensity and pleasantness scores ($F(218,19) = 0.34$, $P=0.731$, partial $\eta^2 = 0.037$; $F(3,38) = 0.37$, $P=0.823$, partial $\eta^2 = 0.035$, respectively). Main effects of salt concentration were found for salt intensity and pleasantness ($F(2,22) = 43.1$, $P<0.001$, partial $\eta^2 = 0.827$; $F(1,18) = 7.05$, $P<0.010$, partial $\eta^2 = 0.414$, respectively). There were no main effects of time for salt intensity and pleasantness scores ($F(1,9) = 0.35$, $P=0.571$, partial $\eta^2 = 0.037$; $F(1,9) = 0.01$, $P=0.910$, partial $\eta^2 = 0.001$, respectively) (**Fig. 2.2 C/D**). No gender interactions or main effects of gender were found for salt intensity and pleasantness measures.

- *Changes in preference for sweetness*

The soft drink intervention increased sweet liking in participants who did not prefer sweet at baseline. Considering the group as a whole (n = 12), the preference for sweeter or less sweet solutions did not change; no significant difference was found between baseline and post-intervention preference scores (50.8 ± 37.5 vs. 57.5 ± 30.5, $t(11) = -0.85$, $P=0.421$, partial $\eta^2 = 0.062$). However, when the participants were split into two groups based on a median split of the baseline preference scores (sucrose-likers, n = 6 and sucrose-dislikers, n = 6), a significant effect of the supplementation was found (time × group interaction, $F(1,10) = 7.04$, $P<0.050$, partial $\eta^2 = 0.413$). There was also a significant main effect of group ($F(1,10) = 18.3$, $P<0.010$, partial $\eta^2 = 0.646$) and no main effect of time ($F(1,10) = 1.13$, $P=0.313$, partial $\eta^2 = 0.101$). Follow-up tests revealed a significantly increased sucrose preference in the initial sucrose-dislikers group ($t(5) = -2.83$,

$P<0.050$, partial $\eta^2 = 0.628$), but no difference in the initial sucrose-likers group ($t(5) = 1.04$, $P=0.348$, partial $\eta^2 = 0.176$) (see **Fig 2.3A**), after the intervention. There was also a trend toward a significant correlation between the baseline sugar intakes of the twelve participants and their baseline preference scores ($n = 12$, $r = 0.54$, $P=0.071$).

- *Implicit attitude toward sweet food*

Automatic attraction to sweet was not changed by the soft drink intervention. IAT scores did not show any alteration with the soft drink intervention (0.95 ± 0.50 vs. 0.97 ± 0.41, $t(11) = -0.11$, $P=0.917$). No statistical difference was observed even when IAT scores were analyzed for the sucrose-likers and sucrose-dislikers subgroups (IAT time × group interaction: $F(1,10) = 0.30$, $P=0.598$, partial $\eta^2 = 0.029$; IAT main effect of time: $F(1,10) = 0.01$, $P=0.920$, partial $\eta^2 = 0.001$ and IAT main effect of group: $F(1,10) = 0.09$, $P=0.775$, partial $\eta^2 = 0.009$). There was no within-subject

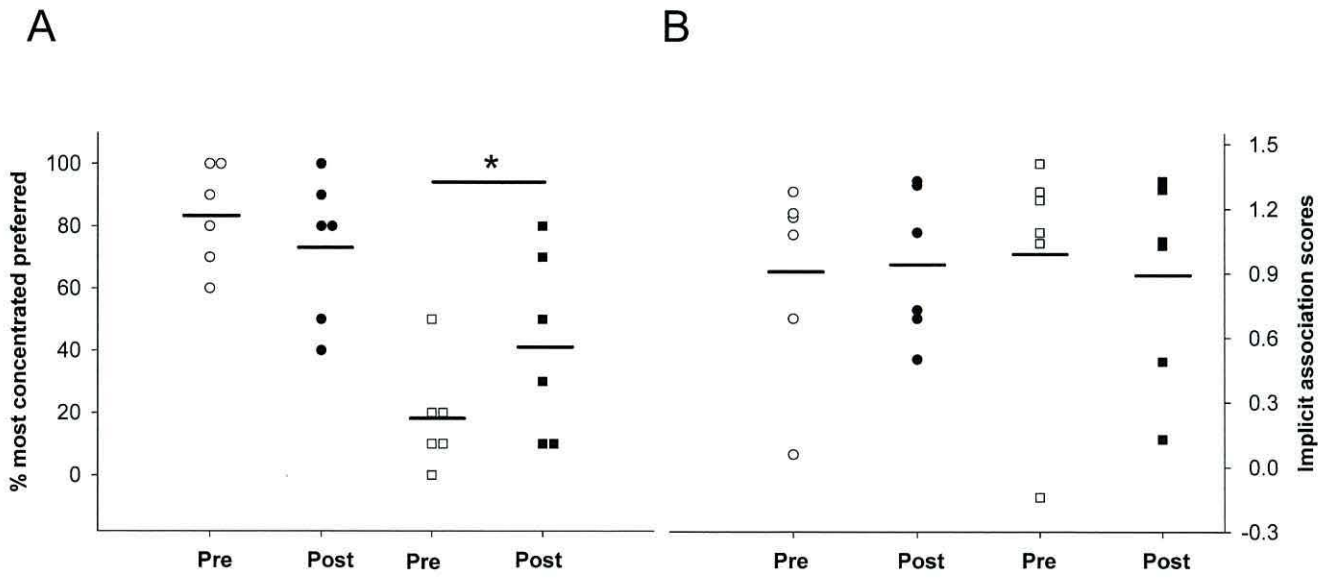


Figure 2.3 Influence of soft drink supplementation on explicit preference (A) and implicit attitude (B) toward sweet. Pre-post sucrose-likers' scores are shown with circles and pre/post sucrose-dislikers' scores with squares. Pre intervention scores are represented with open symbols and post intervention scores with closed symbols. Data points are expressed as mean and SEM.* $P<0.050$. $n = 12$.

correlation between IAT scores and preference scores (n = 12, r = 0.18, t = 0.62, *P*=0.547) (**Fig. 2.3B**).

- *Changes in appetite, adipokines and cytokines*

Out of twelve participants, six reported a smaller overall appetite during intervention compared with before; five reported the same overall appetite and only one a larger appetite. There was a 21% significant increase in post-intervention fasting plasma leptin (6.33 ± 6.23 ng/mL vs. 7.66 ± 6.98 ng/mL; t(8) = -2.23, *P*<0.050, partial η^2 = 0.383). There was a trend toward a significant within-subject correlation between leptin levels and sugar intakes (n = 9, r = 0.60, t = 2.123, *P*=0.067) and between leptin levels and preference taste scores (n = 9, r = 0.57, t = 1.935, P=0.089). Fasting circulating plasma resistin was significantly lower at the post test compared with baseline (4.61 ± 1.87 vs. 3.85 ± 1.67; t(10) = 2.41, *P*<0.050, partial η^2 = 0.368). Fasting plasma cytokines (TNF-α, IL-6 and IL-10) did not significantly change with the intervention (TNF-α: t(9) = 1.50, *P*=0.169; IL-6: t(9) = -0.29, *P*=0.776; IL-10: t(9) = 1.44, *P*=0.184).

Discussion

The purpose of the current investigation was to elucidate the relationship between sweet taste perception and obesity, and to study the implicit attitude of overweight/obese individuals toward sweet food/drinks. Moreover, we aimed to test whether an environmental factor, such as soft drink consumption, could alter sweet taste perception (intensity and pleasantness) and preference, and explicit preferences and implicit attitudes for sweet in healthy, lightly active, lean participants. A further aim was to determine whether leptin levels and sugar consumption, or cytokine levels and changes in macronutrients intake are correlated. We found that there are differences in sweet and salty taste perceptions and implicit attitude toward sweet food/drinks between young overweight/obese individuals and lean controls of the same age, and that sweet taste perception can be altered by one month of soft drink supplementation in young, lean healthy participants. Considering that soft drinks can be associated with various adverse health outcomes (See study 2, **Chapter 3**), soft drink-induced taste alterations and the consequent food preference/selection changes may contribute to the etiology of obesity and metabolic diseases.

Study 1

Taste intensity

We measured sweet (sucrose) and salty (sodium chloride) taste intensity and pleasantness using a gLMS in a group of young overweight/obese individuals and lean controls. Sucrose as well as sodium chloride taste intensity scores were lower in the Ov/Ob group compared with the L group. This indicates that young overweight/obese individuals perceive sweet and salty as being less intense compared to lean persons. The difference in sweet intensity scores is in line with the findings of Bartoshuk *et al.* (2006). The fact that the Ov/Ob group

perceived the salty solutions as less salty than the L group possibly reflects higher dietary levels of salt in the Ov/Ob group diet. In fact, previous studies have shown that salt intensity ratings are dependent upon the level of salt consumed habitually in that higher salt consumption leads to lower intensity scores (Bertino *et al.*, 1982). A similar explanation might also be true for the lower sucrose intensity scores in the Ov/Ob group. Indeed individuals who have a lower sensitivity for sweetness have higher intake of added sugar (Duffy *et al.*, 2003).

Taste pleasantness

We did not find higher pleasantness scores for sweet taste in the Ov/Ob group. This is in accordance with a previous report (Frijters & Rasmussen-Conrad, 1982). However, Bartoshuk *et al.* (2006) found that sweet liking increases with body weight. This discrepancy might be due to differences between our methods. We found that men rate sweet as more pleasant than women. This gender difference is in complete agreement with a previous investigation on pleasantness of sweet taste and alliesthesia (Laeng *et al.*, 1993).

Sodium chloride-salty pleasantness scores, in the present study, did not differ between the Ov/Ob group and the L group. However, a recent study with a greater sample size revealed that normal weight men and overweight women have a higher liking for salty taste than overweight men and normal weight and obese women (Donaldson *et al.*, 2009). The fact that we did not observe any difference associated with BMI might be related to the typology of the test. A previous study analyzing the effect of a low-sodium diet on salt pleasantness showed no effects on hedonic ratings when participants had to rate the pleasantness of salty solutions, but they did report an effect when participants rated the pleasantness of salty soups or crackers (Bertino *et al.*, 1982).

Implicit attitude

We also tried to obtain some insights in the automatic responses related to sweet food and drinks. We demonstrated for the first time that young overweight/obese individuals have a stronger implicit attitude toward sweet food than their lean counterparts. This is in agreement with previous studies on implicit attitude toward food in general. Previous studies have indicated that obese children manifest a more positive implicit attitude toward food, regardless if it is healthy or unhealthy, than non-obese children (Craeynest *et al.*, 2005). Moreover, there is a strong implicit association between unhealthy food and good taste and enjoyment (Raghunathan *et al.*, 2006). Although interestingly, Roefs and Jansen (2002) found that both obese and non-obese people have a negative implicit and explicit attitude toward high-fat foods. Additionally, healthy people had stronger implicit attitude toward food when hungry (Seibt *et al.*, 2007). Taken together, our results and previous findings might suggest that obese people have a larger appetite (central leptin resistance) and therefore a more positive attitude toward food in general (healthy and unhealthy). They might also be more susceptible to the positive reward that good tasting food (e.g., sweet food) gives them than non-obese individuals.

Study 2

In study 2 healthy, lean participants underwent one month of soft drink supplementation. Adverse health outcomes associated with the supplementation were found. Fasting plasma glucose and insulin were increased, fat mass was augmented and the increase in resting respiratory exchange ratio indicates a worsened metabolism (i.e., lower basal fat oxidation) (see study 2, **Chapter 3**).

Taste intensity and pleasantness

We measured sweet and salty taste perception before and after the soft drink supplementation. We hypothesized that dietary levels of sugar

would influence only sweet taste, thus we measured salty taste as a control. We did observe no effect of the intervention in intensity and pleasantness of salty taste.

Importantly, our study showed that the glucose syrup based supplementation did alter sweet taste; sensitivity to sweet was increased for weak concentrations and decreased for stronger concentrations (**Fig. 2.2A**). Interestingly, the reduction in sucrose intensity scores was in a similar range to the intensity scores measured for the soft drink used in this study. The sucrose-pleasantness test showed a reduced liking for the same sweet concentrations after the soft drink intervention. This might be explained by a shift of the post-test pleasantness curve to the right (**Fig. 2.2B**), meaning that liking for sweet was shifted toward higher sucrose concentrations. Unfortunately, this could not be verified in our study since the concentration range did not extend above 1 molar sucrose. The rather rapid alteration in sweet taste found in our intervention study is remarkable. However, taste plasticity has been demonstrated in response to other stimuli, such as a short period of exercise (Cartwright *et al.*, 2010), or at a peripheral level by increases in available serotonin and noradrenalin (Heath *et al.*, 2006).

Preference for sweet

The important implication of the taste change is that environment-induced alteration (i.e. continuous soft drink exposure) could determine an explicit preference change (i.e. greater liking for sweet) and consequently a dietary behavior change (i.e. higher sugar intake). We tried to address this issue by testing sweet preference. We noticed that a subgroup of participants who did not like sweet at baseline (sucrose-dislikers) increased their preference for sweet after the intervention (**Fig. 2.3A**). However, the division into two groups, high and low initial preference, cannot exclude that such a result might be a regression to the mean among the sucrose-dislikers. On the other hand,

if this result is replicated it will have important implications, supporting the hypothesis that regular soft drink use can change explicit sweet preference within a short period of time, at least among those who do not initially like sweet tastes.

Interestingly, we confirmed a strong trend toward a significant correlation between the baseline sucrose preference scores and the baseline dietary sugar consumption as shown previously by Mattes and Mela (1986). All the other nutrients, carbohydrates included, did not correlate at all with the preference scores. An important implication of this is that if soft drink consumption enhances explicit preference for sweet, sugar use could increase as an effect of chronic soft drink consumption. This vicious circle brings us back to the fact that taste is the main determinant in food choice, and that its alteration has a direct effect on eating behavior.

Adipokines and cytokines

Fasting plasma leptin was increased after the soft drink intervention. The increase in fasting circulating leptin could be explained by the 1 kg increase in fat mass (Speakman *et al.*, 2002). Although, the food reports kept by the subjects before and throughout the intervention in this study showed no significant energy intake increase (**Table 2.2**), the increase in fat mass is likely to be due to the energy excess caused by the soft drink supplementation. This discrepancy between body composition changes and diet diary data may be due to inaccuracy in keeping the diet diary and/or probably to reduced energy excretion, which was not measured in this study.

Leptin and insulin are known to function within the central nervous system as satiety signals diminishing food intake when energy levels are met and adipose tissue is restored (Davis *et al.*, 2009b). Recently, a study on mice showed a specific sweet taste inhibition linked to leptin (Kawai *et al.*, 2000). We have found two strong trends toward a

significant positive correlation between leptin levels and dietary sugar intakes and leptin levels and sweet preference scores. Although our outcomes do not provide direct evidence that leptin levels influence sweet preference by changing sweet taste, if this phenomenon took place, leptin would reduce sweet taste sensitivity and reduce sweet-taste-induced reward.

Perhaps individuals with higher leptin levels perceived low sweet taste reward consequently they have to increase sugar intake to experience the same reward as persons with lower leptin levels and a high sweet taste reward. Fasting resistin was reduced by the soft drink intervention, which is likely to be due to the decrease in dietary fat intake observed during the intervention. Despite its name, the relationship between resistin and insulin resistance is not clear (Filkova *et al.*, 2009). It is now thought that resistin might be involved in inflammatory, endocrine, and tumor diseases (Filkova *et al.*, 2009).

We measured circulating proinflammatory cytokines, as we hypothesised that any increase in adiposity, or systemic inflammation, might drive a change in macronutrient intake. Although there was both an increase in fat mass, and a change in macronutrient intake (**Table 2.2**), there were no changes in circulating cytokines, thus refuting this hypothesis.

Implicit attitude

Soft drink supplementation did not affect IAT scores, suggesting that implicit preference to sweet food and drinks in lean healthy participants may not be easily changeable. This does not exclude that a longer period of chronic soft drink consumption might alter central appetite regulation (e.g. leptin resistance) in lean healthy participants and implicit attitudes toward sweet food or food in general.

Implications and conclusions

Brownell *et al.* (2009) suggested that behavioral and biologic mechanisms might explain the link between soft drink consumption and adverse health outcomes. Moreover, they hypothesized that high intake of soft drinks may have chronic adverse effects on taste preferences and food acceptance (Brownell *et al.*, 2009). Our findings give credit to their hypotheses. It is clear that exogenous high-glucose availability, when it is not accompanied by an adequate energy expenditure (e.g. physical exercise), has deleterious effects on people's health. The general positive reward, or in other words, the good taste of sweet food and drinks, combined with their low cost, undoubtedly explains their increasing popularity. Furthermore, it seems that obese people have a stronger implicit attraction to sweet, which might be a result of taste differences, originating from both genetic and environmental factors. The stronger automatic drive of obese individuals to the consumption of sweet food/drinks might also be associated with central leptin resistance. We demonstrated that chronic soft drink consumption changes taste and food preference. Therefore soft drink consumption could be one of the environmental factors leading to obesity.

In conclusion, young, overweight-obese adults showed lower sweet and salty intensity taste scores compared to lean controls. Young men had a higher liking for sweet than women of the same age. Moreover, one month of commercially available soft drink consumption (approximatively 760 mL a day) induced adverse health outcomes (i.e., increased fat mass, and fasting glucose and insulin levles and reduced basal fat oxidation) and altered taste perception of sweet, but did not affect implicit attitude toward sweet in lightly active, lean participants. Finally, a subgroup of sucrose-dislikers showed an augmented preference for sweet after the soft drink supplementation.

CHAPTER III

INFLUENCE OF HIGH GLUCOSE AVAILABILITY ON MONDOA AND TXNIP EXPRESSION IN SKELETAL MUSCLE OF HEALTHY, LEAN INDIVIDUALS *IN VIVO* AND *IN VITRO*

Abstract

Hyperglycemia is a contributing factor for the metabolic alteration seen in insulin resistance and type 2 diabetes mellitus. Recently it was shown that glucose metabolites are sensed by the transcription factor MondoA mediating metabolic gene expression in response to elevated glucose availability in skeletal muscle cells. Moreover, MondoA regulates TXNIP expression which is shown to impair peripheral glucose uptake and is thought to be a key regulator of signals related to radical oxygen species connected to glucose toxicity. Here, we investigated the expression of MondoA and TXNIP in response to chronic high glucose availability in human skeletal muscle cells *in vitro* and to periodic high glucose availability in human skeletal muscle *in vivo* as well as metabolic responses. Periodic high glucose availability was mediated by a 4 weeks soft-drink intervention in healthy lean participants without former history of chronic soft-drink consumption. Muscle cell cultures increasingly expressed MondoA and TXNIP in response to chronic high glucose availability, and elevated expression of glycolytic enzymes, as well as revealing a reduced insulin response. Periodic high glucose availability *in vivo* elevated MondoA expression and altered substrate oxidation towards increased carbohydrate and reduced fat oxidation in skeletal muscle with a reduced signalling response to insulin. Overnight fasting glucose and insulin resistance were increased without alteration in blood lipids. This shows for the first time that periodic and chronic high glucose availability influences glucose dependent signals of MondoA and TXNIP and leads to the metabolic alterations seen in skeletal muscle of insulin resistant and type 2 diabetics.

Introduction

Obesity caused by overnutrition and an inactive lifestyle are the major risk factors for type 2 diabetes mellitus (T2DM) (Chopra *et al.*, 2002). T2DM is characterized by defects in insulin secretion, elevated hepatic glucose output, and impaired glucose uptake in target tissue, especially skeletal muscle (Kahn, 1994). Moreover, the metabolic phenotype of skeletal muscle in T2DM is associated with an impaired capacity to increase fat oxidation upon increased fatty acid availability, and to switch between fat and glucose as the primary fuel with a strong preference for glucose (Corpeleijn *et al.*, 2009). Impaired glucose uptake in skeletal muscle seems to be an early feature in the development of insulin resistance and T2DM (Eriksson *et al.*, 1989). The impact of elevated blood glucose and lipids seem to be substantial for the progression of the disease while the mechanisms are still not fully understood.

Although the role of lipid metabolites like ceramide for the impairment of cellular insulin response is well established (Ellis *et al.*, 2000), there is some debate about their contribution (Muoio, 2010). However, knowledge about the mechanisms of the contribution of glucose and associated metabolites for the development of T2DM is still sparse. Recently, it was shown that high glucose availability, in the form of hyperglycaemia, can influence signalling pathways regulating peripheral glucose uptake and metabolism (Parikh *et al.*, 2007; Stoltzman *et al.*, 2008). These signalling pathways seem to include mechanisms for the detection of glucose and related metabolites for their signal transduction. Glucose sensing in skeletal muscle cells, as part of the mechanisms for the maintenance of cellular energy homeostasis, was demonstrated to be strongly dependent on the transcription factor MondoA (Stoltzman *et al.*, 2008) which may also be involved in maladaptative processes to high glucose availability converging into T2DM. MondoA seems to be a master-regulator of glycolytic genes and is presumably responsible for a wider range of

metabolic gene regulation (Sans *et al.*, 2006). MondoA, building a heterodimeric pair with the transcription factor Mlx, is localized on the outer mitochondrial membrane and shuttles between mitochondria and nucleus in response to the elevation of extracellular glucose. The major intracellular metabolite which is 'sensed', mediating the nuclear shuttle of MondoA:Mlx, is glucose-6-phosphate. Glycolytic gene expression is highly upregulated in response to MondoA shuttle/activation under conditions of high glucose availability (Stoltzman *et al.*, 2008). A further important target of the MondoA:Mlx complex is the promoter of thioredoxin interacting protein (TXNIP). TXNIP transcription is strongly elevated by MondoA:Mlx activity (Stoltzman *et al.*, 2008). Moreover, TXNIP expression is reported to be enhanced by hyperglycemia in beta cells (Cha-Molstad *et al.*, 2009), L6 muscle cell line (Stoltzman *et al.*, 2008) and endothelial cells (Li *et al.*, 2009). Functionally, TXNIP, via binding to thioredoxin, causes inhibition of thioredoxin activity reducing cystine groups in proteins. Consequently, the TXNIP driven inhibition of thioredoxin leads to an enforcement of radical stress by reactive oxygen species (ROS) in cells (Nordberg & Arner, 2001). ROS concentration is elevated during hyperglycemia and is hypothesized to be a key factor in the mechanism of glucose toxicity (Green *et al.*, 2004) as well as in diabetic cardiovascular disease (Ceolotto *et al.*, 2007).

Interestingly, a lack of TXNIP protects against glucotoxicity related apoptosis in beta cells (Shalev, 2008), and it has recently been demonstrated that oxidative stress leads to TXNIP shuttling into the mitochondria causing mitochondrial apoptosis in INS-1 cells (Saxena *et al.*, 2010). Decisively, TXNIP is shown to regulate peripheral glucose metabolism in adipocytes and skeletal muscle cells. In view of a possible contribution of TXNIP to T2DM, overexpression of TXNIP reduces glucose uptake in several cell types including muscle, while its expression is negatively regulated by insulin.

Additionally, a consistent elevation of TXNIP mRNA transcripts in skeletal muscle samples from subjects in a prediabetic and diabetic state has recently been reported (Parikh *et al.*, 2007). TXNIP seems to link redox circuitry to glucose control and is thought to be an important diabetogenic mediator (Muoio, 2007). While chronically elevated glucose availability, like hyperglycemia, can enhance MondoA – TXNIP expression and action in cells, it is possible that even periods of high glucose availability, like chronic high soft-drink consumption in sedentary people, could lead to an enhanced expression of these signalling molecules, thereby interfering with glucose homeostasis. Chronic soft drink consumption is a common feature of Westernized diet and has been shown to be highly correlated with the development of obesity and T2DM (Bray *et al.*, 2004; Gross *et al.*, 2004; Dhingra *et al.*, 2007). Accordingly, periods of high glucose availability could increase the expression of MondoA and TXNIP and then contribute to the development of metabolic alterations known to be present in a prediabetic state as a metabolic preference for carbohydrate, increased glycolytic gene expression, reduced insulin response and aerobic capacity.

Consequently, we assumed that high glucose availability, whether chronic or periodical, can elevate MondoA and TXNIP expression as well as alter metabolic enzyme expression and insulin response in skeletal muscle. We therefore hypothesized that periods of high glucose availability induced by soft drink consumption for 4 weeks can cause elevation of the expression of MondoA – TXNIP and glycolytic enzymes in skeletal muscle, an elevation in fasting blood glucose, reduction of insulin sensitivity, and preference of carbohydrate in metabolism.

Additionally, primary muscle cell cultures based on participants muscle biopsies were exposed to chronic hyperglycemia to analyse MondoA and TXNIP response as well as metabolic enzyme expression and insulin signalling response in comparison to the *in vivo* approach.

Here we hypothesise that periods of high glucose availability, like soft drink consumption for 4 weeks, could influence MondoA expression in skeletal muscle *in vivo* similarly to what is achieved via exposure of muscle cells to hyperglycemia *in vitro* as well as concomitant alterations in metabolism and insulin response.

Methods

Ethical approval

The departmental research Ethics Committee approved this study design in agreement with the Helsinki Declaration. All participants involved in this study were given a participant information sheet and signed an informed consent form prior to testing.

In vitro study:

- *Muscle biopsies*

After local anaesthesia (1% lignocaine) four needle muscle biopsies were collected at a mid distance between the great trochanter and the femorotibial joint on the vastus lateralis (VL) of the left leg of healthy male volunteers also taking part in the *in vivo* study (see below) (age 37.5 ± 13.44 yrs, height 1.79 ± 0.06 m, weight 72 ± 5.66 kg, 44.5 ± 17.9% MHC IIa, 55.5 ± 17.9% MHC I). Biopsies were taken with a 14 gauge needle (14ga x 10cm, Tru•Core® II Biopsy Instrument, Angiotech Gainesville, FL, USA).

- *Primary cell cultures*

After muscle biopsies were taken, tissue was immediately transferred to RT Skeletal Muscle Growth Medium (SMGM) with 5% Foetal Calf Serum (FCS), 1% L-Glutamine with Penicillin/Streptomycin and supplements (PromoCell, Heidelberg, Germany) and washed in medium twice. Followed by disintegration with surgical blades with 0.05% trypsin (PAA, Pasching, Austria) in PBS for 30 minutes, trypsination was stopped by trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA) with a final concentration of 0.14 mg/ml. After centrifugation at 800 × g for 5 minutes at 10°C pellet was resuspended in accutase (PAA, Pasching, Austria) and additionally incubated for 20 min at 37 °C. After sedimentation, the cell suspension was centrifuged again at 800 × g for 5 minutes at 10°C and the cell pellet was resuspended in SMGM

with 5% FCS, 1% L-Glutamine with Penicillin/Streptomycin plus supplements (PromoCell, Heidelberg, Germany). Cell suspension was transferred into 75cm^2 TC-flasks (Greiner, Frickenhausen, Germany) and incubated at 37 °C, 5% CO_2 and 95% humidity until passage. Half medium change was performed every second day. Three passages were completed before initiation of differentiation (**Fig. 3.1**). Samples of human primary myotubes were tested for creatine kinase activity after initiation of differentiation in DMEM (PAA, Pasching, Austria), with 2% FCS for 4 days. For the cultivation of differentiated myocytes grown on microcarriers, 5 x 10^6 cells per 10 mL medium were seeded on microcarriers in suspension (0.015 g micorcarriers, CultiSpher-GL; Percell Biolytica, Astorp, Sweden) in 25 cm^2 flasks. Flasks were placed on a circular shaker (53 rpm) to guarantee adequate O_2 supply to the cells and to prevent the cells and microcarries from settling down.

After 12 days of differentiation in DMEM, with 2% FCS, myocytes were exposed to high-glucose; DMEM with 2% FCS, 15 mM D-glucose (Sigma-Aldrich, St. Louis, MO, USA) and 10 µg/mL of insulin (Actrapid, Novo Nordisk A/S, Bagsvaerd, Denmark). Control myocytes were cultivated in DMEM with 2% FCS, 5 mM D-glucose and 10 µg/mL insulin. Exposure time to high glucose or control conditions was 7 days until harvest. For the insulin dependent signalling response (serine-threonine kinase Akt), additional cultures were exposed to high-glucose (15 mM D-glucose) or 5 mM D-glucose (control) in DMEM with 2% FCS without addition of insulin. After 7 days myocytes were stimulated with 100 nM insulin and harvested at baseline, 10 and 30 min after the insulin stimulus. The localisation of total GSK in myotubes nuclei was observed in the four different conditions: high-glucose/high-insulin (15 mM D-glucose, 10 µg/mL insulin), normal-glucose/high-insulin (5 mM Dglucose, 10 µg/mL insulin), high-glucose/no-insulin, and normal-glucose/no-insulin.

Picture of satellite muscle cells were acquired with a light inverted microscope (Eclipse TS100, Nikon, Kawasaki, Japan) equipped with 20x/0.40 and 40x/0.55 Ph1 ADL objectives.

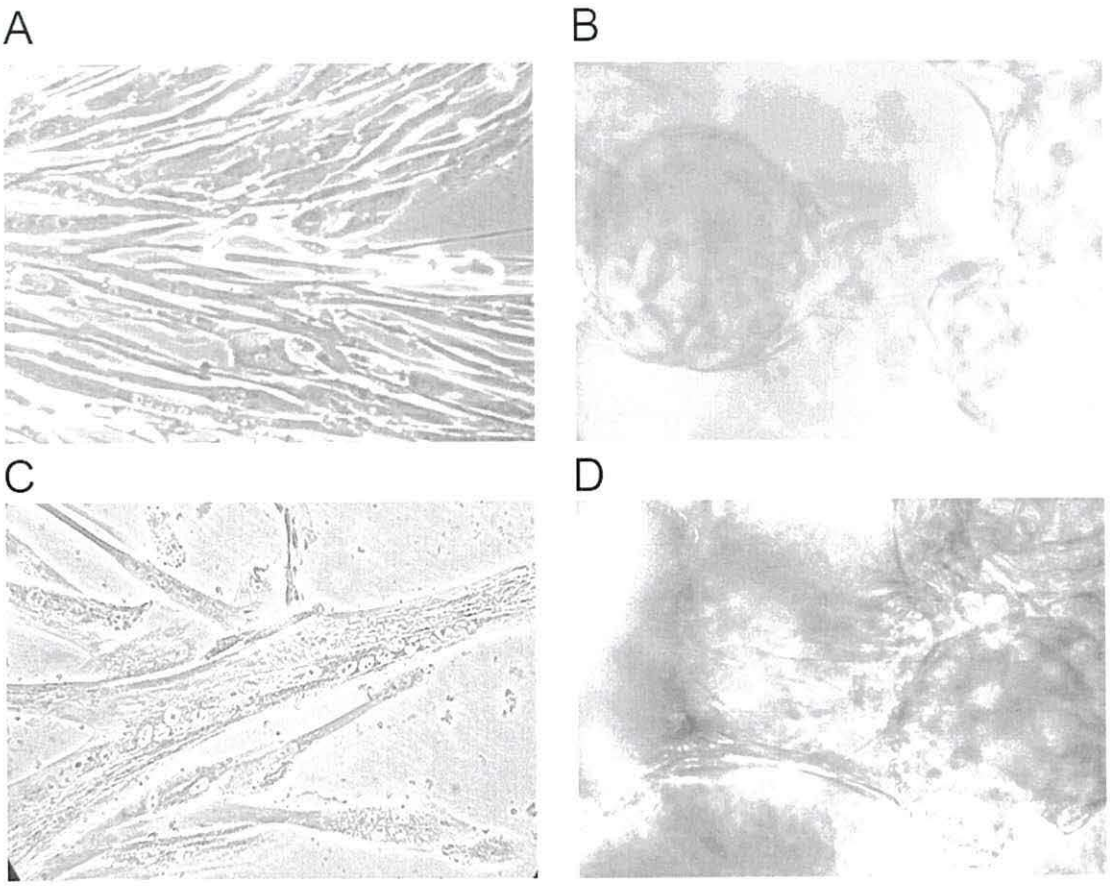


Figure 3.1 Myocytes grown on microcarriers for 14 days in culture, photographed at 20X magnification (B,D) and seeded in conventional culture flasks, 40X magnification (A,C).

- *Cell harvest and Western Blotting*

Myocytes on microcarriers were washed with PBS, lysed and denatured with SDS-PAGE sample buffer at 95°C for 3 minutes, followed by cooling on ice. Samples were cleared by centrifugation at 16,000 x g in Qia-Shredder columns (Qiagen, Hilden, Germany). Eluates were frozen in liquid nitrogen and kept at -80°C until use. After protein estimation by the modified Lowry method (SIGMA), equal amounts of total protein per lane were separated electrophoretically in 10% or 5% SDS-PAGE and then transferred to a nitrocellulose membrane (Hybond ECL

6x8 cm, GE Healthcare, Amersham, Slough, UK). Loading control was performed using India Ink (Pelikan AG, D3000, Hanover, Germany) staining of the nitrocellulose membranes after blotting and normalization based on alpha-actin signal levels. After blocking in PBS containing 0.2% Tween and 5% low fat dry milk, blots were probed with primary antibodies for 4 h at a dilution of 1:250, 1:2,000, and 1:10,000 for acetyl-CoA carboxylase (ACC) (H-76) (sc-30212), fumarate hydratase (FUM) (J-13) (sc-100743), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (0411) (sc-47724) respectively (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA).
However, glycogen synthase kinase -3β (GSK-3β) (1:3000), p-GSK-3α/β (Ser21/9) (1:3000), serine-threonine kinase Akt (AKT) (1:3000), p-AKT (Thr308) (1:1000) (Cell Signaling Technology Inc, Beverly, MA, USA), α-Mondo-A (1:500) (Sans *et al.*, 2006) and thioredoxin-interacting protein (TXNIP) (1:1000) (MBL, Naka-ku Nagoya, Japan), were incubated overnight. As secondary antibodies, goat anti-rabbit IgG-HRP 1: 15,000 (Sigma-Aldrich, St. Louis, MO, USA), or goat anti-mouse IgG-HRP 1:5,000 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) were used. ECL (Amersham Hyperfilm ECL, 18 × 24 cm, GE Healthcare Life Sciences, Little Chalfont, UK) detection was carried out using the SuperSignal West Pico or Femto ECL kit (Pierce, Rockford, IL, USA). If necessary, membranes were stripped for 45 min at 50°C in stripping buffer (10% SDS, 0.5 M Tris pH 6.8, and 0.08% β-mercaptoethanol), washed for 1 h and re-probed. Densitometry was then used to quantify protein bands (Gel Doc 2000 and software Quantity One-4.6.3, Bio-Rad, Hercules, CA, USA).

Nuclear and cytoplasmic fractions from myocytes grown on microcarriers for 12 days followed by exposure to high glucose (15mM) and control conditions (5mM) in DMEM with 2% FCS, with or without 10 μg/mL insulin for 7 days were prepared according manufacture's protocols using a Nuclear Extraction Kit (Kit No400010&No40410, Activemotif, Rixensart, Belgium).

In vivo study:

- *Participants and study design*

A pre- post-test within subject design was used to test our research hypothesis. An initial screening for lifestyle and soft drink consumption was executed via qualitative questionnaires (for details refer to **Chapter 2**). Healthy people with low physical activity and consuming less than one pint of soft drink per week were considered eligible to take part in this study.

Eleven participants (**Table 3.1**) took part in this study, after reading the study information and signing a written informed consent. Participants were informed that upon completion of testing they would receive £100 money as compensation for their time. Before and after the intervention period, participants attended our laboratories for two testing sessions. Although all the participants included in this study revealed a low physical activity, they were asked to refrain from exercise for 24 h prior to all tests. Post-tests were conducted 36 hours after the last soft-drink supplementation.

One subject was not able to attend the post intervention oral glucose test and the muscle biopsy because of a viral infection.

Table 3.1 Participants' characteristics, body composition, HOMA and lipid profile.

Parameters (units) (n)	Baseline	4 weeks Intervention	t(df)	*P* level	partial η^2
Age (yrs) (11)	26 ± 7				
Height (m) (11)	1.74 ± 0.09				
Weight (kg) (11)	65.9 ± 10	66.8 ± 11	-1.674 (10)	0.125	0.219
BMI (kg/m^2) (11)	21.6 ± 1.5	22.0 ± 1.8	-1.883 (10)	0.089	0.240
Bone Mineral Content (kg) (11)	2.60 ± 0.59	2.60 ± 0.60	0.000 (10)	1.000	0.000
Fat Mass (kg) (11)	15.2 ± 5.1	16.2 ± 4.7	-2.637 (10)	0.025*	0.410
Lean Mass (kg) (11)	48.1 ± 12.6	48.0 ± 13.0	0.130 (10)	0.904	0.002
%B (%) (10)	76.6 ± 28.4	81.3 ± 21.8	-0.588 (9)	0.571	0.037
%S (%) (10)	158 ± 47	126 ± 32	2.138 (9)	0.061#	0.337
IR (10)	0.68 ± 0.19	0.84 ± 0.20	-1.981 (9)	0.079#	0.304
Total Cholesterol (mmol/L) (10)	4.27 ± 0.89	4.42 ± 0.73	-1.070 (9)	0.312	0.113
Triglycerides (mmol/L) (10)	1.01 ± 0.36	1.02 ± 0.33	-0.162 (9)	0.875	0.003
HDL (mmol/L) (10)	1.30 ± 0.21	1.29 ± 0.22	0.182 (9)	0.860	0.004
LDL (mmol/L) (10)	2.52 ± 0.75	2.67 ± 0.64	-0.919 (9)	0.382	0.086

%B = steady state beta cell function; %S = insulin sensitivity; IR = insulin resistance * $P < 0.05$, # $P < 0.10$

- *Study intervention*

Participants underwent a four week soft-drink (Lucozade, GlaxoSmithKline plc, Harlow, UK) supplementation on top of their habitual diet. The soft drink bottles were provided by the experimenters. Supplementation was carried out on the basis of a daily carbohydrate intake of ≈ 2.0 g/kg body weight. Post-test analysis determined a carbohydrate intake of 2.1 ± 0.2 g/kg body weight per day. This corresponded on average to 760 mL soft-drink per subject a day. In order to increase compliance of the participants to the supplementation, the participants were told that their urine samples would have been tested for markers of soft-drink consumption. Participants' urine was collected throughout the intervention period without prior notice on random days. Moreover, empty soft drink bottles were collected.

- *Diet diaries*

Participants were introduced to the diet diaries via standard instruction (Gibson, 1993) and were warned about the importance of the accuracy and precision of their reports as crucial requirements of this study. Then, participants were asked to keep a seven day diet diary for a week before the intervention started. The accuracy and precision of their records were checked at the pre-test sessions and an adequate feedback about the standards required was given. Since a diet record necessitates time and high commitment, keeping a diet diary on a daily basis could induce superficial recording of participants' food and drinks consumed. Therefore, during the supplementation month participants were asked to keep a fourteen day diet diary. The fourteen days were randomly chosen over the intervention period of four weeks. The diet analysis was conducted using the USDA food search for windows, Version 1.0, database version SR21 in combination with food nutritional information labels.

- *Body composition*

Lean mass, fat mass, and bone mineral content were assessed by dual energy X-ray absorptiometry (DXA, QDR1500, software version 5·72; Hologic, Waltham, MA, USA). The post-test DXA scan was carried out at the same time of day as the pre-test scan. Participants wore shorts, socks, underwear (no bra) and t-shirt. The manufacturer's recommendations for whole-body examination (daily quality control, subject positioning, scanning, and analysis with Hologic software) were followed. As shown in a previous publication, the coefficient of variation for appendicular lean mass by DXA measured in our lab was 1.8% (Marcora *et al.*, 2006).

- *Indirect calorimetry*

Participants were asked to attend this test session in an overnight (8-12 h) fasted condition. The meal prior to the indirect calorimetry and the blood and muscle sampling met the WHO recommendations for an oral glucose tolerance test (WHO, 1999). Respiratory exchange ratio (RER) was measured by indirect calorimetry (MetaLyzer 3B, Cortex Biophysik, Leipzig, Germany), following da Rocha *et al.*'s recommendations (2006). Ambient temperature of 20°C and 40% humidity were kept constant throughout this test which was carried out in a climate chamber. The steady state coefficient of variation for our indirect calorimetry system was below 5%. Participants with a coefficient of variation above this value were excluded from the statistical analysis.

Substrate oxidation rates were calculated based on indirect calorimetry, assuming a constant urinary nitrogen excretion (n) equal to 0.01 g / min. The equations used to calculate the substrate oxidation rate were:

$$c = 4.55\ VCO_2 - 3.21\ VO_2 - 2.87\ n$$

$$f = 1.67\ VO_2 - 1.67\ VCO_2 - 1.92\ n$$

where c and f are the grams of carbohydrate and fat oxidised per minute, respectively (Frayn, 1983).

- *Analytical procedures*

An overnight fasting venous blood sample (4 mL) was collected in heparinised vacutaners from the antecubital vein of each subject. Then, a 75 g oral glucose load was administrated to the participants and after 1 hour a new venous blood sample (4 mL) was drawn. Plasma glucose was analyzed for each sample by immobilized enzymatic assay (YSI 2300 STAT, Incorporated Life Sciences, Yellow Springs, OH, USA). Lipid profile was analyzed from the fasting plasma samples by optic enzymatic assay (Reflotron®, Roche Diagnostics, Mannheim, Germany). The plasma samples were then stored at -40°C for later plasma insulin analysis. Plasma insulin was analyzed by ELISA (Ultrasensitive human insulin ELISA-kit, Mercodia, Uppsala, Sweden). Homeostasis model assessment (HOMA) calculator, version 2.2.2., was used to compute steady state beta cell function (%B), insulin sensitivity (%S) and insulin resistance (IR) (http://www.dtu.ox.ac.uk/homa).

- *Muscle biopsies*

The biopsy collection procedure was carried out as described in the *in vitro* section.

After 1 hour of the glucose load, a needle muscle biopsy was taken from each subject. The biopsy for the post-test was taken about 0.5-1 cm from the pre-test biopsy. Muscle specimens were snap-frozen and stored in liquid nitrogen until further analysis. Frozen muscle biopsies (15.6 ± 0.30 mg) and 150 µL of frozen Buffer containing 10% PBS, 5% protein phosphatase inhibitors, 0.1% 1 M DTT, 0.05% protease inhibitor and 0.1% detergent (Activemotif, Rixensart, Belgium) were pulverized (1900 rpm for 15 s) at liquid nitrogen temperature by using a micro-dismembrator (Sartorius-Stedim Biothec, Goettingen, Germany). The protein content of the muscle samples was assessed by Lowry protein assay (Sigma-Aldrich, Saint Louis, MO, USA).

- *Western Blotting*

Pulverized muscle samples were thawed on ice and centrifuged at 4°C (20,000 × g for 5 min). The supernatants were used to assess ACC, FUM, GAPDH, GSK-3β, p-GSK-3α/β, AKT, p-AKT, TXNIP expression levels. Pellets were resuspended in SDS-sample buffer and used to assess glucose transporter 4 (GLUT4), insulin receptor substrate-1 (IRS-1) and MondoA expression levels. Parts of the pellets were used for myosin extraction without resuspension in SDS-sample buffer (see below). Western blotting procedure was performed as described above. GLUT4 (1:200) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), IRS-1 (1: 500) (Cell Signaling Technology Inc, Beverly, MA, USA) primary antibodies were incubated overnight.

- *Two-step real-time RT-PCR*

Total RNA was isolated using RNeasy® Fibrous Tissue Kit (Qiagen Venlo, Netherlands) following the kit instructions. Briefly, liquid nitrogen frozen muscle biopsy samples were pulverised as described above together with lysis buffer and once at RT the homogenised samples were incubated at 55°C for 10 minutes in presence of Proteinase K. The samples were centrifuged at 10,000 × g for 3 min and the supernatants were mixed with ethanol and spun through RNA spin columns. DNA was removed by incubating samples with DNase for 15 min. DNase was then washed away and total RNA was eluted in RNase-free water. Total RNA content was determined by spectrophotometry (U-2800A, Hitachi High- Technologies, Tokyo, Japan) using the following formula [RNA] = 44 µg/mL · $Absorbance_{260}$ · dilution factor. Total RNA concentrations averaged around 108 ± 37 µg/mL. Reverse transcription (RT-step) was performed using Sensiscript® RT Kit (Qiagen Venlo, Netherlands) in a gradient thermocycler (Auto Q Server Gradient Thermal Cycler, Quanta Biotech, West Byfleet, UK). Complementary DNA (cDNA), produced by the RT-step, was amplified via real-time PCR (7900HT Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA) to assess the gene expression of MondoA (forward

TGACTTTGGCCTACAGTGGG; reverse TTGCGCTTCTCCAGATACTGC) and TXNIP (forward CTGGCGTAAGCTTTTCAAGG; reverse AGTGCACAAAGGGGAAACAC) (Eurofins, MWG/Operon, Ebersberg, Germany). SYBR-Green (Qiagen Venlo, Netherlands) was used to perform and quantify the cDNA amplification, 18 s ribosomal RNA (forward GTAACCCGTTGAACCCCATT; reverse CCATCCAATCGGTAGTAGCG) (Eurofins, MWG/Operon, Ebersberg, Germany) was used for normalisation, and the mRNA levels were determined by relative quantification using standard curve method.

- *Myosin heavy chain (MHC) extraction and electrophoresis*

Pellets from muscle sample centrifugation were extracted on ice by means of ultrasound (3 s pulses followed by 3 s cooling; 10 cycles with 5 min pause on ice between each cycle) (Ultrasonic Processor VCX 130, Sonics & Materials INC, Newtown, CT, USA) with myosin extraction buffer (0.6 M KCl, 1 mM EGTA, 10 mM sodium phosphate dibasic, 1 mM PMSF, pH 6.8) at 0°C. Extracts were centrifuged at 20,000 × g for 20 min at 4°C and supernatants diluted 1:10 (with ice cold H_2O, 1 mM PMSF) and incubated overnight at 0°C for precipitation of actomyosin. The suspension was centrifuged the next day at 20,000 × g for 20 min at 4°C. Pellets were resuspended in 20-50 µL extraction buffer. MHCs were separated with a SDS-PAGE (separating gel: T = 9%, C = 1.3% and 34% Glycerol, with a maximum voltage of 400 V for 36 h, at 12 mA) followed by silver staining and densitometry (Gel Doc 2000 and software Quantity One-4.6.3, Bio-Rad, Hercules, CA, USA) (**Appendix**, **Fig. A.5**).

Statistics

The statistical analysis was performed using SPSS 11.5 standard version. Unless otherwise specified, all data are reported as means ± standard deviations. The *in vitro* outcomes were analysed with non-parametric two-independent samples Mann-Whitney U tests. Total

GSK-3 nuclear factions were analysed by Kruskal-Wallis test (non-parametric ANOVA). While the outcomes of the *in vivo* study were analysed with Student's paired-samples t-tests. The significance level was set at 0.05 (two-tailed). The significant level for trends was set at 0.10.

Results

In vitro study

- *MondoA and TXNIP protein expression is elevated in hyperglycemia in human primary muscle cell cultures*

To investigate whether MondoA and TXNIP expression are driven by hypergycemia, in human myocytes, in culture, primary muscle cell cultures were grown on microcarriers for 14 days. Cell cultures were based on quadriceps femoris biopsies, taken from participants of the soft-drink intervention. After differentiation (**Fig. 3.1**) myocytes were exposed for 7 days to hyperglycemia (15mM glucose) or control conditions (5mM glucose) both with 10 µg/mL insulin. Western Blot analysis of cell homogenates revealed that expression levels of MondoA as well as TXNIP were strongly elevated in response to high glucose availability after 7 days (190 ± 30% and 352 ± 47% of control; $Z = -2.121$, $P < 0.05$) (**Fig. 3.2 A/B**). The expression of TXNIP was highly correlated with MondoA (**Fig. 3.2C**), which underlines the transcriptional control of TXNIP by MondoA (Stoltzman *et al.*, 2008). Additionally, nuclear and cytoplamic fractions derived from the cultured myocytes showed that MondoA is increasingly expressed under hyperglycaemic condition while being more localized in the nuclear fraction than in the cytoplasm (**Fig. 3.2A**).

- *Glycolytic and lipogenic enzymes are upregulated by hyperglycemia; insulin response is diminished by hyperglycemia*

Western Blot analysis of glycolytic, oxidative and lipogenic marker enzymes from myocytes exposed to hyperglycemia for 7 days showed a significant increase in glycolytic GAPDH and lipogenic ACC expression compared to controls (173 ± 32%

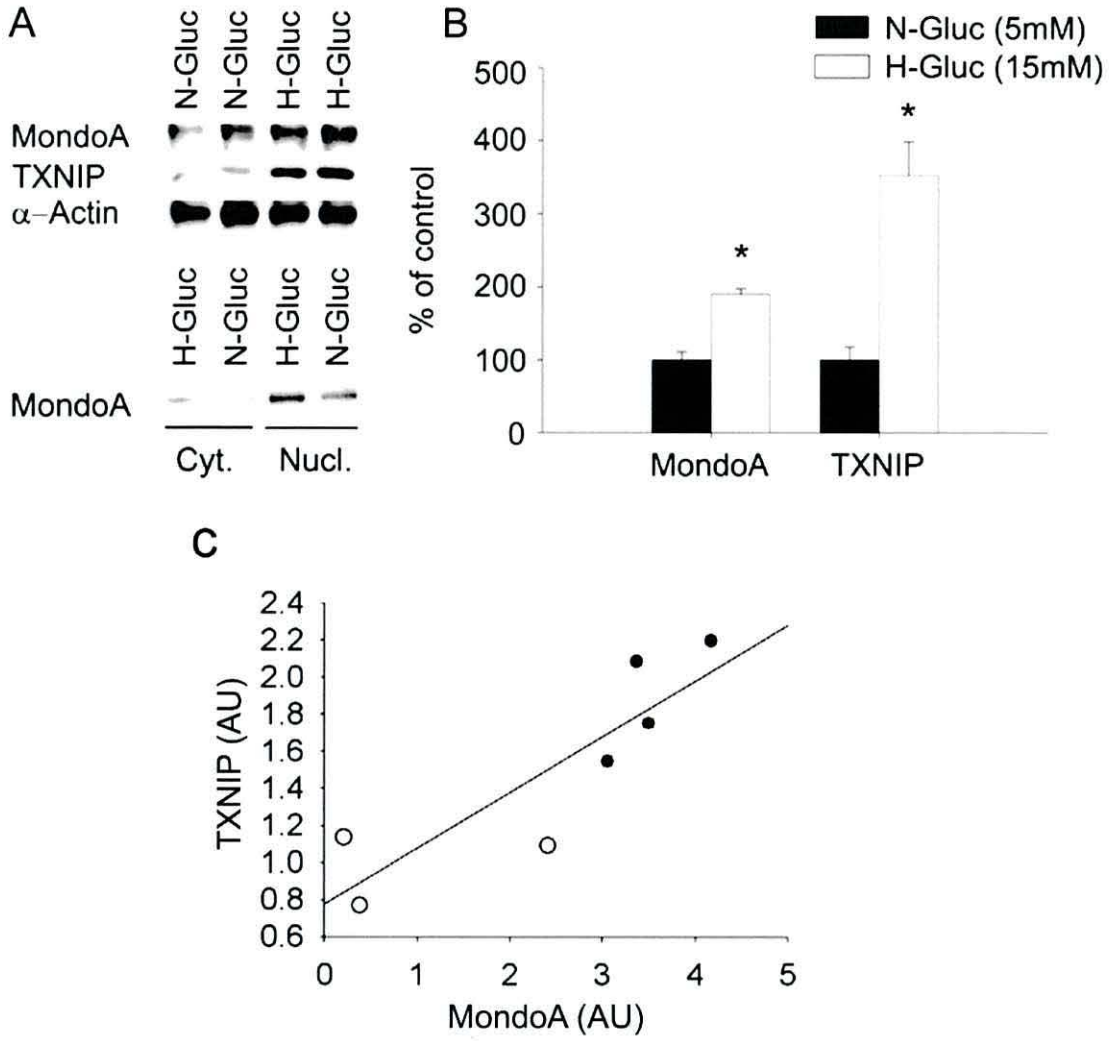


Figure 3.2 A. Western blot analysis of MondoA and TXNIP expression in primary muscle cell cultures under conditions of hyperglcaemia (15 mM) and control (5 mM) – 12 days plus 7 days exposure. Alpha-actin was used as loading control. MondoA cytoplasmic (Cyt.) and nuclear (Nucl.) fractions were derived from same cultures. B. Densitometric quantification of Western blots for MondoA and TXNIP, expressed as percentage of control. Results are presented as means ± SEMs. * $P < 0.05$ for differences between glucose conditions. C. Pearson correlation between MondoA and TXNIP, opened dots are myotubes cultures exposed to normal glucose (5 mM), closed dots are myotubes cultures exposed to high glucose (15 mM) ($r = 0.87$; $P < 0.011$; $n = 7$).

and 177 ± 18% of control, Z = -2.121 and Z = -1.903 respectively; $P < 0.05$) (**Fig. 3.3**). Oxidative marker enzyme FUM did not respond to the treatment (91 ± 30% of control, Z = -0.775, $P = 0.44$) (**Fig. 3.3**). Furthermore, exposure to hyperglycemic condition for 7 days diminished Akt phosphorylation (thr 308) in response to insulin (100 nM) (**Fig. 3.4**). A two by three way ANOVA showed a main effect for group ($P < 0.01$) and when followed up by a non-parametric two-independent samples Mann-Whitney U test, a trend towards ($P < 0.10$) a difference at 30 min was present.

- *Increase in total GSK-3 nuclear fraction with high-glucose*

P-GSK, total GSK3 and p-GSK3/GSK3 did not differ between the two glucose conditions (H-Gluc vs. N-Gluc: 111 ± 14% vs. 100 ± 30%; 86 ± 12% vs. 100 ± 17%; 1.32 ± 0.28 vs. 1.04 ± 0.45, respectively) (**Fig. 3.5 A**). The Kruskal-Wallis test (non-parametric ANOVA) showed a significant, Chi-square = 9.176; P=0.027 for total GSK-3 contents in the nuclear fractions. Post hoc Mann-Whitney Tests indicated that GSK-3 was significantly less in the normal-glucose/no-insulin condition in comparison with all the other conditions, (P<0.05) (**Fig. 3.5B/C**); and that the high-insulin conditions accumulate significantly more GSK-3 in the nucleus than no-insulin conditions regardless of the glucose levels, (P<0.05 (**Fig. 3.5B/C**).

In vivo study

- *Periodic high glucose availability for 4 weeks elevates MondoA but not TXNIP expression in skeletal muscle biopsies*

Healthy lean participants without a history of chronic soft-drink consumption consumed soft drinks (~2g sugar/kg body weight per day) for 4 weeks (**Table 3.2**).

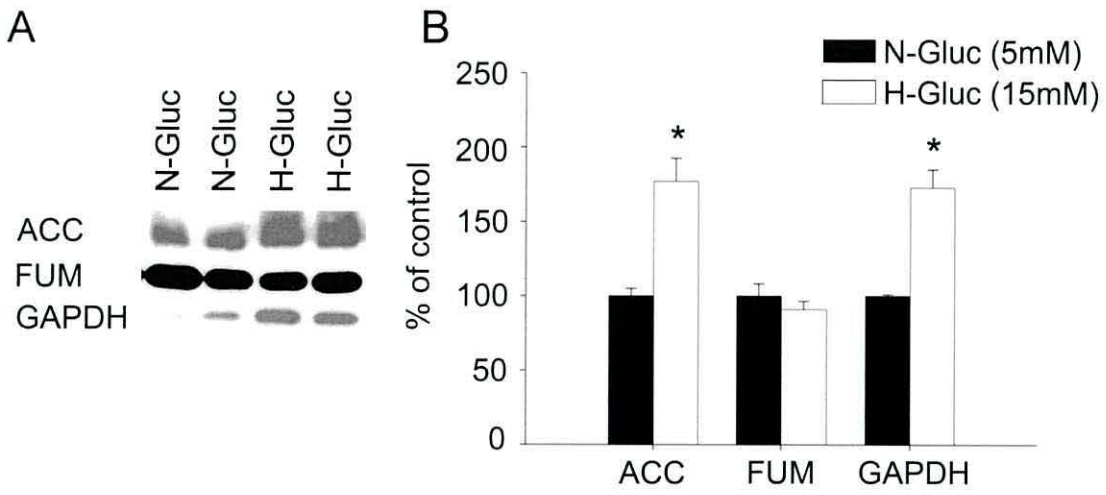


Figure 3.3 A. Western blot analysis of metabolic markers, acetyl CoA Carboxylase (ACC), fumarate hydratase (FUM) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in primary muscle cell culture, under conditions of hyperglycaemia (15 mM) and control (5 mM) for 7 days. B. Densitometric quantification of Western blots for ACC, FUM and GAPDH, expressed as percentage of control. Results are presented as means ± SEMs. * $P < 0.05$ for differences between glucose conditions (n = 7).

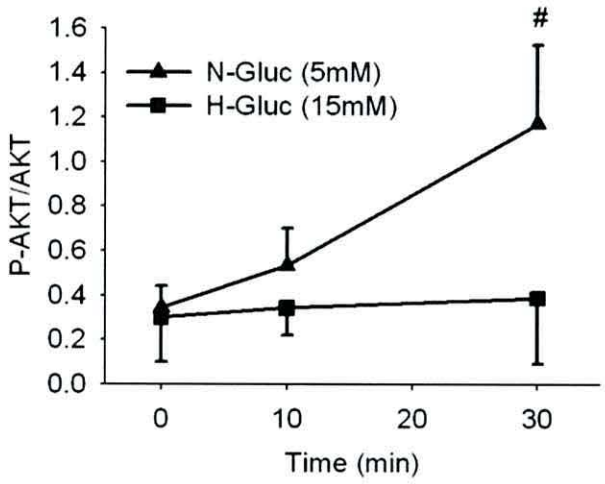
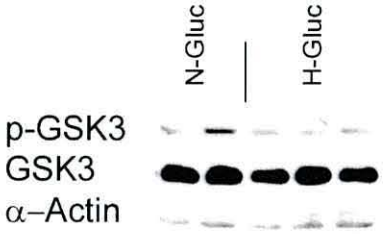
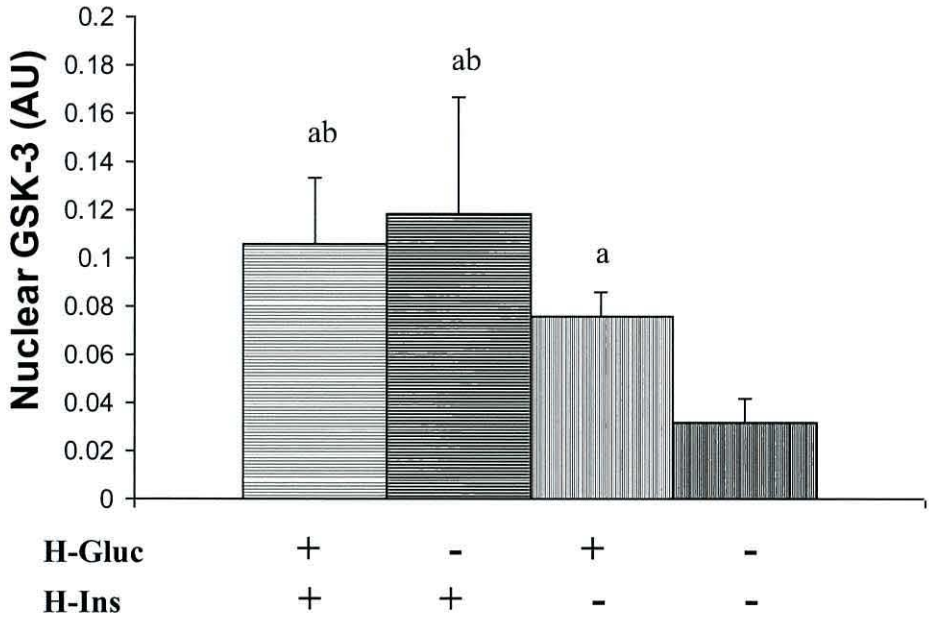


Figure 3.4 Insulin-dependent Akt (Thr 308) phosphorylation in primary muscle cell cultures under conditions of hyperglycaemia (15 mM D-glucose) and control (5 mM D-glucose) for 7 days exposed to insulin (100 nM). Samples were retrieved before insulin, after 10 minutes and after 30 minutes. Both hyperglycaemia and control conditions were compared after quantification of western blotting analysis. Results are presented as means ± SEMs. # $P < 0.10$ for trends between glucose conditions (n = 7).

A



B



C

Figure 3.5 A) Western blots of p-GSK3 and total GSK3 myotubes exposed to 7 days in High-glucose (H-Gluc, 15 mM D-glucose), or Normal-glucose (N-Gluc, 5 mM D-Glucose), both cultures were exposed to 10 µg/mL insulin. P-GSK, total GSK3 and p-GSK3/GSK3 did not differ between the two glucose conditions. B) Differences in GSK3 nuclear contents. Myotubes were exposed 7 days to 15 mM D-glucose and 10 µg/mL insulin (+/+); 5 mM D-glucose and 10 µg/mL insulin (-/+); 15 mM D-glucose without insulin (+/-); and 5 mM D-glucose, no insulin (-/-). Post hoc Mann-Whitney Tests showed: [a] significant differences from NGnoI, $P<0.05$, [b] significant differences from HGnoI, $P<0.05$. C) Corrispondent western blots of nuclear GSK-3 at the four different conditions.

Table 3.2 Average daily and macronutrient intake at baseline and during the 4 weeks of intervention

Parameters (units) (n = 11)	Baseline	4 weeks Intervention	t(df = 10)	*P* level	partial η^2
Energy (kcal/d)	2383 ± 654	2463 ± 538	-0.431	0.675	0.02
Protein (g/d)	83.4 ± 21.1	71.0 ± 23.2	3.300	0.008**	0.52
Carbohydrate (g/d)	264 ± 72	347 ± 65	-3.586	0.005**	0.56
Sugars (g/d)	98.6 ± 40.2	183.9 ± 32.1	-8.972	< 0.001***	0.89
MUFA (g/d)	40.6 ± 15.7	33.3 ± 17.6	1.698	0.120	0.22
PUFA (g/d)	10.7 ± 5.1	7.4 ± 4.6	2.431	0.035*	0.27
SFA (g/d)	41.8 ± 19.0	31.7 ± 16.9	1.937	0.082#	0.27
Dietary fibre (g/d)	19.6 ± 5.9	18.5 ± 5.8	1.125	0.287	0.11

# $P < 0.10$, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

Pre and post intervention muscle biopsies from vastus lateralis were taken precisely 1 hour after ingestion of 75 g glucose solution to secure activation of insulin signalling pathways. Myosin heavy chains electrophoresis and quantification showed no difference in fibre type composition pre and post intervention, confirming that results were not confounded by variability in origin of the muscle biopsies (**Table 3.3**). Western blot analysis of MondoA protein content, in muscle samples, showed a strong trend towards an elevated expression of this transcription factor (pre: 1.00 ± 1.08 AU, post: 2.58 ± 2.46 AU, t(9) = -1.959, P=0.082, partial η^2 = 0.299) (**Fig. 3.6A/B**). However, TXNIP expression did not change (pre: 1.00 ± 0.38 AU, post: 1.24 ± 0.49 AU, t(9) = -1.337 P = 0.214, partial η^2 = 0.166) (**Fig. 3.6C/D**). Alterations of TXNIP expression were significantly correlated with the alterations of MondoA expression (**Fig. 3.6E**) which shows that the functional relationship between MondoA and TXNIP also exists in skeletal muscle *in vivo*. Protein expression results were confirmed by the gene expression results. MondoA mRNA showed a strong trend towards an

increase (t(9)= -2.152, P=0.057, partial η^2 = 0.316), whereas mRNA levels of TXNIP were not altered by the soft drink intervention (t(9)= 0.952, P=0.364, partial η^2 = 0.083) (**Fig. 3.7**, see also **Appendix**, **Fig. A.4**).

- *Influence of 4 weeks soft-drink intervention on muscular insulin signalling pathway and metabolic enzyme expression*

Signalling response to high glucose and insulin of skeletal muscle was investigated by Western blot analysis of Akt phosphorylation (Thr308) and GSK 3beta (Ser21/9) phosphorylation. Phosphorylation level of GSK 3beta was lower after 4 weeks soft-drink intervention (p-GSK 3β, pre: 1.00 ± 0.33 AU, post: 0.79 ± 0.31 AU, t(9) = 2.126, P=0.062, partial η^2 = 0.334; total-GSK 3β, pre: 1.00 ± 0.56 AU, post: 0.96 ±

Table 3.3 Myosin heavy chain phenotypes, metabolic markers and insulin signalling markers

Parameters (units) (n =10)	Baseline	4 weeks Intervention	t(df = 9)	P level	partial η^2
MHC I (%)	52.2 ± 17.0	50.4 ± 15.5	0.453	0.661	0.022
MHC IIa (%)	45.4 ± 19.3	46.8 ± 17.6	-0.357	0.729	0.014
MHC IIx (%)	2.4 ± 7.6	2.8 ± 8.9	-1.000	0.343	0.100
FUM (AU)	1.00 ± 0.35	0.79 ± 0.41	1.456	0.179	0.191
GAPDH (AU)	1.00 ± 0.24	0.93 ± 0.37	0.898	0.393	0.082
ACC (AU)	1.00 ± 0.55	0.84 ± 0.47	1.035	0.328	0.106
GLUT 4 (AU)	1.00 ± 0.55	1.04 ± 0.46	-0.294	0.775	0.010
IRS-1 (AU)	1.00 ± 0.32	1.01 ± 0.28	-0.092	0.929	0.001

For the explaination of the acronyms see abbreviations on page XVII.

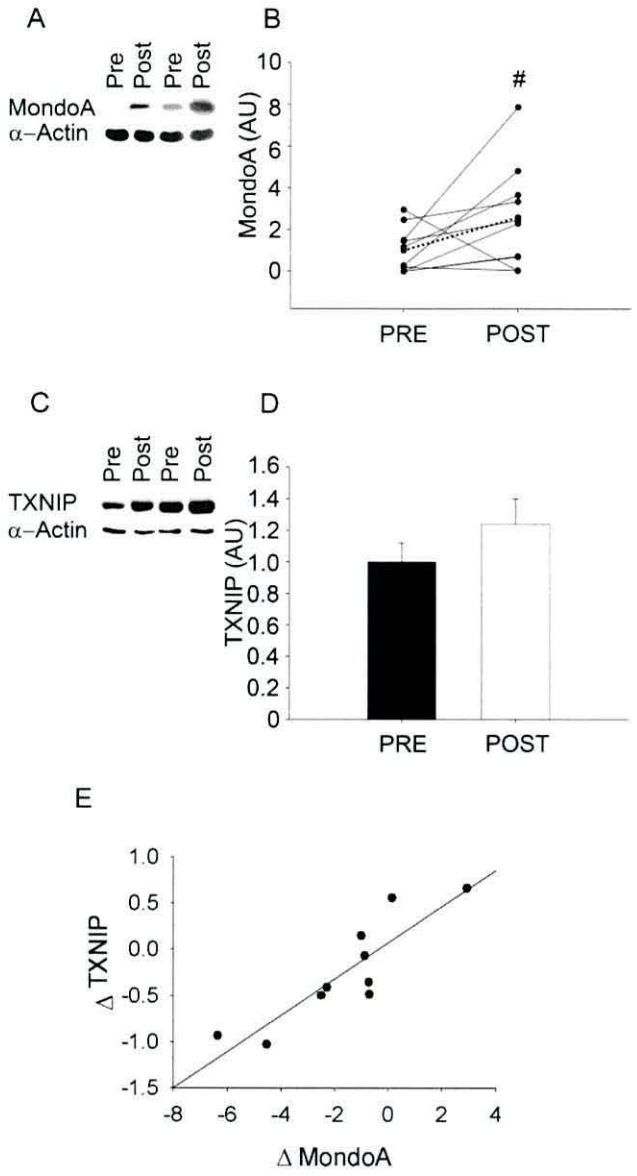


Figure 3.6 A. Western blot analysis of glucose sensitive protein MondoA in muscle biopsies, before and after 4 week soft drink supplementation – loading control by alpha-actin. B. Densitometric quantification of western blots for MondoA, pre intervention and post intervention for all participants. C. Western blot analysis of TXNIP in muscle biopsies, before and after 4 week soft drink supplementation; loading control alpha-actin. D. Densitometric quantification of Western blots for TXNIP pre and post intervention. E. Correlation between MondoA changes and TXNIP changes (*in vivo*) ($r = 0.88$, $P < 0.01$, $n = 10$). Results are presented as means ± SEMs. # $P < 0.10$ for differences between pre and post 4 week soft drink supplementation. ($n = 10$).

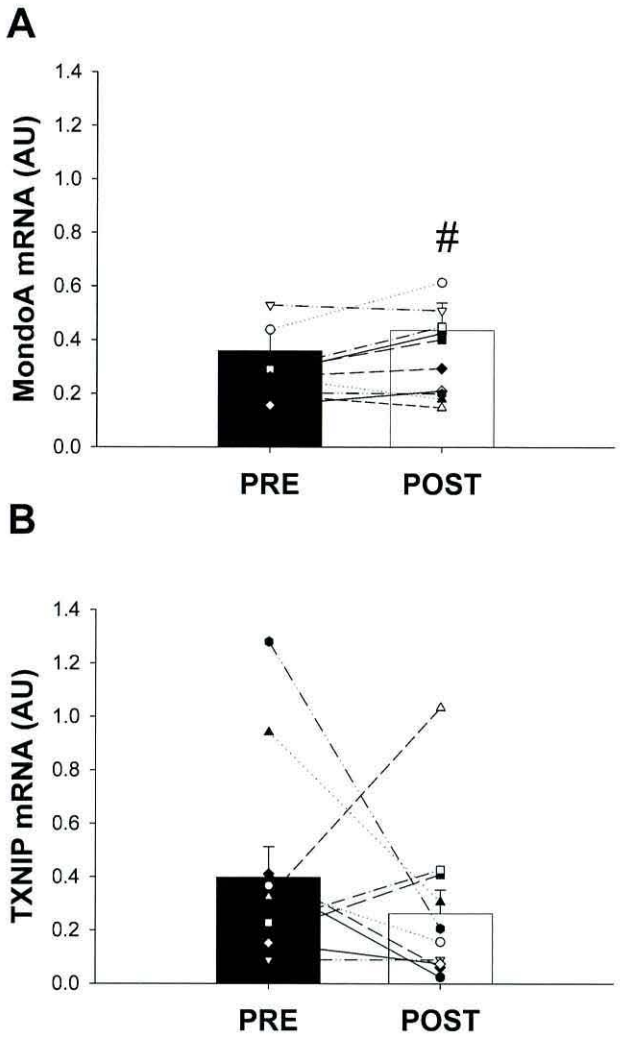


Figure 3.7 A. MondoA individual and group gene expressions and B. TXNIP individual and group gene expressions in response to soft drink supplementation. Results are presented as means ± SEMs (n = 10).

0.53 AU, t(9) = 0.507, P=0.625, partial η^2 = 0.028), whilst the level of Akt phosphorylation was not altered post intervention (p-AKT, pre: 1.00 ± 0.60 AU, post: 1.14 ± 0.48 AU, t(9) = -0.873, P=0.405, partial η^2 = 0.078; total-AKT, pre: 1.00 ± 0.57 AU, post: 0.88 ± 0.57 AU, t(9) = 1.379, P=0.201, partial η^2 = 0.174, respectively) (**Fig. 3.8**). On the other hand, changes of GSK 3beta phosphorylation were significantly correlated with the changes of Akt phosphorylation representing the functional connection of the signalling responses (**Fig. 3.8C**). Analysis of metabolic marker enzymes FUM, ACC and GAPDH revealed no significant changes in expression. Moreover, protein levels of GLUT4 and IRS1 did not change (**Table 3.3**, and **Appendix**, **Fig. A.3**).

- *Insulin sensitivity, resting glucose levels and metabolic substrate preference are altered after periodic high glucose availability for 4 weeks*

Fasting plasma glucose levels increased significantly while fasting plasma insulin levels showed a clear trend towards an elevation (pre: 4.83 ± 0.43 mmol/L, post: 5.13 ± 0.38 mmol/L; $P<0.050$, t(9) = -2.875, partial $\eta^2 = 0.479$; pre: 4.95± 1.90 mU/L, post 6.40 ± 1.62 mU/L, t(9) = -1.874, $P=0.094$, partial $\eta^2 = 0.281$; respectively) (**Fig 3.9**). Blood lipids (**Table 3.1**) did not change after the 4 weeks intervention. However, oral glucose tolerance test revealed that insulin sensitivity (%S) diminished and insulin resistance (IR) increased, based on the homeostasis model assessment (HOMA2), statistical testing showed p values close to significance (**Table 3.1**). Otherwise, beta cell function (%B) showed no response to the periodic high glucose availability (**Table 3.1**). Analysis of resting metabolism after over-night fast measured by indirect calorimetry revealed a significant increase in VCO_2/VO_2 (pre: 0.75 ± 0.09, post: 0.87 ± 0.08; t(7) = -2.530, $P<0.050$, partial $\eta^2 = 0.478$, n = 8) (**Fig. 3.10B**); calculation of preferred substrate oxidation in metabolism demonstrates that metabolism changed from the expected high level of fat oxidation to a strong preference of carbohydrate oxidation after only 4 weeks intervention (**Fig. 3.10A**). Additionally, body composition, analysed by DXA, was altered towards higher body fat content while lean mass did not change (**Table 3.1**).

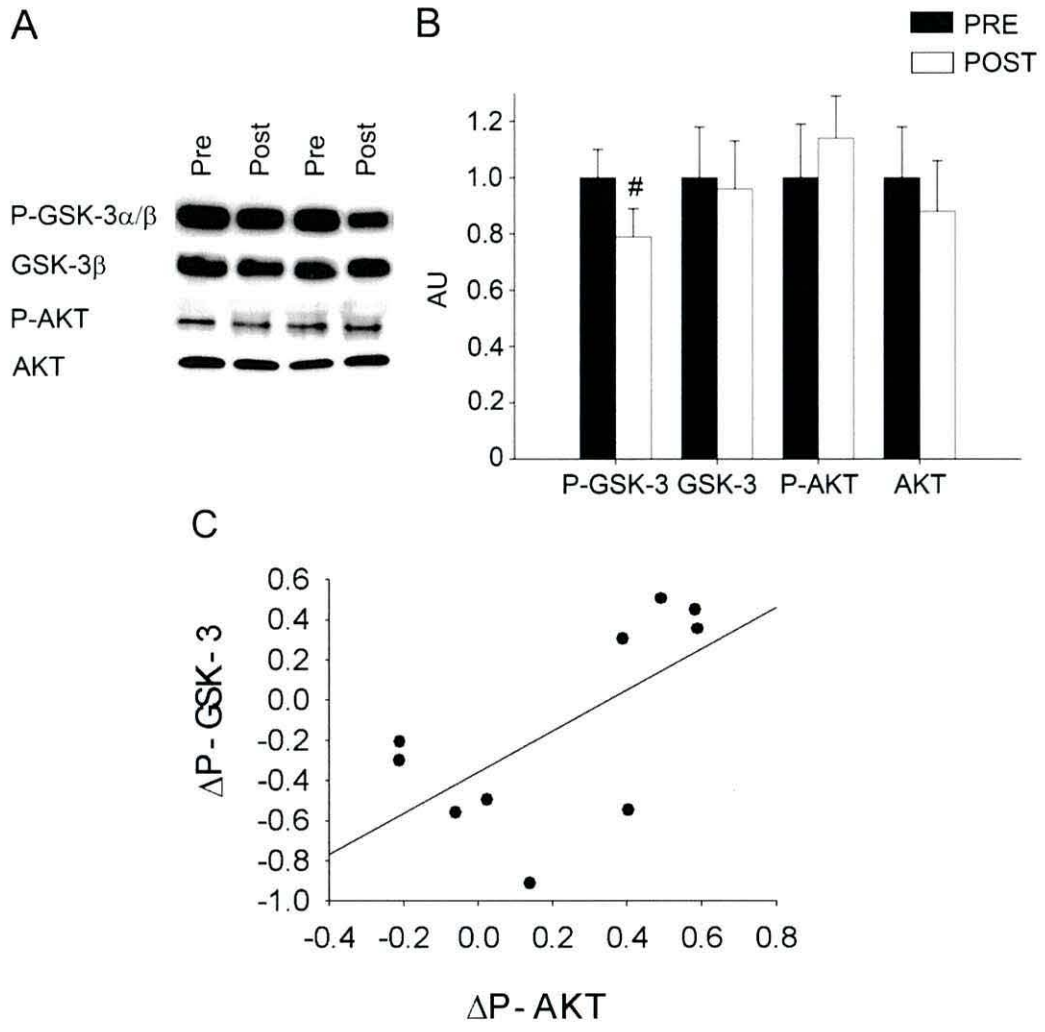


Figure 3.8 Western blot analysis of glycogen synthase kinase 3-beta (GSK 3β), Akt and phosphorylated GSK 3β (Ser 21/9) and Akt (Thr 308), in muscle biopsies, before and after 4 week soft drink supplementation. B. Densitometric quantification of Western blots for Akt, Akt phosphorylation, GSK 3β and GSK 3β phosphorylation, from pre and post intervention. Results are presented as means ± SEMs. # $P < 0.10$ for differences between pre and post 4 week soft drink supplementation (n = 10). C. Correlation between p-AKT (Thr 308) changes and p-GSK 3-β (Ser 21/9) changes (r = 0.64, $P < 0.05$, n = 10).

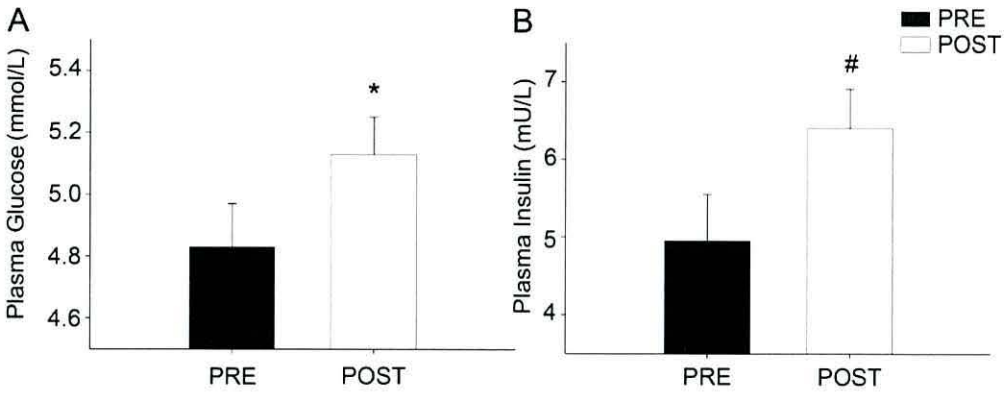


Figure 3.9 A. Analysis of resting blood samples after over-night fasting for plasma glucose and B. for plasma insulin levels before and after 4 week soft drink intervention. Results are presented as means ±SEMs. * $P < 0.05$ and # $P < 0.10$ for differences between pre and post 4 week soft drink supplementation. (n = 10)

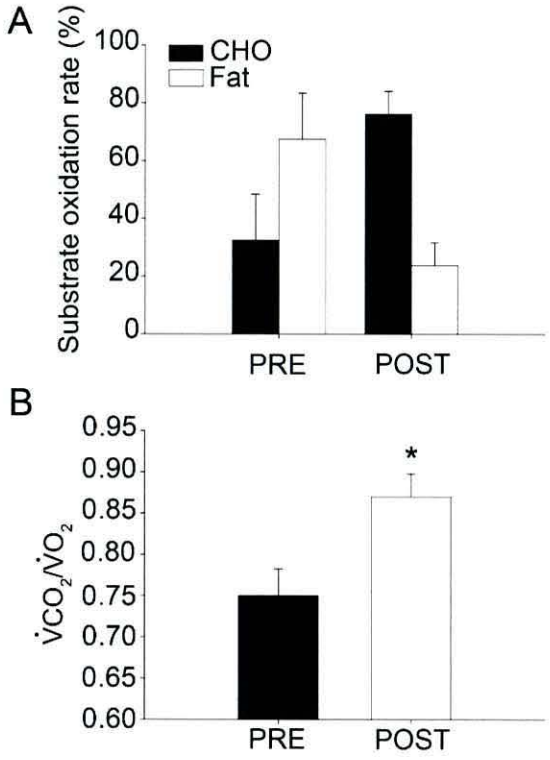


Figure 3.10 A. Substrate oxidation rate was derived from VCO_2/VO_2 before and after 4 week soft drink supplementation by indirect calorimetry after overnight fast. B. VCO_2/VO_2 measured before and after 4 week soft drink supplementation by indirect calorimetry after overnight fast. Results are presented as means ± SEMs. * $P < 0.05$ for differences between pre and post 4 week soft drink supplementation (n = 8).

Discussion

Alterations of MondoA and TXNIP expression

In this study we have shown, for the first time, that MondoA protein and gene expression in human skeletal muscle cells can be elevated via chronic hyperglycaemic conditions *in vitro* but also influenced by periods of high glucose availability *in vivo*. The importance of this finding lies in the fact that MondoA has recently been recognized as a glucose metabolite sensing transcription factor regulating glycolytic genes and possibly a much wider range of metabolic responses to high glucose availability. Moreover, MondoA has been recognised as the transcription factor up-regulating the expression of TXNIP (Stoltzman *et al.*, 2008). TXNIP is involved in peripheral glucose uptake inhibition, especially in skeletal muscle (Parikh *et al.*, 2007) and is a critical regulator in glucose production in the liver (Chutkow *et al.*, 2008) as well as a possible mediator of apoptosis in glucose toxicity of beta cells (Shalev, 2008). Moreover, TXNIP is shown to be increasingly transcribed in skeletal muscle of prediabetics and T2DM subjects and is suggested to contribute to the development of T2DM. It has also been used as possible prediabetic marker and target for treatment of T2DM (Muoio, 2007; Parikh *et al.*, 2007). In our *in vitro* study, chronic exposure to high glucose augmented TXNIP expression, but this was not confirmed *in vivo*. Perhaps, an average of two 380 mL bottles of glucose syrup based drinks per day (i.e. ~2 to 4 hours of hyperglycaemia over 24 hours) for 4 weeks were not sufficient to activate MondoA long enough to induce significant changes in TXNIP. However, this result does not exclude that longer and greater soft drink consumptions would not also increase TXNIP expression *in vivo*.

In support of a close functional connection of MondoA and Txnip expression, it is not only shown that MondoA knockdown results in a complete block of TXNIP expression *in vitro* (Stoltzman *et al.*, 2008), but also that the human TXNIP promoter contains ChoRE which are

controlled by ChREBP/MondoB conferring strong transactivation of the TXNIP promoter (Cha-Molstad *et al.*, 2009). MondoA is a paralog of ChREBP/MondoB. MondoA is mainly expressed in skeletal muscle, whereas ChREBP/MondoB is highly expressed in liver (Postic *et al.*, 2007). Our *in vitro* work with human skeletal muscle cell cultures grown on microcarriers supports the findings of Stoltzman *et al.* (2008); from experiments with L6 cells that hyperglycaemia leads to increased activity in the MondoA-TXNIP signalling axis which accounts for the coordinated adaptive transcriptional response to chronic high glucose availability. In the current study we found an increased expression of MondoA and TXNIP as well as a highly correlated expression of MondoA and TXNIP in human skeletal muscle cultures (**Fig 3.2C**). Moreover, the results of our soft-drink intervention can now partially extend this finding to human skeletal muscle under conditions of periodic high glucose availability *in vivo* – MondoA expression was increased and TXNIP changes and MondoA changes were highly correlated (**Fig 3.6E**).

These findings lead to two suggestions, firstly, that skeletal muscle cells seem to integrate periods of high glucose availability by increasing MondoA expression and its activity for the adaption of metabolic gene expression and glucose uptake to elevated nutrient availability. The reasons why periods of high glucose availability can achieve a similar response, like chronic hyperglycaemia, might be found on various levels. Firstly, elevated MondoA expression levels and its translocation into nuclei may happen with a faster import than export kinetics leading to an enrichment of MondoA in nuclei similar to what it is shown for calcium-calcineurin-NFATc1 signals in muscle cells (Kubis *et al.*, 2003). Certainly, this needs to be addressed in future research. Secondly, a rather applied suggestion, which results from these findings, is that chronic soft-drink consumption even in lean sedentary people can activate signalling systems which are reported to be involved in the mediation of metabolic alteration, leading to a prediabetic state and might contribute to the development

of insulin resistance and T2DM. The amount of soft-drink the participants in this study consumed on top of their usual diet for four weeks represents approximately the mean value of soft-drink consumption per capita per day in the UK (BSDA, 2009). Therefore, many people consume much more and for a much longer time than our participants with the possible consequence of stronger effects on MondoA and possibly on TXNIP.

Moreover, the selection of people without a history of chronic soft-drink consumption and the fact that the selected participants have been lean, young individuals with low physical activity levels underpins the importance of our finding – there have been no risks of confounding effects related to overweight or obesity or former adaptation to chronic sugar-sweetened beverage consumption. Certainly, for the suggestion that periodic high glucose availability pertains a possible health risk even for these subjects, metabolic alteration *in vivo* should be detectable to justify this notion.

Metabolic alteration and insulin response in skeletal muscle cells exposed to chronic and periodical hyperglycaemia in vitro and in vitro

MondoA was shown to contribute to 75% of glucose-induced gene expression in HA1ER cells using Agilent 44K human microarrays (Stoltzman *et al.*, 2008) and is both necessary and sufficient for glycolytic enzyme expression as demonstrated in C2C12 and K562 cells (Sans *et al.*, 2006). In the present study, we focussed on metabolic marker enzymes of glycolytic, lipogenic and oxidative pathways to investigate the metabolic adaptation of human skeletal muscle cells *in vitro* and *in vivo* to high glucose availability. Exposure of human skeletal muscle cell cultures to hyperglycaemia (15 mM) for 7 days resulted in an upregulation of glycolytic (GAPDH) and lipogenic (ACC) enzyme expression while no change was detected in the mitochondrial marker enzyme FUM (**Fig. 3.3**). These alterations

towards higher glycolytic and lipogenic capacity in skeletal muscle cells are typical for the metabolic phenotype seen in T2DM (He & Kelley, 2004). Also, glycolytic and lipogenic enzymes have been shown to possess ChoRE in their promoter sequences (Kabashima *et al.*, 2003; Merla *et al.*, 2004). Additionally, Hanke *et al.* (2008) recently showed that oxidative and glycolytic enzyme transcription in primary skeletal muscle cells from rabbit is heavily influenced by glucose availability. However, in contrast to the current results in cell culture we could not detect significant alterations in metabolic enzyme expression in the human muscle biopsies post intervention possibly due to the short duration of the intervention.

While hyperglycaemia in T2DM may be the result of processes related to impaired beta cell function (Pick *et al.*, 1998), adipokine signalling (Arner, 2005) and influence of lipid metabolites (Ellis *et al.*, 2000), the contribution of glucose depending signalling for the development of the metabolic phenotype found in skeletal muscle in T2DM seems highly plausible. The development of metabolic inflexibility could be enhanced by MondoA response to periodic high glucose availability, regulating metabolic gene expression as well as TXNIP influence on glucose uptake. Moreover, in the present study metabolic alterations post intervention *in vivo* show that after 4 weeks of soft-drink consumption fasting metabolism in rest, based on the significantly elevated overnight fasting RER (**Fig. 3.10B**), shifted from preference for fatty acid oxidation towards preference for carbohydrates. These findings are consistent with the metabolic preference in individuals with insulin resistance and T2DM (Kelley & Mandarino, 2000). A possible influence of an altered blood lipid profile on skeletal muscle metabolism as a possible contributor to our results can be excluded because no alterations in this parameter could be detected (**Table 3.1**). However, the influence of intramuscular lipids cannot be excluded. However, the importance of ceramide for the reduction of insulin sensitivity and loss of aerobic capacity in skeletal muscle was recently questioned (Skovbro *et al.*, 2008).

The significant elevation of resting blood glucose and insulin (**Fig. 3.9A/B**) derived from oral glucose tolerance tests show that skeletal muscle adapts to the periodic high glucose availability in a way similar to what is expected in muscle from insulin resistant obese individuals on a lower absolute level. The importance of soft-drink consumption for the prevalence of obesity is strongly supported by its high correlation with obesity and diabetes (Bray *et al.*, 2004; Gross *et al.*, 2004; Dhingra *et al.*, 2007; Hu & Malik, 2010). Moreover, longitudinal studies could detect alterations in serum insulin and glucose levels in healthy and hyperinsulinemic subjects after elevated sucrose intake (Reiser *et al.*, 1979; Reiser *et al.*, 1981). Kiens and Richter (1996) performed a high glycemic diet intervention for 30 days but did not detect alteration of resting glucose and insulin. However, the subjects in their study were physically active individuals. The factor of physical activity might be relevant considering the difference to our study. Physical activity is a strong factor contributing for the activation of signalling systems increasing insulin sensitivity and aerobic capacity in muscle (Fluck & Hoppeler, 2003; Yeo *et al.*, 2008). The combination of periods of high glucose availability with physical activity might ameliorate the glucose dependent effects on metabolism and insulin response seen in our study with physically inactive participants. As an example, exercise driven inhibition of nuclear import ChREBP/MondoB and therefore a reduction in the expression of glycolytic enzymes could be mediated by phosphorylation via protein kinase A and AMP dependent protein kinase (Kawaguchi *et al.*, 2001; Kawaguchi *et al.*, 2002).

Indeed, insulin signalling response was influenced by high glucose availability both *in vitro* and *in vivo* in the current study (**Figs. 3.4** and **3.8**). Akt phosphorylation (Thr308) showed a trend towards lower levels in response to insulin after exposure of skeletal muscle cells to hyperglycaemia for 7 days (**Fig. 3.4**). Akt (Thr308) phosphorylation in response to insulin was shown to be impaired *in vitro* in L6 muscle

cells exposed to high glucose, high insulin (Huang *et al.*, 2002), and in skeletal muscle of obese insulin resistant females (Hojlund *et al.*, 2008). Hyperglycaemia itself can reduce Akt phosphorylation in skeletal muscle of rats (Steiler *et al.*, 2003). Additionally, GSK-3 phosphorylation was reduced in the muscle biopsies 1 hour after glucose ingestion (**Fig. 3.8**) while no difference in Akt phosphorylation was detectable. GSK-3 is phosphorylated by Akt in response to insulin in skeletal muscle (Markuns *et al.*, 1999; Sakamoto & Goodyear, 2002). Moreover, GSK-3, besides regulating glycogen synthesis, is shown to be involved in the mediation of insulin resistance (Nikoulina *et al.*, 2000). Elimination of one allele of GSK-3β in insulin resistant mice enhanced insulin sensitivity and glucose disposal in skeletal muscle (Tanabe *et al.*, 2008). In **Figure 3.5B** GSK-3 is accumulated in the nuclei of the muscle cells when high insulin levels are present regardless the glucose levels. However, when insulin is not present, high glucose availability *per se* increases GSK-3 content in the nuclei. This is important because the presence of GSK-3 in the nucleus has been associated with apoptosis (Jope & Johnson, 2004). The insulin independent effect of glucose could be due to the high glucose gradient that is applied continuously to the muscle cell, *in vitro*, and that perhaps increases GLUT1 basal glucose transport into the muscle cells. The contribution of GLUT1 *in vitro* could also explain why we found some discrepancy *in vivo*. Perhaps, TXNIP and GAPDH and ACC failed to show a significant increase *in vivo*, not only because of the sort duration of supplementation, but also because of the limited contribution of GLUT 1 *in vivo*.

The lack of a clear effect on Akt phosphorylation *in vivo* might be related to the transient manner of Akt phosphorylation in response to insulin. While the phosphorylation (Thr308) peaks after 20 minutes and levels off after 40 minutes to sustained insulin in rat skeletal muscle (Sharma *et al.*, 2009), faster and shorter phosphorylation transients are reported for Ser473 (Markuns *et al.*, 1999). In our experiments we took biopsies only at one time point after glucose ingestion. Therefore we

might have missed detecting the peak in Akt-phosphorylation and consequently any possible differences pre and post intervention. Otherwise, it is shown in skeletal muscle from rats that periods of high glucose availability can cause insulin resistance without affecting Akt phosphorylation (Hoy *et al.*, 2007). However, there is some indication that periodic high glucose availability might still have an impact on Akt level in our study *in vivo*; since GSK-3 changes were well correlated with Akt phosphorylation changes, and GSK-3 phosphorylation reduction is likely based on Akt deactivation during insulin response. It is possible that the altered Akt activity in response to insulin seen after periodic and chronic high glucose availability is mediated by mechanisms related to elevated TXNIP expression. TXNIP deficiency in mice protects against diabetes and beta-cell apoptosis and it was accompanied by enhanced Akt activity (Chen *et al.*, 2008).

In summary, we found that chronic and periodical high glucose availability increases expression of MondoA in skeletal muscle *in vitro*, as demonstrated with human primary skeletal muscle cells grown on microcarriers, and *in vivo*, based on muscle biopsies taken pre and post soft-drink consumption for 4 weeks. Exposure to high glucose availability affected metabolic phenotype and substrate preference as well as insulin sensitivity and signalling in human skeletal muscle *in vitro* and *in vivo*. We suggest that periodical high glucose availability, like chronic soft-drink consumption, can contribute to alterations in metabolism and insulin response in skeletal muscle in lean healthy individuals with low physical activity which are symptomatic in skeletal muscle from insulin resistant and T2DM individuals. Additionally, we propose that MondoA and TXNIP are involved in the mediation of these adaptive responses to high glucose availability in skeletal muscle.

CHAPTER IV

HIGH-INTENSITY EXERCISE AND CARBOHYDRATE-REDUCED ENERGY-RESTRICTED DIET IN OBESE INDIVIDUALS

Abstract

Continuous high glycemic load and inactivity challenge glucose homeostasis and fat oxidation. Hyperglycemia and high intramuscular glucose levels mediate insulin resistance, a precursor state of type 2 diabetes. The aim was to investigate whether a carbohydrate (CHO)-reduced diet combined with high-intensity interval training (HIIT) enhances the beneficial effects of the diet alone on insulin sensitivity and fat oxidation in obese individuals. Nineteen obese participants underwent 14 days of CHO-reduced and energy-restricted diet. Ten of them combined the diet with HIIT (4 min bouts at 90% VO_{2peak} up to 10 times, 3 times a week). Oral glucose insulin sensitivity (OGIS) increased significantly in both groups [diet–exercise (DE) group: pre 377 ± 70, post 396 ± 68 mL min^{-1} m^{-2}; diet (D) group: pre 365 ± 91, post 404 ± 87 mL min^{-1} m^{-2}; $P < 0.001$]. Fasting respiratory exchange ratio (RER) decreased significantly in both groups (DE group: pre 0.91 ± 0.06, post 0.88 ± 0.06; D group: pre 0.92 ± 0.07, post 0.86 ± 0.07; $P = 0.002$). VO_{2peak} increased significantly in the DE group (pre 27 ± 5, post 32 ± 6 mL kg^{-1} min^{-1}; $P < 0.001$), but not in the D group (pre 26 ± 9, post 26 ± 8 mL kg^{-1} min^{-1}). Lean mass and resistin were preserved only in the DE group ($P < 0.05$). Fourteen days of CHO-reduced diet improved OGIS and fat oxidation (RER) in obese participants. The energy-balanced HIIT did not further enhance these parameters, but increased aerobic capacity (VO_{2peak}) and preserved lean mass and resistin.

Introduction

Obesity has reached epidemic proportions worldwide and is associated with increased morbidity and mortality. It has been recognised as a major cause of death in the USA (Allison *et al.*, 1999). Moreover, visceral obesity is linked to insulin resistance (Bjorntorp, 1991), which is a precursor state of T2DM (Martin *et al.*, 1992). Genetic as well as environmental factors, such as physical inactivity and unbalanced diet, are known to be implicated in the pathophysiology of insulin resistance (Diamond, 2003).

Skeletal muscle is the principal tissue involved in glucose disposal (Katz *et al.*, 1983) and it plays a major role in energy balance (Storlien *et al.*, 2004). Oxidative capacity is reduced in obese individuals and patients with type 2 diabetes mellitus (Kelley & Mandarino, 1990; Kelley *et al.*, 1999). It is suggested that low oxidative capacity contributes to insulin resistance (Kelley *et al.*, 1999). Moreover, it is well established that continuous high glycemic load and insufficient energy expenditure (sedentarism) challenge glycemic regulation (Manson *et al.*, 1992; Salmeron *et al.*, 1997) and oxidative capacity (Brand-Miller *et al.*, 2002). Hyperglycemia (Yki-Jarvinen *et al.*, 1987) and high intramuscular glucose levels (Tomas *et al.*, 2002) play a major role in the onset of insulin resistance. Therefore, a treatment to prevent insulin resistance should target these two factors.

A common method to reduce exogenous carbohydrate availability (glycemia) is a low-carbohydrate diet. There is some evidence that low-carbohydrate diets reduce fasting plasma insulin and glucose concentrations in overweight and obese individuals with insulin resistance and in patients with T2DM (Garg *et al.*, 1988; Dansinger *et al.*, 2005). Moreover, *in vitro* studies on insulin-resistant muscle cell cultures have shown that insulin sensitivity is restored when the glucose concentration in the medium is reduced to normal levels (Zierath *et al.*, 1994). Hanke *et al.* (2008) have recently demonstrated

in primary rabbit skeletal muscle cell cultures that oxidative capacity is improved by a reduction in glucose availability.

An effective way to reduce endogenous carbohydrate availability (glycogen) is through high-intensity interval exercise (MacDougall *et al.*, 1977). It has been suggested that training in a low-glycogen state and incomplete carbohydrate feeding may result in greater training adaptation, such as increased oxidative capacity (Hansen *et al.*, 2005; Yeo *et al.*, 2008; Morton *et al.*, 2009). Fell *et al.* (1982) have shown in rats that glucose uptake is significantly higher (60–80%) for the same insulin concentration, when muscle glycogen is kept low than when glycogen is raised by carbohydrate feeding. In fact, cycling exercise in a low muscle glycogen state has a positive acute effect on muscle glucose uptake (Richter *et al.*, 2001).

Furthermore, high-intensity interval training (HIIT) is known to increase antioxidant enzyme activity (Criswell *et al.*, 1993) and activate CAMK and AMPK, which effectively stimulate mitochondrial biogenesis and GLUT4 expression (Ojuka, 2004). Recent studies support the use of HIIT to improve insulin sensitivity (Tjonna *et al.*, 2008; Babraj *et al.*, 2009). As was concluded in a recent review on low-carbohydrate nutrition and metabolism, there is a need for further research on exercise combined with low-carbohydrate diets (Westman *et al.*, 2007).

The aim of this study was therefore to test whether the addition of HIIT to a carbohydrate (CHO)-reduced diet leads to greater improvements in insulin sensitivity compared to CHO-reduced diet alone. Consequently, we have combined a CHO-reduced and energy-restricted diet with HIIT. We compared the combined diet exercise intervention with a diet only group as control to discriminate the specific effects of the training. Importantly, the two groups had the same energy deficit. This allowed us to dissociate the specific effects of high-intensity exercise on insulin sensitivity from the already known

energy deficit related benefits of exercise on insulin sensitivity (Black *et al.*, 2005). We hypothesized that high-intensity exercise would amplify the increase in insulin sensitivity that the diet has on its own.

Methods

Participants and study design

A two-group pre-test/post-test randomized design was used to test our research hypothesis. Participants were recruited via newspaper advertisements and posters. **Figure 4.1** shows the enrolment flowchart. The diet and exercise (DE) group consisted of eight females and two males aged 37 ± 10 years, with a BMI = 32 ± 4. The diet (D) group consisted of six females and three males aged 41 ± 14 years, BMI = 32 ± 3. Age and BMI did not significantly differ between the two groups. None of the participants had been treated for insulin resistance. Participants with resting systolic and diastolic blood pressure ≥140/90 mmHg were excluded from the study. One of the participants in the D group was being treated for hypertension with an ACE inhibitor. This participant was not excluded because both his resting and exercise blood pressure values were within the normal range.

The experimental protocol was approved by the local NHS Research Ethics Committee. Written informed consent was obtained from the participants prior to testing. Before and after the intervention period, participants attended our laboratories for three or four testing sessions. All tests described below were carried out during the pre-test as well as the post-test (48 h after last exercise session). Participants were asked to refrain from heavy physical exercise 24 h prior to each test.

ECG stress test and peak oxygen uptake

During the first visit, medical and physical activity questionnaires were administered and anthropometric measures were obtained. Next, resting standard 12-lead

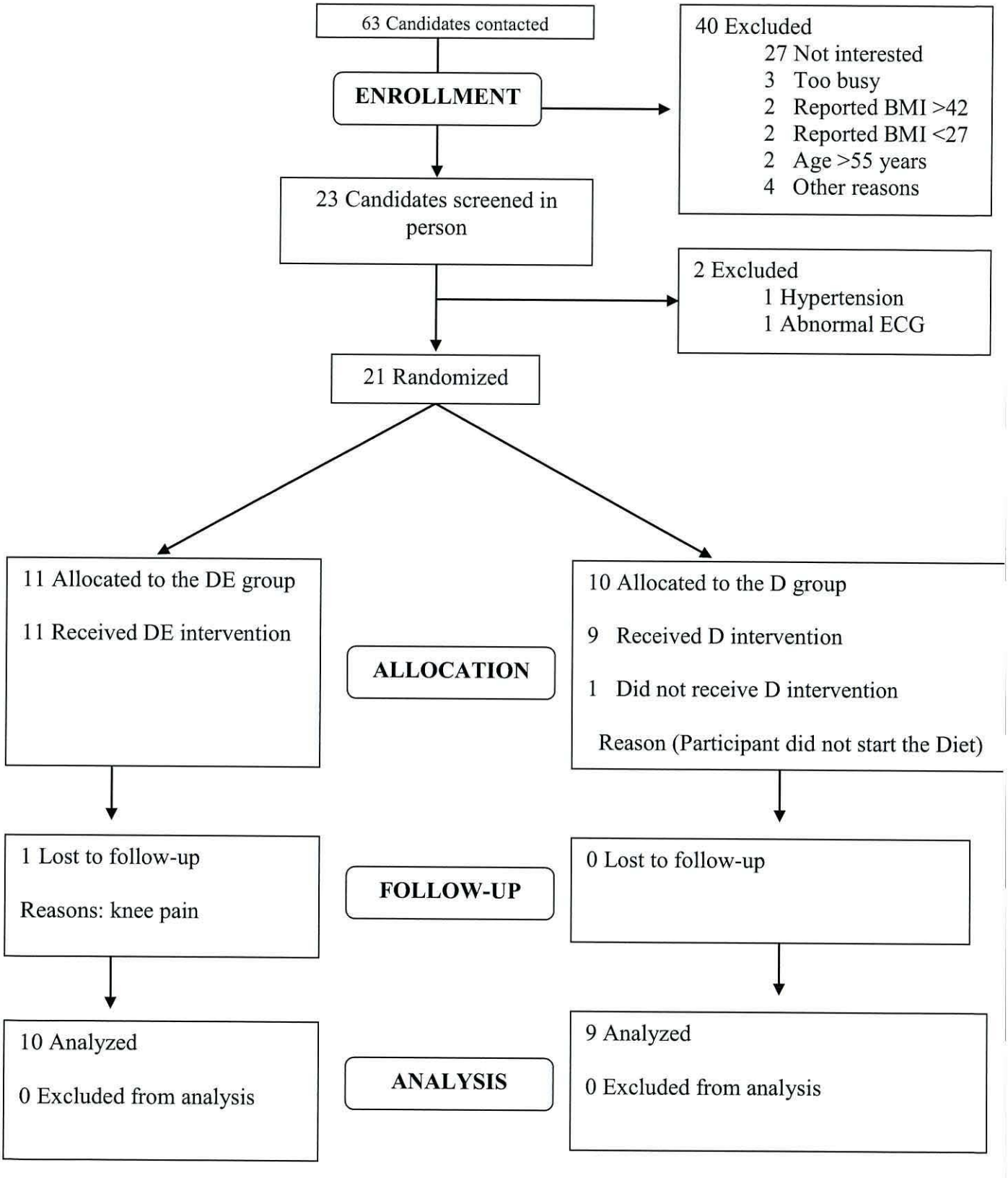


Figure 4.1 Study enrolment flow-chart. DE is the diet-exercise group and D is the diet only group.

electrocardiogram (ECG) traces were recorded (ZAN 600 CPET, Meßgeräte, Germany), while participants were lying in a supine position. Resting systolic and diastolic blood pressure (Tango SunTech, Morrisville, NC, USA) were measured with the participants seated. Subsequently, participants moved to a cycle ergometer (Corival 400, Lode, Groningen, The Netherlands) to perform an incremental cycling test to exhaustion (1 min at 50 + 20 W increments every minute). ECG electrode placement was modified to enable the participants to cycle, and ECG traces were monitored throughout the exercise. Blood pressure was measured every minute during the incremental test. Participants with abnormal resting or exercise ECG traces or blood pressure were excluded from further participation in the study. Oxygen uptake was measured breath by breath throughout the incremental test (ZAN 600 CPET, Meßgeräte, Germany) to establish peak oxygen uptake (VO_{2peak}).

Indirect calorimetry and oral glucose tolerance test

Participants were asked to attend a second visit under overnight (8–12 h) fasting condition. Resting energy expenditure and respiratory exchange ratio (RER) were measured by means of indirect calorimetry (ZAN 600 CPET, Meßgeräte, Germany) following da Rocha *et al.*'s (2006) recommendations. We determined that the coefficient of variation for our indirect calorimetry system was below 5%.

A 2-h 75 g oral glucose tolerance test (OGTT) was then executed following the WHO recommendations (WHO, 1999). Plasma glucose was analyzed for each sample by immobilized enzymatic assay (YSI 2300 STAT, Incorporated Life Sciences, Yellow Springs, OH, USA). Lipid profile was analyzed from the fasting plasma samples by optic enzymatic assay (Reflotron®, Roche Diagnostics, Mannheim, Germany). The plasma samples were then stored at -40°C for later plasma insulin analysis. Plasma insulin was analyzed by ELISA

(ultrasensitive human insulin ELISA kit, Mercodia, Uppsala, Sweden). Oral glucose insulin sensitivity (OGIS) was calculated by using Mari *et al.*'s (2001) formula. Cytokines (IL-10, IL-6 and TNF-α) and adipokines (Leptin and Resistin) were also analyzed from fasting plasma samples by ELISA (Bender MedSystems GmbH, Vienna, Austria and BioVendor, Laboratoní medicína, Modrice, Czech Republic, respectively).

Body composition

During the third visit, lean mass, fat mass and bone mineral content were assessed by dual energy X-ray absorptiometry (DXA, QDR1500, software version 5.72; Hologic, Waltham, MA, USA). The post-test DXA scan was carried out at the same time of day as the pre-test scan. Participants were wearing shorts, socks, underwear (no bra) and t-shirt. The manufacturer's recommendations for whole-body examination (daily quality control, subject positioning, scanning and analysis with Hologic software) were followed.

Muscle biopsies

Five participants of the D group and three of the DE group agreed to have needle muscle biopsies taken. These participants all came in for a fourth visit after overnight fasting. After local anaesthesia (1% lignocaine), two muscle biopsy samples were taken from the middle part of the vastus lateralis of the left leg. Biopsies were taken with a 14 ga needle (14 ga × 10 cm, Tru·Core® II Biopsy Instrument, Angiotech Gainesville, FL, USA). The two biopsies for the post-test were taken about 0.5–1 cm from the pre-test biopsy and 48 h after the last training session for the DE group. Muscle specimens were snap-frozen and stored in liquid nitrogen until further analysis. Frozen muscle biopsies (54.2 ± 0.01 mg) and 150 µL of frozen Cell- disruption Buffer (PARIS kit, Ambion, Austin, TX, USA) were pulverized (1,900

rpm for 15 s) at liquid nitrogen temperature by using a micro-dismembrator (Sartorius-Stedim Biothec, Goettingen, Germany).

Protein assay and glycogen assay

The protein content of the muscle samples was assessed by Lowry protein assay (Sigma–Aldrich, Saint Louis, MO, USA). Muscle glycogen content was determined as described in Hanke *et al.* (2008).

Myosin heavy chain (MHC) extraction and electrophoresis

After centrifugation of the homogenized samples, pellets were extracted on ice by means of ultrasound (3 s pulses followed by 3 s cooling; 10 cycles with 5 min pause on ice between each cycle) (Ultrasonic Processor VCX 130, Sonics & Materials INC, Newtown, CT, USA) with myosin extraction buffer (0.6 M KCl, 1 mM EGTA, 10 mM sodium phosphate dibasic, 1 mM PMSF, pH 6.8) at 0°C. Then, samples were thoroughly vortexed and placed on ice for 20 min. Extracts were centrifuged at 20,000×*g* for 20 min at 4°C and the supernatants were diluted 1:10 (with ice cold H2O plus 1 mM PMSF) and incubated overnight at 0°C for precipitation of actomyosin. The suspension was centrifuged the next day at 20,000×*g* for 20 min at 4°C. Pellets were resuspended in 20–50 µL extraction buffer. MHCs were separated with an SDS–PAGE (separating gel: $T = 9\%, C = 1.3\%$ and 34% glycerol, with a maximum voltage of 400 V for 36 h, at 12 mA) and were silver stained. Densitometry was then used to quantify protein bands (Gel Doc 2000 and software Quantity One-4.6.3, Bio-Rad, Hercules, CA, USA).

Interventions

Participants were asked to record a 7-day diet diary before the pre-tests (Gibson, 1993). They were told that it was important to report their eating habits very precisely. Both groups underwent a 2-week moderately low-carbohydrate and high-unsaturated fat diet. Carbohydrates represented 35% of the total energy intake; fat and protein accounted for 50 and 15%, respectively. Dietary fat was 33%

unsaturated and 17% saturated (**Table 4.1**). A daily ingestion of around 150–200 g of carbohydrate was used in this study to avoid possible risk of starvation ketosis. A reduction in calorie intake to 75% of the daily energy expenditure was applied. This calorie restriction was used to assess participant's compliance to the diet and to increase the ecological validity of the study. It was also used to test the sensitivity of our measures to an improvement in insulin sensitivity, since a 500 kcal restriction has been proven to improve insulin sensitivity in 6 days in overweight/obese people (Black *et al.*, 2005). An activity correction factor was used to account for individual habitual physical activity. Moreover, the prescribed dietary intakes for the DE group were increased via the activity correction factor to compensate for the energy costs of the training.

The food to be consumed during the diet intervention was provided to the participants by the research staff (**Appendix**, **Fig. A.8**). Participants were asked to report any alterations they had made to the imposed diet. If participants reported changes, on the following days their diets were adjusted to keep the average diet values unaltered. In addition to the CHO-reduced diet, the DE group performed HIIT during the 2-week intervention period. The training was carried out in the laboratory under supervision of the research staff on cycle ergometers (Ergomedic 874E, Monark, Vansbro, Sweden). Participants trained three times a week performing 4 min bouts at 90% VO_{2peak} with 2–3 min rest, up to ten times (Talanian *et al.*, 2007). Heart rate and rating of perceived exertion were used throughout the training to ensure that the required exercise intensity was kept. One week before the diet and training began, participants carried out a week of training familiarization. The aim of the familiarization week was to make sure that the participants felt at ease with cycling exercise and the prescribed intensity. During the familiarization week, the participants were not required to complete full training sessions. Indeed, they did not perform more than four bouts.

Table 4.1 Average daily energy and macronutrient intake.

	Baseline	CHO-reduced diet	Effect of time F (df1,df2)	Effect of time *P* level	Effect of time Partial η^2
Energy ($kcal \cdot d^{-1}$)					
DE	2363 ± 452	1886 ± 345*	31.432	<0.001	0.649
D	2317 ± 581	1662 ± 316*	(1,17)		
Carbohydrate ($g \cdot d^{-1}$)					
DE	304 ± 57	163 ± 30*	134.286	<0.001	0.888
D	305 ± 63	147 ± 25*	(1,17)		
Dietary fiber ($g \cdot d^{-1}$)					
DE	25.2 ± 11.9	23.6 ± 3.3			
D	20.4 ± 8.3	22.5 ± 1.7			
Unsaturated fat ($g \cdot d^{-1}$)					
DE	44.9 ± 17.5	63.8 ± 12.6*	23.502	<0.001	0.580
D	37.8 ± 14.5	55.8 ± 12.6*	(1,17)		
Saturated fat ($g \cdot d^{-1}$)					
DE	35.7 ± 11.8	33.5 ± 8.0			
D	28.7 ± 11.0	29.9 ± 6.9			
Protein ($g \cdot d^{-1}$)					
DE	91.7 ± 26.2	70.9 ± 9.8†	13.074	0.002	0.435
D	90.4 ± 16.7	62.7 ± 10.2†	(1,17)		

Data are presented as means ± standard deviations. * Significant main effect of time ($P < 0.001$). † Significant main effect of time ($P < 0.01$). DE is diet-exercise group and D is diet only group (DE group $n = 10$, D group $n = 9$).

Statistical analysis

Unless otherwise noted, all data are shown as mean ± standard deviations. Statistical analyses were conducted with SPSS 11.5. For the analysis of the pre- and post-glucose curves, three-way ANOVA was used and pre- and post-insulin curves were analysed with a three-way ANCOVA, with the baseline value as the covariate. All the other measures were analyzed using two-way mixed ANOVAs with within-participants factor time (pre vs. post) and between-participants factor treatment (DE vs. D). Tests of simple main effect of time were

conducted as follow-up. Pearson correlations for the difference between pre- and post-tests were conducted. Muscle biopsy data are shown for qualitative purposes, since the small sample size did not allow inferential use. The significance level was set at 0.05 (two-tailed). The significant level for trends was set at 0.10 (two-tailed).

Results

Diets

Baseline diet and diet intervention details are shown in **Table 4.1**. No group × time interaction or main effect of group was found for daily energy intake and macronutrient intake, even though the DE group had their diet increased by an activity factor accounting for the exercise. A type two error might explain why no interaction was found for these parameters (partial $\eta^2 = 0.044$; observed power = 0.132). Energy, carbohydrate and protein intake were significantly decreased during the intervention in both groups (main effect of time, all $P < 0.01$). Unsaturated fat intake was significantly higher during the intervention than at baseline (main effect of time, $P < 0.001$). The percentage of carbohydrates in the diet decreased from 54% on average at baseline to 35% of the CHO-reduced diet for both groups. The percentage of proteins remained unaltered (17% at baseline vs. 15% during intervention). Unsaturated fat was considerably lower in the baseline diet for both groups than during the intervention (17% at baseline vs. 33% during intervention). Finally, saturated fat was slightly higher in the intervention diet (12% at baseline vs. 17% during intervention).

Exercise training and aerobic exercise capacity

The participants in the DE group trained at a mean intensity of 90 ± 0.5% VO_{2peak} for 234 ± 7 min. The oxygen uptake values of the incremental maximal cycling tests showed a group × time interaction for VO_{2peak} ($F(1,17) = 16.156$, $P=0.001$, partial $\eta^2 = 0.487$). Follow-up tests revealed that cardiovascular fitness was significantly increased in the DE group (pre 27 ± 5, post 32 ± 6 mL kg^{-1} min^{-1}; simple main effect of time, $t(9) = -4.855$, $P=0.001$, partial $\eta^2 = 0.724$), while the D group did not change (pre 26 ± 9, post 26 ± 8 mL kg^{-1} min^{-1}). The participants in the DE
group increased their VO_{2peak} on average by 16% (**Fig. 4.2**).

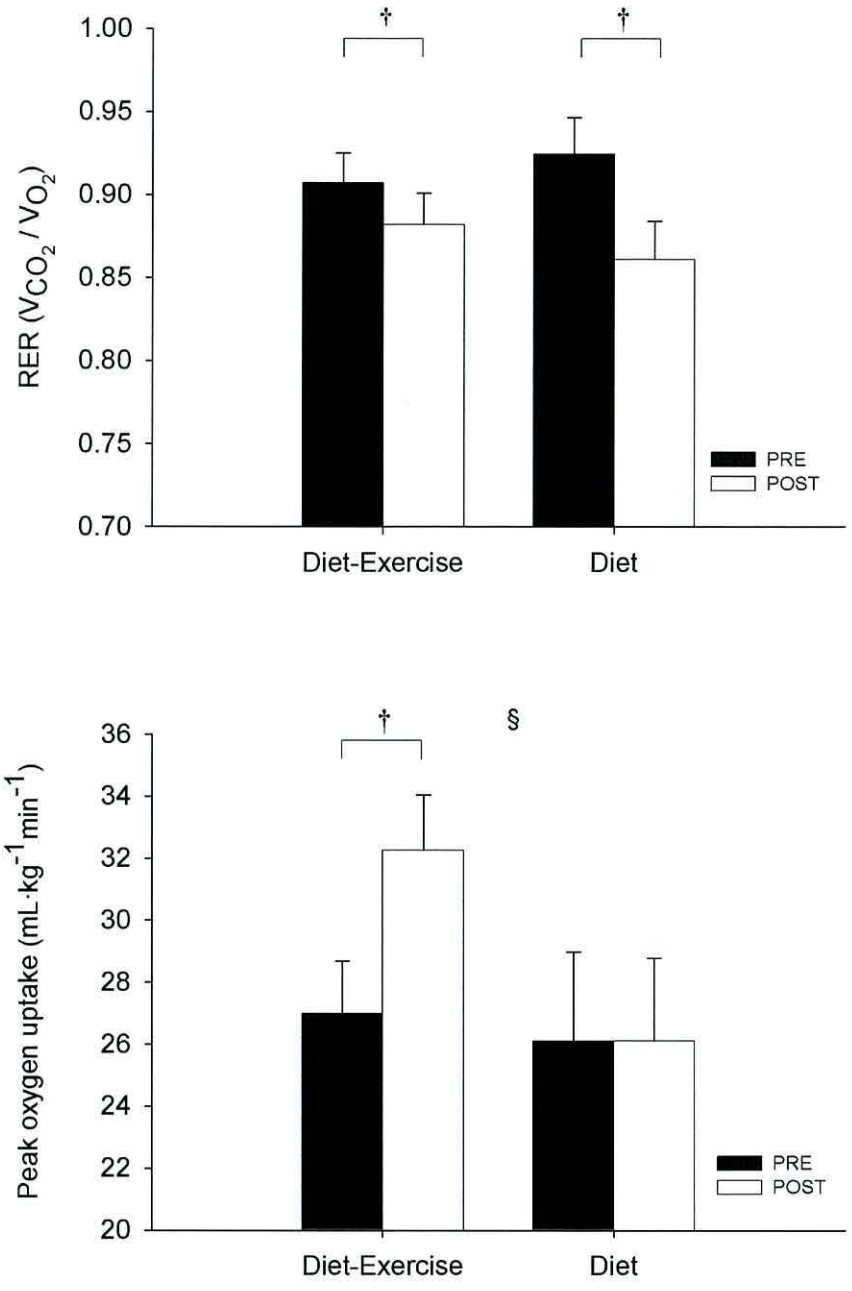


Figure 4.2 Respiratory exchange ratio (*upper panel*) and peak oxygen uptake normalized for body weight (*bottom panel*). Data are presented as mean ± SEMs of the mean. [§]Significant group × time interaction ($P < 0.01$), [†] significant effect of time ($P < 0.01$). DE group $n = 10$, D group $n = 9$.

Energy balance

The energy balance was estimated from measurements of changes in body composition by using Elia *et al.*'s (2003) method. Post-test

analyses revealed no significant divergence between the negative energy balances of the two groups. In fact, from the indirect calorimetry data we estimated that the DE group's total energy expenditure excluding HIIT training was 32,368 kcal over 14 days of intervention. Based on a mean VO_2 of 2.7 L min^{-1} (90% of VO_{2peak}) and an energy expenditure during exercise of ~5 kcal L^{-1} O_2, mean exercise energy expenditure during the HIIT was 13.5 kcal min^{-1}. The total energy needed for the HIIT intervention was therefore 3,159 kcal (average total training time was 234 min). Consequently, the target energy intake for the DE group, to achieve a 25% energy restriction, was 26,654 kcal over 14 days. Hence, the 14 day caloric intake of 26,404 kcal (1,886 kcal day^{-1}) reported by the DE group did indeed match the target energy intake.

Body composition

Body composition data are shown in **Table 4.2**. The changes in total body weight were significantly larger for the D group than for the DE group. Indeed, a group × time interaction was found for total body weight ($F(1,17) = 5.811$, $P=0.028$, partial $\eta^2 = 0.255$); post hoc tests revealed a significant weight loss for the D group (simple main effect of time, $t(8) = 5.881$, $P < 0.001$, partial $\eta^2 = 0.609$) and a trend ($P=0.102$; partial $\eta^2 = 0.269$; observed power = 0.370) for the DE group. Both groups significantly lost fat mass at the same rate (no interaction; main effect of time, $F(1,17) = 12.323$, $P = 0.003$, partial $\eta^2 = 0.420$). Trunk fat percentage was also significantly reduced (main effect of time, $F(1,17) = 5.099$, $P=0.037$, partial $\eta^2 = 0.231$), no interaction for this parameter was found probably due to the small sample size ($F(1,17) = 2.140$, $P=0.162$, partial $\eta^2 = 0.112$; observed power = 0.282). A significant group × time interaction was found for lean mass ($F(1,17) = 8.583$, $P=0.009$, partial $\eta^2 = 0.335$), showing a significant decrease only in the D group (simple main effect of time, $t(8) = 3.164$, $P = 0.013$, partial $\eta^2 = 0.556$).

Table 4.2 Body weight and body composition at baseline and after two weeks of intervention.

	Baseline	Post intervention
Body weight		
(kg)	91 ± 15	90 ± 16[§]
DE	91 ± 18	89 ± 18[§*]
D		
Fat mass (kg)		
DE	39 ± 10	38 ± 11[†]
D	41 ± 9	40 ± 8[†]
Lean mass (kg)		
DE	48 ± 11	49 ± 11[§]
D	47 ± 13	46 ± 13[§‡]
Trunk fat (%)		
DE	44.8 ± 7.7	43.7 ± 8.0[#]
D	45.9 ± 13	45.7 ± 6.7[#]

Data are presented as means ± standard deviations. [§] Significant group×time interaction ($P < 0.05$). [†] Significant main effect of time ($P < 0.01$). [#] Significant main effect of time ($P < 0.05$). [*] Significant simple main effect of time ($P < 0.001$). [‡] Significant simple main effect of time ($P < 0.05$). DE is diet-exercise group and D is diet only group (DE group $n = 10$, D group $n = 9$).

OGTT and insulin sensitivity

OGTT data are presented in **Table 4.3** (see also **Appendix**, **Fig. A.6**). Insulin sensitivity (OGIS) was significantly improved after the intervention in both D and DE groups (no group × time interaction; main effect of time, $F(1,17) = 25.209$, $P<0.001$, partial $\eta^2 = 0.597$) (**Fig. 4.3**), while changes in plasma glucose and insulin separately did not reach significance. The area under the glucose curve (AUGC) was not significantly altered. A trend toward a significant Pearson correlation between fasting plasma glucose changes and fat mass

changes was found (r = 0.43, P = 0.061, n = 19). No significant Pearson correlation was found between OGIS and fat mass (r = -0.34, n = 19).

Table 4.3 Plasma glucose and insulin at baseline and after two weeks of intervention.

	Baseline	Post intervention
Fasting plasma glucose		
($mmol \cdot L^{-1}$)	5.6 ± 1.3	5.4 ± 0.8
DE	5.1 ± 0.4	5.0 ± 0.4
D		
2h plasma glucose ($mmol \cdot L^{-1}$)		
DE	6.6 ± 3.5	6.1 ± 3.3
D	5.3 ± 1.0	5.2 ± 0.9
Fasting plasma insulin		
($pmol \cdot L^{-1}$)	59.0 ± 32.5	54.2 ± 34.2
DE	87.6 ± 59.6	73.0 ± 53.5
D		
2h plasma insulin ($pmol \cdot L^{-1}$)		
DE	438 ± 356	270 ± 249
D	541 ± 418	476 ± 638
AUGC ($mmol \cdot L^{-1} \cdot min^{-1}$)		
DE	887 ± 366	835 ± 249
D	788 ± 132	756 ± 101

Data are presented as means ± standard deviations.

DE is diet-exercise group and D is diet only group (DE group n = 10, D group n = 9). OGIS is oral glucose insulin sensitivity and AUGC is area under the glucose curve.

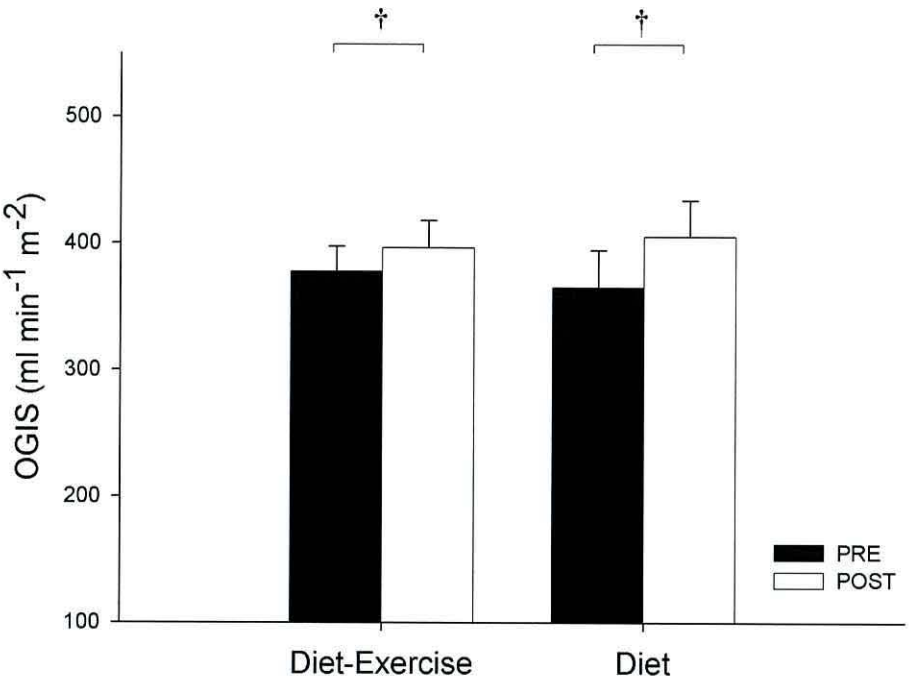


Figure 4.3 Oral glucose insulin sensitivity (OGIS). Data are presented as mean ± SEM. † Significant effect of time ($P < 0.001$). DE group $n = 10$, D group $n = 9$.

Lipid profile

Lipid profile data are shown in **Table 4.4**. Both groups improved their lipid profile during the interventions. Total plasma cholesterol decreased (main effect of time, $F(1,17) = 12.425$, $P=0.003$, partial $\eta^2 = 0.442$) and so did the triglycerides (main effect of time, $F(1,17) = 9.9193$, $P=0.008$, partial $\eta^2 = 0.351$) and LDL (trend toward a main effect of time, $F(1,17) = 4.312$, $P=0.053$; partial $\eta^2 = 0.202$; observed power = 0.500). HDL did not change significantly. A significant Pearson correlation was found between fat mass changes and total cholesterol changes ($r = 0.69$, $P = 0.001$, $n = 19$) and between fat mass changes and LDL changes ($r = 0.67$, $P = 0.002$, $n = 19$).

Cytokines and adipokines

Cytokine and adipokine data are shown in **Table 4.4**. There were no significant changes in cytokines (IL-10, IL-6, TNF-α). Leptin was significantly reduced in both groups after the interventions (main effect of time, $F(1,17) = 18.964$, $P<0.001$, partial $\eta^2 = 0.527$) and resistin showed a significant interaction ($F(1,15) = 5.613$, $P=0.032$, partial $\eta^2 = 0.272$) and a significant simple main effect of time for the D group ($t(7) = -3.794$, $P = 0.007$, partial $\eta^2 = 0.673$) (see **Table 4.4**). A trend toward a significant negative Pearson correlation was found between VO_{2peak} changes and resistin changes ($r = -0.43$, $P = 0.084$, $n = 17$).

Fat oxidation

The RER, derived from resting indirect calorimetry, did not show a significant interaction or main effect of group. However, there was a significant main effect of time for this variable ($F(1,17) = 12.706$, $P=0.002$, partial $\eta^2 = 0.428$). Both groups reduced RER and therefore increased their basal fat oxidation (DE group: pre 0.91 ± 0.06, post 0.88 ± 0.06; and D group: pre 0.92 ± 0.07, post 0.86 ± 0.07) (**Fig. 4.2**). The decreased fasting RER suggests that both groups had a reduced carbohydrate oxidation and an augmented fatty acid oxidation at rest. A trend toward a significant Pearson correlation was found between RER changes and LDL changes ($r = 0.41$, $P = 0.085$, $n = 19$). No Pearson correlation was found between RER changes and fat mass changes ($r = 0.30$, $n = 19$).

Muscle glycogen

Individual and group overnight fasting muscle glycogen contents, assessed by enzymatic determination from vastus lateralis muscle biopsy samples are shown in **Figure 4.4**. Overall, the decrease in muscle glycogen was 20%. A trend toward a significant Pearson correlation was found between muscle glycogen changes and fasting plasma glucose changes ($r = 0.67$, $P = 0.070$, $n = 8$).

Table 4.4 Lipid profile, cytokines and adipokines at baseline and after two weeks of intervention.

	Baseline	Post intervention
Total plasma cholesterol		
($mmol \cdot L^{-1}$)	4.60 ± 0.94	4.24 ± 0.83[†]
DE	5.00 ± 0.76	4.49 ± 0.88[†]
D		
Plasma triglycerides ($mmol \cdot L^{-1}$)		
DE	1.44 ± 1.10	1.05 ± 0.74[†]
D	1.33 ± 0.37	0.97 ± 0.25[†]
Plasma HDL ($mmol \cdot L^{-1}$)		
DE	0.98 ± 0.29	1.01 ± 0.35
D	0.97 ± 0.29	0.89 ± 0.34
Plasma LDL ($mmol \cdot L^{-1}$)		
DE	2.97 ± 0.74	2.76 ± 0.66 [t]
D	3.43 ± 0.63	3.17 ± 0.69 [t]
Plasma IL-10 ($pg \cdot mL^{-1}$)		
DE	1.18 ± 0.67	1.41 ± 1.11
D	0.95 ± 0.07	1.17 ± 0.64
Plasma IL-6 ($pg \cdot mL^{-1}$)		
DE	0.86 ± 0.72	1.14 ± 0.80
D	0.95 ± 0.65	0.94 ± 0.58
Plasma TNF-α ($pg \cdot mL^{-1}$)		
DE	48.4 ± 11.6	48.3 ± 20.0
D	42.2 ± 19.3	42.6 ± 15.8
Plasma Leptin ($ng \cdot mL^{-1}$)		
DE	26.2 ± 14.5	19.8 ± 15.8[*]
D	25.3 ± 11.7	18.2 ± 11.6[*]
Plasma Resistin ($ng \cdot mL^{-1}$)		
DE	5.22 ± 3.18	4.78 ± 2.20[§]
D	3.73 ± 1.00	5.45 ± 1.94[§‡]

Data are presented as means ± standard deviations. [§] Significant group×time interaction ($P < 0.05$). [*] Significant main effect of time ($P < 0.001$).

† Significant main effect of time ($P < 0.01$). ‡ Significant simple main effect of time ($P < 0.01$). ˈ Trend towards a main effect of time ($P < 0.1$). DE is diet-exercise group and D is diet only group (DE group $n = 10$, D group $n = 9$). For IL-10, IL-6 and DE group $n = 10$, D group $n = 8$; for TNF-α and Resistin DE group $n = 9$, D group $n = 8$.

Myosin heavy chains

Myosin heavy chains (MHCs) were measured to ensure that the pre- and post-test muscle biopsies were comparable. The statistical analysis for the muscle biopsy MHC extracts was conducted after pooling the data of the two groups. A large proportion of MHC IIa and IId/x was found. No significant difference was observable in MHC isoforms before and after the interventions (MHC IId/x pre 13.8 ± 14.2%, post 11.8 ± 10.9%; MHC IIa pre 47.6 ± 15.6%, post 51.2 ± 10.7%, MHC I pre 38.6 ± 16.4%, post 37.0 ± 14.9%). The homogeneity of the myosin heavy chain phenotypes within and between the participants proves an excellent repeatability of the biopsy parameters giving solidity to the glycogen results. Secondly, it confirms the low physical activity level of the participants (**Appendix**, **Fig. A.5**).

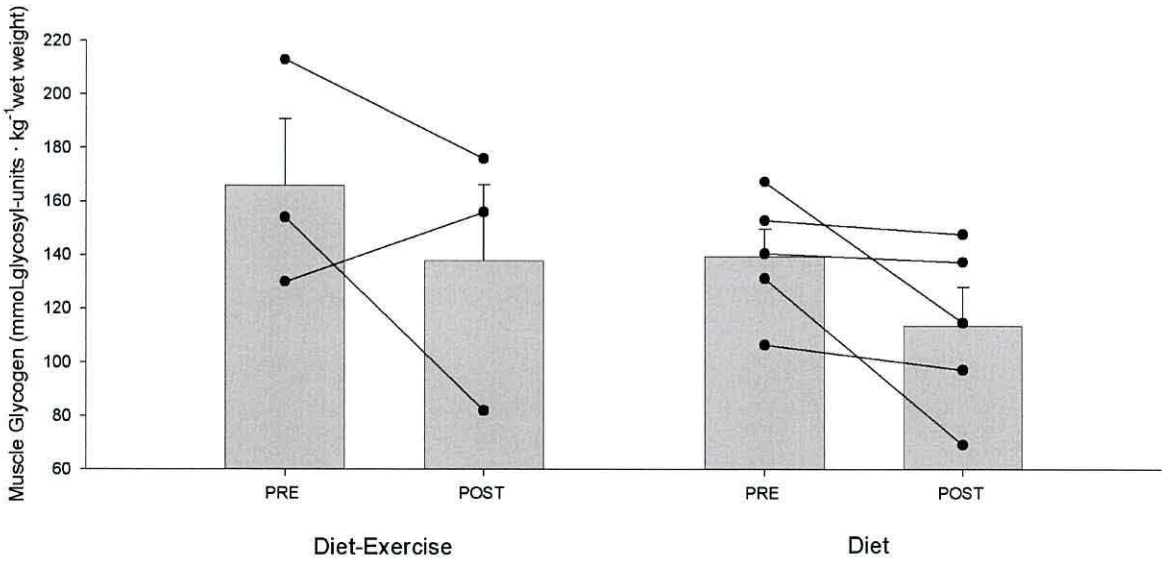


Figure 4.4 Changes in muscle glycogen content. Data are presented as mean ± SEM. DE group $n = 3$, D group $n = 5$.

Discussion

Our study was the first to combine a CHO-reduced diet with high-intensity interval training. The novel finding is that the positive effect of a 14-day carbohydrate- and energy-reduced diet on insulin sensitivity and fat oxidation was not enhanced by high-intensity exercise training in sedentary, obese individuals. However, specific benefits of the energy-balanced HIIT were lean mass and resistin preservation and improved aerobic capacity.

The specific effects of high-intensity exercise training on insulin sensitivity were isolated by controlling for the exercise- induced energy deficit, which is known to improve insulin response (Black *et al.*, 2005). Glucose clearance during high-intensity cycling exercise is twice as high when muscle glycogen content is low than when it is normal (Richter *et al.*, 2001). Consequently, there was additional glucose clearance during the exercise in the DE group. We therefore expected an additional improvement in insulin sensitivity in the DE group. However, we have found that
regardless of the transient endogenous carbohydrate reduction (glycogen depletion), the main contributor to enhanced chronic insulin sensitivity was the energy and/or exogenous carbohydrate deficit, which was the same in both groups. These findings are in agreement with Black *et al.* (2005) who used moderate aerobic exercise and did not find an effect on insulin sensitivity when energy balance was preserved. The improvement in insulin sensitivity in the present study is in line with what has been found in obese, type 2 diabetic patients (Accurso *et al.*, 2008) and non-diabetic
obese individuals (Samaha *et al.*, 2003; Black *et al.*, 2005).

As shown in **Figure 4.4**, the fasting muscle glycogen content seemed to be reduced by the CHO-reduced diet in both groups, although the small number of muscle specimens did not allow us to be conclusive. We have also found a reduction in fasting RER in both groups, which

demonstrates an improvement in fat oxidation, which is usually low in obese people (Kelley *et al.*, 1999). Our results are in line with the finding that fasting RQ decreased significantly after just 2 days of low-carbohydrate diet (Smith *et al.*, 2000). We believe that the reduction in RER could be attributed to low-carbohydrate availability, rather than calorie restriction, since it has been shown that fasting leg RQ was not affected in obese people after severe calorie restriction (Kelley *et al.*, 1999). The inclusion of a low-carbohydrate eucaloric diet group in the present study could have directly clarified this. We did not find a difference in fasting RER between the D group and the DE group. However, this could simply be because, in this study, the effect of HIIT was concealed by the strong effect of carbohydrate and/or energy restriction.

Moreover, our data show that the blood lipid profile was improved to the same extent in both groups revealing that carbohydrate and/or energy restriction might have a major influence on this parameter. In particular, LDL seemed to correlate with the enhancement in fat oxidation, although this did not reach statistical significance. These findings are in line with studies showing that low-carbohydrate diet improves lipid profile in both obese diabetic and non-diabetic individuals (Garg *et al.*, 1988; Parillo *et al.*, 1992; Volek *et al.*, 2009). This is very important, considering the high correlation between blood lipids and cardiovascular disease (Wallace & Anderson, 1987).

Body composition was changed by the 14 days intervention in both groups. The loss in total body weight and body fat during our CHO-reduced diet is in line with previous findings (Brehm *et al.*, 2003). It is often believed that changes in body composition could fully explain increased insulin response, but several studies have demonstrated that improvements in insulin sensitivity could be independent of changes in fat mass (Black *et al.*, 2005). More likely, exercise and diet improved insulin response via carbohydrate and/or energy restriction. We have found significant correlations between loss in fat mass and improved

lipid profile, but not between fat mass changes and RER changes and fat mass changes and OGIS changes. Therefore, our study seems to confirm that the mechanism behind the improvement in insulin sensitivity may be carbohydrate and/or energy related. An added benefit of combining HIIT to CHO-reduced diet might be a quicker decrease in visceral fat. However, the small sample size did not allow us to clearly see this effect.

As was expected, we found a decrease in leptin in both groups (Friedman & Halaas, 1998). The decrease in leptin does not seem to be associated with the specific effects of HIIT. Although the study design does not allow us to differentiate between the contribution of carbohydrate restriction and the contribution of energy restriction on leptin decrease, other investigations have reported a reduction in leptin with negative energy balance regardless of the carbohydrate availability (Brehm *et al.*, 2003). Strikingly, we found an increase in resistin in the D group, while in the DE group it was unaltered. Considering that a decrease in resistin has been shown in type 2 diabetic patients after a high-intensity exercise training (Kadoglou *et al.*, 2007), possibly the increase in resistin in our study was counteracted by HIIT. To support this possibility, we found a trend toward a significant negative Pearson correlation between the changes in VO_{2peak} and in resistin. Although resistin was first believed to be a factor contributing to insulin resistance, there is now uncertainty about this relation (Filkova *et al.*, 2009). However, there is growing evidence for the involvement of resistin in inflammatory, endocrine and tumor diseases (Filkova *et al.*, 2009).

Our CHO-reduced and energy-restricted diet was well tolerated, since all 19 participants who underwent the diet finished it without reporting negative aspects. Moreover, the fact that the diet included a moderate weight loss adds ecological validity to this study. Although a single dietary element is often identified as the cause of metabolic and cardiovascular diseases (e.g., saturated fat causes heart disease), there

is growing evidence that practically all western world diseases have multifactorial dietary causes (Cordain *et al.*, 2005). In the present diet intervention, carbohydrate was greatly reduced (from 54 to 35%) and replaced mainly
with unsaturated fat (from 17 to 33%), while percentages of saturated fat, proteins and dietary fibres remained unaltered. The current results show that in the groups studied,
there were improvements in insulin response, fat oxidation, blood lipids and body composition despite the fact that there was no reduction in the absolute dietary intake of saturated fat. All those improvements are benefits possibly associated with the partial replacement of carbohydrate with unsaturated fat. In fact, from numerous surveys carried out on Siberian Chukotka and westernized Alaskan Natives (Nikitin *et al.*, 1991; Nobmann *et al.*, 1994), it is clear that a diet high in carbohydrate and low in unsaturated fat increases their risk of metabolic and cardiovascular diseases.

Although the HIIT did not magnify the loss in fat mass seen in the D group, a clear benefit associated exclusively with the HIIT was the preservation of lean mass shown in the DE group. It is well established that diet alone often leads to loss in lean mass as well as fat mass (Chaston *et al.*, 2007). The addition of physical exercise is crucial, if not to increase the systemic benefits already induced by the diet, to preserve metabolically active tissue. However, not all types of exercise are suitable for preserving lean mass. Indeed, Grediagin *et al.* (1995) proved that high-intensity exercise (80% VO2max) increases the lean mass more than twice as much as low-intensity exercise (50% VO2max). Therefore, as we show in this study, HIIT can successfully maintain metabolically active tissue during a short-term diet regime, which would reduce lean mass when applied on its own. Furthermore, HIIT was effective in increasing aerobic exercise capacity by 16% (VO_{2peak}) in only 2 weeks of training. Talanian *et al.* (2007) found a similar (12%) increase in their study also only in 2 weeks.

Importantly, this study does not disqualify exercise from being a good way to counteract metabolic and cardiovascular diseases, but it shows the importance of the combination with a diet that does not compensate for the energy expended via the exercise. Moreover, this study underlines the additional benefits attributable solely to HIIT, such as lean mass and resistin preservation and a rapid increase in cardiovascular fitness. Although our intervention lasted only 2 weeks, long-term studies have revealed beneficial effects of high-intensity physical activity on reducing diabetes risk in overweight individuals with impaired glucose tolerance (Lindstrom *et al.*, 2003). Additionally, a 20% carbohydrate diet with some caloric restriction has been shown to have long-lasting (22 months) positive effects on bodyweight and glycemic control in obese diabetic patients (Nielsen & Joensson, 2006). However, more long-term studies are needed, especially because the advantages of low-carbohydrate diets over low-fat diets at 6 months seem to recede at 12 months (Hession *et al.*, 2009). Moreover, the small sample size of the present study might have concealed some of the differences between the two groups.

Conclusions

In conclusion, 19 sedentary obese individuals improved their oral glucose insulin sensitivity, fat oxidation and lipid profile by undergoing 14 days of CHO-reduced and energy-restricted diet. The addition of energy-balanced HIIT was not sufficient to further enhance these parameters. However, HIIT improved aerobic capacity and preserved lean mass and resistin. Therefore, this study suggests that carbohydrate and/or energy-restricted diets are effective in reducing the risk of type 2 diabetes in obese, sedentary individuals in the short term. However, longer and larger intervention studies are needed to confirm these outcomes and to ensure that the benefits are long lasting.

CHAPTER V

REVERTING TYPE 2 DIABETES MELLITUS BY COMBINING LOW-CARBOHYDRATE DIET WITH HIGH-INTENSITY INTERVAL TRAINING: A CASE REPORT

Abstract

Background: Chronic hyperglycaemia is associated with insulin-resistance and T2DM. This project aims to reduce high extracellular glucose levels (hyperglycaemia) and intracellular glucose availability (glycogen) *in vivo*.

Case Report: An untreated, overweight (BMI 32) 39 year old female with T2DM (fasting glucose 9.0 mmol/L, insulin 18.9 mU/L) underwent a 4 week low-carbohydrate diet (35% carbohydrates, 15% proteins, 50% fats, 33% unsaturated) combined with high-intensity interval training (three times a week, 10 bouts of 4 min 90% $V_{O_{2peak}}$, 3 min rest) to reduce glucose availability and activate muscle glycogen breakdown. Oral glucose tolerance tests revealed that post intervention fasting glucose had dropped to 6.9 mmol/L and fasting insulin to 17.0 mU/L. Insulin-Sensitivity-Index increased from 1.53 to 1.96. Intramuscular glycogen levels (from the vastus lateralis muscle using biopsies) declined in two weeks from 154 to 82 mmol glucosyl U/kg wet weight. Conclusions: In this patient our intervention of carbohydrate restriction combined with exercise-induced glycogen depletion could reverse T2DM to an insulin resistant state within 4 weeks.

Introduction

Chronic hyperglycaemia is linked to insulin-resistance and T2DM (Rossetti *et al.*, 1987). *In vitro* studies on insulin-resistant cells have shown that insulin-sensitivity is restored when the glucose concentration in the medium is reduced to normal levels (Davalli *et al.*, 1991; Zierath *et al.*, 1994; Buren *et al.*, 2003)]. As in **Chapter 4,** this study aims to reduce high extracellular glucose levels (hyperglycaemia) and intracellular glucose availability (glycogen) *in vivo*. A 39 year old female with T2DM underwent a low-carbohydrate diet (Low-CHO) and exercise-induced glycogen depletion to reduce glucose availability and activate muscle glycogen breakdown. We hypothesised that the combined effect of these interventions would amplify the recovery of insulin-sensitivity in T2DM patients. We also tried to assess the feasibility of using this combined intervention in diabetic patients.

The case

The subject was diagnosed with impaired glucose tolerance (fasting glucose: 6.6 mmol/L and 2h: 8.8 mmol/L) at the age of 34. She had elevated blood cholesterol and triglycerides (6.20 mmol/L and 4.44 mmol/L). When she first attended our laboratory, she was overweight (39 of age, BMI: 32) and sedentary. Her diet consisted of 50% carbohydrates, 19.6% proteins and 29.4% fats (9.7% saturated and 19.7% unsaturated). Her average energy intake was 2144 kcal/day (measured as per **Chapter 4**). Fasting and 2 hour plasma glucose levels during an oral glucose tolerance test (OGTT) (for details refer to **Chapter 4**) were high (9.0 mmol/L and 16.0 mmol/L, respectively) and so was the fasting insulin level (18.9 mU/L). Insulin-sensitivity index (ISI) (Matsuda & DeFronzo, 1999) was low (1.53) and plasma cholesterol, triglycerides, HDL and LDL were abnormal (6.06 mmol/L; 3.81 mmol/L; 0.87 mmol/L and 3.47 mmol/L). Her body composition was 44.5% fat and 52.6% lean mass (measured by DXA). ECG traces throughout a stress-test on a cycle-ergometer were normal and $V_{O_{2peak}}$ was 21.6 $ml \cdot kg^{-1} \cdot min^{-1}$.

After a weeklong familiarization with the high-intensity interval-exercise, a four week diet and training intervention started. Three times a week the subject trained on a cycle-ergometer: 10 bouts of 4 minutes at 90% $V_{O_{2peak}}$ (Talanian *et al.*, 2007) with 3 minutes of rest between each bout (same as **Chapter 4**). This was combined with a Low-CHO diet with an energy restriction to 75% of resting energy expenditure (1655 kcal/day). Food was provided following a diet composed of 35% carbohydrates, 15% proteins and 50% fats (17% saturated and 33% unsaturated). The subject reported high adherence to the diet and completed all training sessions successfully. The OGTT, indirect calorimetry, DXA and stress ECG test were repeated after the 4 week intervention. A needle muscle biopsy was collected before and half way through the intervention at 2 weeks.

The OGTTs revealed that fasting and 2 hour plasma glucose levels were considerably reduced from 9.0 to 6.9 mmol/L and from 16.0 to 11.2 mmol/L after 4 weeks intervention (**Fig. 5.1**). Fasting insulin levels were decreased from 18.9 to 17.0 mU/L and ISI increased from 1.53 to 1.96. Fasting plasma cholesterol and triglycerides decreased from 6.06 to 5.67 mmol/L and from 3.81 to 2.24 mmol/L respectively. The intervention resulted in a 2.9 kg weight loss, while body fat decreased by 3.2 kg and lean body mass increased by 0.3 kg. $V_{O_{2peak}}$ increased by 6 $ml \cdot kg^{-1} \cdot min^{-1}$.

Needle muscle biopsies were taken from the right Vastus Lateralis muscle (VL) in fasting conditions. From the baseline muscle biopsy specimen, MHCs composition as analysed by SDS-PAGE was 20% MHC IId/x, 45% MHC IIa and 35% MHC I. Two weeks of intervention resulted in a reduction of muscle glycogen from 154 to 82 mmol glucosyl U/kg wet weight.

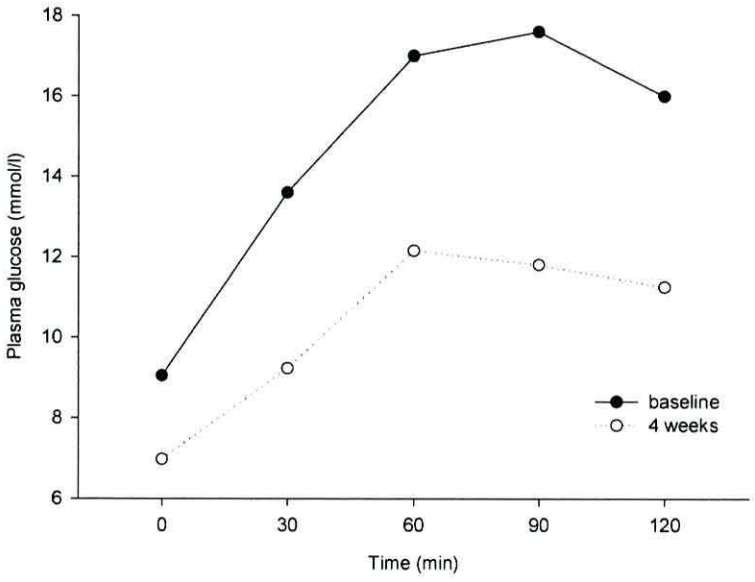


Figure 5.1 OGTT curves before and after four weeks of training and diet intervention.

Discussion

In this case report it has been shown that in an untreated female with T2DM a simple, low cost intervention of Low-CHO diet combined with high-intensity interval exercise can drastically reverse the main features of T2DM. Fasting glucose decreased, insulin sensitivity increased and there was a reduction in blood lipids. This confirms that a reduction of glucose availability can improve insulin-sensitivity (Garg *et al.*, 1988; Parillo *et al.*, 1992) even in diabetic patients. The increase in glucose clearance seems to be correlated with a reduction in muscle glycogen. Fell *et al.* (1982) have demonstrated that glucose uptake at the same insulin concentration was significantly higher (60-80%), when muscle glycogen was kept low, than when glycogen was raised by carbohydrate feeding. In accordance to this, by adding exercise-induced glycogen depletion to the Low-CHO diet in this case study, the glycogen storage was diminished by 35%, in only two weeks. The reduction in blood lipids and body fat found in this study agrees with other Low-CHO diets on healthy and diabetic people (Brehm *et al.*, 2003; Samaha *et al.*, 2003). Moreover, the improved lipid profile found in the subject in the post intervention examination may also be due to an increased exercise-induced fat oxidation in skeletal muscle. In fact, Talanian *et al.* (2007) measured a significant increase in muscle mitochondrial β-hydroxyacyl-CoA dehydrogenase and citrate synthase activity after only two weeks of the same type of high-intensity interval training. This has additionally been confirmed by an increase in the $V_{O_{2peak}}$ of the subject, which can be attributed to an improvement in cardiovascular fitness and skeletal muscle oxidative capacity.

In terms of high abundance of muscle fibre type IIx/d, which is shown to be correlated with sedentarism and type 2 DM (Venojarvi *et al.*, 2005) we also found a high percentage of MHC IIx (20%) in the VL of the subject.

Therefore, considering the results achieved within a short period of time in this subject; this combination of Low-CHO diet and high-

intensity interval training could be an economic and effective alternative to the conventional drug therapies for diabetes. In fact, the cardiovascular improvement due to this intervention can not be achieved by means of drug therapy. Indeed, Herman *et al.* (Herman *et al.*, 2005) found that the costs per quality adjusted life year are approximately $1,100 for the lifestyle intervention (diet & physical activity) and $31,300 for the Metformin intervention, showing the considerable advantage for the NHS in adopting an economical treatment for insulin resistance and or diabetes such as diet combined with exercise.

CHAPTER VI

GENERAL DISCUSSION

Summary of main findings

In **Chapter 2** we have shown that obese people not only have a reduced taste perception of sweet and salty, they also have a higher implicit attraction to sweet. Moreover, continuous exposure to sugary drinks changes sweet taste perception, and this may change food preference. Indeed, a subgroup of lean participants, who did not prefer sweet before the soft drink exposure, increased their liking for sweet after the exposure.

In **Chapter 3** we focused on the metabolic consequences of high glucose availability. We used an *in vitro* model, based on isolated human skeletal muscle satellite cells. We exposed these myotubes to high glucose availability for one week and measured metabolic markers as well as glucose and insulin signalling proteins. *In vitro*, skeletal muscle metabolism was altered by high glucose exposure. In fact, glycolysis (GAPDH) and de novo lipogenesis (ACC) were enhanced by high glucose exposure. Protein expression in the skeletal muscle tissue of the glucose sensing transcription factor MondoA was increased and so was the expression of the radical stress and insulin resistance related protein TXNIP. Activation of Akt, a key molecule in the insulin signalling pathway, was diminished by high glucose availability. These results were partially confirmed *in vivo*. Although skeletal muscle metabolic markers did not change significantly with soft drink exposure on protein level, increases in fat mass and RER indicated an increase in glycolytic flux and lipogenesis. MondoA indicated a clear trend towards an increase associated with the glucose syrup based drink supplementation. GSK-3 phosphorylation measured in the skeletal muscle was reduced, possibly as a result of Akt deactivation. Importantly, whole body insulin sensitivity was shown to be reduced by the soft drink consumption.

With the exercise-diet study presented in **Chapter 4** we tried to reduce the risk of T2DM (i.e., insulin resistance reduction) in obese, sedentary

people by restricting CHO ingestion and storage. The outcomes of this study show that a low-CHO and -energy diet of only 2 weeks duration is sufficient to improve insulin sensitivity, and that there is no additional benefit of performing high intensity exercise on this parameter. However, high intensity exercise is important to counteract body composition alterations (e.g., lean mass loss) caused by the diet and to increase cardiovascular fitness.

In **Chapter 5** we confirmed in a T2DM patient the positive effect of the lifestyle intervention tested in obese people (**Chapter 4**) over a period of 4 weeks. The strikingly positive effects of low-CHO diet and exercise on insulin sensitivity and the absence of side effects reported by the patient must lead to a larger and longer study involving T2DM patients.

Changes in taste lead to changes in food preference

With the studies presented in **Chapter 2**, the relation between the human passion for sweet tasting food/drinks and obesity has been clarified. From the previous literature it was not clear whether obese individuals have an altered taste perception of sweet or not (Donaldson *et al.*, 2009). The sample of overweight/obese young adults that we tested in this thesis clearly showed that this group of people perceives sweet taste less intense than their lean counterparts. Importantly, as already shown in previous investigations (Frijters & Rasmussen-Conrad, 1982) and in **Chapter 2**, overweight/obese individuals perceived sweet taste as pleasant as the normal weight controls did. Therefore, overweight/obese people find sweet taste pleasant in the same way as lean people do, but they cannot taste it as well (or, as intense), and this may generate a hedonic satisfaction gap in obese persons (**Fig. 6.1**). We define hedonic satisfaction as the magnitude of the hedonic reward response generated by a certain positive stimulus. We suggest that in order to fill this hedonic satisfaction gap, produced by lower sweet taste sensitivity, obese people may consume more

sugary food/drinks (**Fig. 6.1**). The stronger implicit attraction to sweet, found in the obese group, might be an indication of this need to ingest more sugary food/drinks to fill the hedonic satisfaction gap. It is very likely, considering the existing literature (Bertino *et al.*, 1982; Duffy *et al.*, 2003), that the lower taste intensity ratings shown by the obese participants are a consequence of years of high sweet food/drinks exposure. This means that consuming sweet food/drinks, in the long run, creates the need for more and more sugary products to maintain the same level of hedonic satisfaction. This may lead to intermittent excessive intake of sweet food/drinks, which has been shown to be addictive (Avena *et al.*, 2008).

The study conducted in **Chapter 2** on obese and lean participants did not investigate directly the effects of sugary food/drink consumption on taste and food preference. To study these direct effects we tested lean and fairly sedentary participants with little or no former soft drink consumption; this because obese people generally have a high soft drink consumption. The exposure to soft drinks did alter sweet taste intensity and pleasantness perceptions, proving that constant soft drink consumption has an important impact on food acceptance. Those changes also strengthened the suggestion that obese people have a reduced sensitivity to sweet taste because of their large consumption of sweet sugary products. From our soft drink intervention study we also

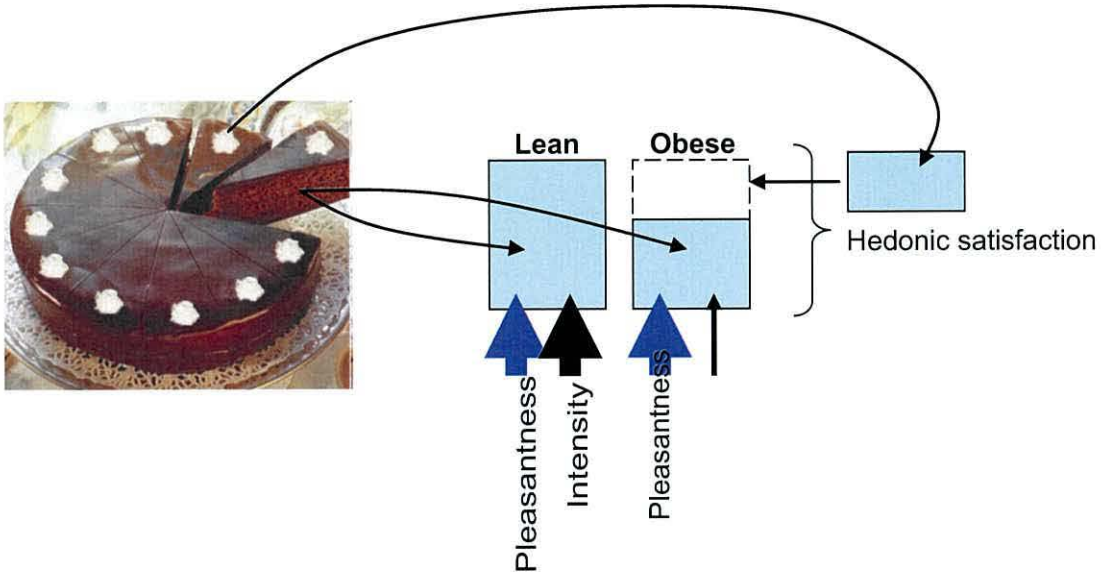


Figure 6.1 Hedonic satisfaction gap hypothesis.

gathered a direct insight into how soft drinks can change food preference, probably via changing taste perception. Sucrose-dislikers increased their explicit preference for sweet. Whilst, sucrose-likers did not increase their liking for sweet with the supplementation, perhaps because of a ceiling effect.

Although eleven out of twelve participants in the soft drink intervention study had a positive implicit preference towards sweet to start with, this was not increased at the end of the exposure period. Perhaps the fact that pleasantness changed along with intensity perception of sweet did not generate a hedonic satisfaction gap, also because sweet taste intensity scores seemed to be disturbed rather than decreased. That is to say that intensity scores after the intervention were higher for lower sucrose concentrations and lower for higher sucrose concentrations, instead of being consistently higher or

consistently lower for all sucrose concentrations. Additionally, implicit attitude scores could change in relation to leptin sensitivity. In fact, hunger increases implicit attitude towards food in general (Seibt *et al.*, 2007), and leptin resistance results in an increased appetite (Scarpace & Zhang, 2009). The soft drink supplementation raised the plasma leptin levels, but one month of high leptin levels may not be enough to induce central leptin resistance in lean subjects. The hypothesis that leptin could influence sweet food consumption by altering taste for sweet food is fascinating. Our study was not designed to test this hypothesis, yet we found a trend towards a significant within-subject correlation between leptin levels and sugar intake and between leptin levels and preference for sweet. The data presented in **Chapter 2** seem to give credit to the hypothesis that soft drink consumption induces behavioural and physiological alterations which are linked to obesity and metabolic disorders.

Glucose sensing system and insulin resistance

As just described, a high consumption of refined CHO and sugar may have addictive effects. In parallel, high refined CHO and sugar consumption has negative health effects, leading to obesity and metabolic dysfunction (Malik *et al.*, 2006; Dhingra *et al.*, 2007; Palmer *et al.*, 2008). The combined approach using primary human satellite cell cultures (*in vitro*) and muscle biopsy specimens (*in vivo*) in **Chapter 3** focused on investigating whether high glucose availability induces changes to the metabolism of glucose in skeletal muscle tissue. The advantage of using an *in vitro* approach is that the experimental conditions are better controlled, and that long term *in vivo* adaptations can be obtained after only a few days (e.g., 7 days) of exposure. On the other hand, the limitation of having only an *in vitro* approach is that the results obtained are often not sufficient to draw definite conclusions and these results need to be confirmed *in vivo*. Thus, the combination of these two approaches is appropriate to answer our research questions concerning the effects of glucose availability on

muscle metabolism. This approach is presented in **Chapter 3** and in the **Appendix, Fig. A.2**.

The muscle satellite (stem) cells were all gathered from healthy lightly active participants. The cells were differentiated using specific conditions (microcarriers), which have previously been shown to improve differentiation (Kubis *et al.*, 1997), and therefore the quality of the muscle fibres grown *in vitro* (myotubes). The *in vitro* glucose availability experiments indicated that skeletal muscle cells respond to the abundance of energy/glucose by increasing their glycolytic flux and glucose storage (Acheson *et al.*, 1988), although muscle glycogen can only increase to a certain extend beyond which de novo lipogenesis increase occurs (Acheson *et al.*, 1988). The parallel increases in GAPDH and ACC found in **Chapter 3** suggests this scenario. Considering previous *in vitro* investigations on high or low glucose availability from rabbit myotube cultures (Hanke *et al.*, 2008), a decrease in oxidative capacity in response to high-glucose exposure was expected. However, the marker that we selected to monitor TCA cycle function, FUM, did not change. This marker was chosen because its changes are known to alter the TCA cycle (Ramakrishna *et al.*, 2001). One reason could be that, by measuring the protein level of this marker, changes in the cell need to be very large to be detected. Presumably, the deactivation of AMPK and therefore PGC1α [mitochondrial biogenesis stimulator (Liang & Ward, 2006)] consequent upon an increased glucose flux and glycogen storage needed longer exposure time. This might suggest that a reduction in oxidative capacity is not a primary response to high glucose availability, but perhaps it occurs after other metabolic modifications have taken place, or this might also be related to a slow turnover of the oxidative marker analysed. Citrate Synthase (CS) could have been a more sensitive enzyme to monitor the TCA cycle (Hanke *et al.*, 2008), but the poor quality (in respect to its low sensitivity) of the primary antibodies on the market did not allow reliable quantification (see **Appendix, Fig. A.1**).

Clearly, skeletal muscle cells are programmed to sense rising glucose levels and promptly respond to this rise. **Chapter 3** showed for the first time in primary human myotubes that MondoA responds to high glucose levels. The Western blots of nuclear and cytoplasmic protein fractions confirmed the accumulation of MondoA in the nucleus with high glucose availability (Stoltzman *et al.*, 2008), but also showed an overall cellular increase in MondoA in this condition. If we compare GSK-3 and MondoA nuclear contents when insulin is high, we notice that GSK-3 responds primarily to insulin and that MondoA responds primarily to glucose. MondoA is responsible for the regulation of the majority (~75%) of glucose related genes (Stoltzman *et al.*, 2008). It is also known to increase the expression of glycolytic enzymes, such as HK, and the anaerobic glycolytic enzyme LDH. Hypothetically, high glucose-dependent MondoA activation may be behind the shift in fibre distribution, from slow-oxidative to fast-glycolytic, as seen in obese people and T2DM patients (Simoneau *et al.*, 1995; Simoneau & Kelley, 1997; Oberbach *et al.*, 2006), and the higher lactate production in obese individuals (Lovejoy *et al.*, 1990), but this needs further investigations. In response to high-glucose, MondoA promotes metabolic modifications that equip the cell with the appropriate enzymatic phenotype. That is to say, MondoA senses higher glucose levels and therefore over-expresses glycolytic ezymes. Moreover, MondoA, by increasing the transcription of TXNIP and perhaps other molecules, seems to reduce glucose uptake. TXNIP is certainly associated to ROS accumulation, which could lead to mitochondrial dysfunction (Saxena *et al.*, 2010). However, by inducing insulin resistance (Parikh *et al.*, 2007) in healthy cells, TXNIP can remove the cause of the energy/glucose excess. Since TXNIP suppression requires intact insulin signalling (Parikh *et al.*, 2007), in healthy subjects TXNIP could function as a homeostatic switch that integrates glucose sensing and insulin signalling to control cellular energy status (Muoio, 2007). In insulin resistant persons however, TXNIP levels remain high, exposing the muscle cells to radical stress and cellular damage.

In our *in vitro* and *in vivo* studies the effects of high-glucose exposure on insulin signalling in skeletal muscle cells was mainly assessed by Akt and GSK-3 phosphorylation, which determines their activation and deactivation respectively. Following an insulin stimulus, Akt is activated by phosphorylation and in turn it deactivates GSK-3 also by phosphorylation (Cross *et al.*, 1995). By acutely stimulating our primary human myotubes, which were cultured in high glucose without insulin, following addition of 100 nM insulin, we confirmed the reduction in Akt phosphorylation previously observed by Huang *et al.*, (2002) in L6 muscle cells exposed to high glucose and insulin (25 mM; 100 nM, respectively). Bearing in mind that Akt is a key molecule for GLUT4 translocation, acting on AS160, high glucose-induced Akt deactivation indicates that high energy/glucose availability can lead to insulin resistance.

A possible mechanism to explain our finding could be that glucose influences Akt phosphorylation via TXNIP activity, since enhanced Akt activity has been shown in TXNIP deficient mice (Chen *et al.*, 2008). Reduced Akt activity decreases GSK-3 phosphorylation and this may have worsened GS function therby inducing insulin resistance (Hojlund *et al.*, 2009) or increased the detrimental action of GSK-3 directly on IRS-1 (Eldar-Finkelman & Krebs, 1997). However, several other stimuli, such as $p90^{RSK}$, lead to GSK-3 inactivation through S21/9 phosphorylation (Lavoinne *et al.*, 1991). The studies in **Chapter 3** cannot rule out the effects of these alternative insulin stimulated molecules. It seems that total and phosphorylated GSK-3 contents were not elevated by glucose abundance. Nevertheless, in an additional set of *in vitro* experiments we have manipulated not only glucose but also insulin concentration. We observed that regardless the glucose levels, when insulin was high there was a high presence of GSK-3 in the nucleus. However, in the absence of insulin, high-glucose availability is sufficient to increase GSK-3 nuclear presence. These outcomes could explain why we did not find an overall difference in p-GSK-3 or total

GSK-3 when myotubes exposed to two different glucose concentrations, at the same high insulin level. Qualitatively we confirmed these results by an additional immunofluorescence experiment (**Appendix, Fig. A.2**). We observed the highest presence of p-GSK-3 in the high glucose, high insulin condition, and a virtual absence of p-GSK-3 with normal glucose levels, without insulin. GSK-3 phosphorylates more than 40 proteins (Jope & Johnson, 2004). In the nucleus, GSK-3 regulates many transciption factors and it has been associated with apoptosis (Jope & Johnson, 2004). However, GSK-3β-knockout mice die after around 14 days by TNF-α induced hepatocyte apoptosis (Hoeflich *et al.*, 2000). Therefore, it appears that GSK-3 levels have to be maintained within an appropriate range. The GSK-3 in the nucleus inhibits cyclic-AMP response element binding protein (CREB) (Grimes & Jope, 2001), which is activated by AMPK and CaMKII and modulates PGC1α (Egan *et al.*, 2010). Hence, the results from **Chapter 3** seem to suggest indirectly that AMPK and CaMKII deactivation occurs in myotubes cultured in high glucose medium. This, in the long term, could contribute to a reduction in oxidative capacity.

We have tried to corroborate the *in vitro* findings with the outcomes gathered from muscle biopsy specimens collected from healthy, lean participants who underwent four weeks of soft drink supplementation. At a systemic level our results confirmed Reiser *et al.*'s (1979) outcomes that 2 g of sugar per kg body weight per day increase fasting glucose and insulin levels. Additionally, drinking soft drinks on top of their habitual diet worsened participant's body composition, but not their blood lipid profile. Additionaly, the whole body metabolism of these participants was negatively influenced by the intervention. Indeed, their basal CHO oxidation rate was increased to the detriment of basal fat oxidation, which in turn was diminished. However, generally the metabolism in healthy, young and lean individuals is flexible (Storlien *et al.*, 2004), and the increase in resting RER does not necessarily imply that enzymatic alterations took place in the

skeletal muscle tissue. On a protein level we could not confirm the glycolysis up-regulation (GAPDH increase) or de novo lipogenesis enhancement (ACC increase) that we saw *in vitro*. Possibly, these adaptations took place at mRNA level (analyses of this parameter is still to be conducted). Importantly, we confirmed *in vivo* that an increase in protein and gene expression of MondoA occurred with high glucose availability, and thus shows its central role in this condition. Conversely, TXNIP did not seem to respond to the soft drink treatment, either at a protein or mRNA level. The discrepancy between *in vitro* and *in vivo* TXNIP results perhaps indicates that TXNIP needs a continuous and longer high glucose exposure than what was achieved by one month of soft drink supplementation. Probably, *in vitro* the constantly large glucose gradient increases the insulin independent glucose uptake via GLUT1 as well as the insulin dependent glucose uptake mediated by GLUT4. This higher basal intracellular glucose flux constantly activates MondoA and therefore expresses TXNIP. Conversely, an average of two bottles of soft drink a day may not increase GLUT1's basal glucose uptake and consequently basal intracellular glucose flux, applying much less pressure on the MondoA-TXNIP system. The fact that insulin sensitivity was reduced and with it some alterations in the insulin signalling pathway were found (decrease in GSK phosphorylation) reveals that, via yet not completely known mechanisms, high energy/glucose availability can lead to insulin resistance. Longer soft drink intervention studies are needed to corroborate the adaptations found *in vitro*.

Furthermore, recently Kaadige *et al.* (2009) have shown *in vitro* that if high glucose activates MondoA resulting in TXNIP expression, in the presence of glutamine high glucose still activates MondoA, which paradoxically inhibits TXNIP expression. Since insulin stimulates glutamine transport into the muscles (Rennie *et al.*, 1996), and in the soft drink study (**Chapter 3**) we found elevated fasting insulin levels, it is likely that the higher intramuscular glutamine could be behind the *in vitro*/*in vivo* discrepancy for TXNIP. Consistently, in the diet-

exercise study, although we did not find changes in MondoA expression, we found an increase in TXNIP (**Appendix**, **Fig. A.7**). This is in line with Varnier *et al.*'s (1995) findings that exercise and lack of food can decrease skeletal muscle glutamate and glutamine concentrations. Our data seem to confirm the model proposed by Kaadige *et al.* (2009), which is schematically reported in **Figure 6.2**.

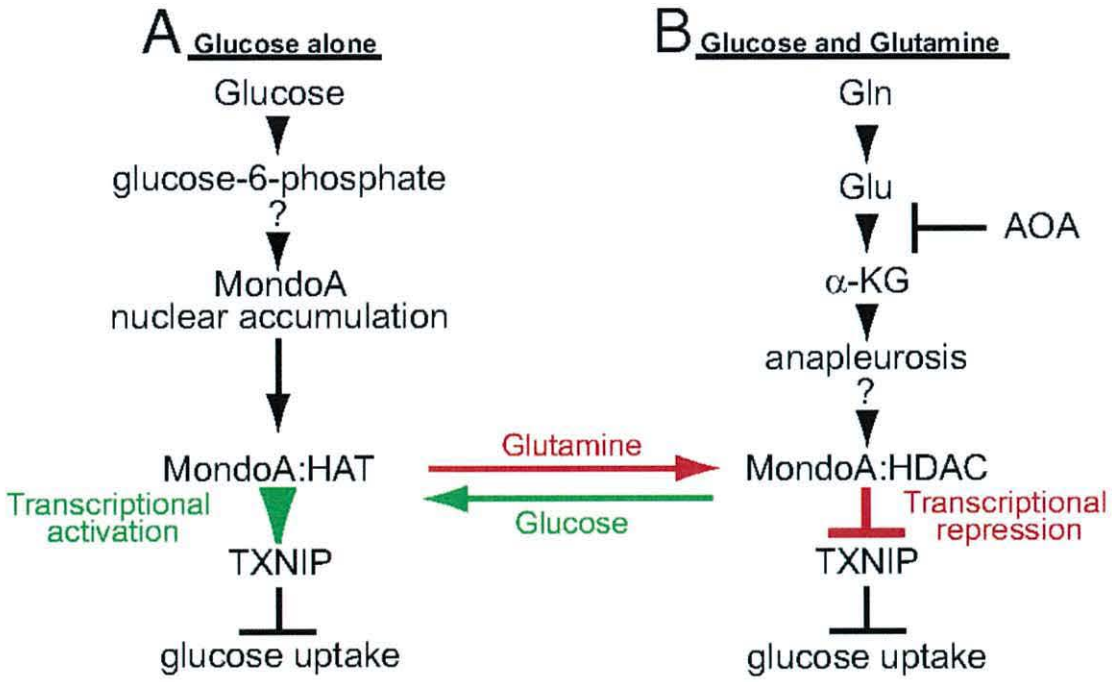


Figure 6.2 MondoA is a nutrient-dependent transcription factor. As indicated, the mechanisms controlling how MondoA accumulates in the nucleus in response to glucose and how glutamine-dependent mitochondrial anapleurosis (metabolic reaction that refills the TCA cycle) converts MondoA to a transcriptional repressor are not known. HAT, histone acetyl-transferase; HDAC, histone deacetylase; α-KG, α-ketoglutarate; AOA, aminooxyacetate; Gln, glutamine; Glu, glutamate. (from Kaadige *et al.*, 2009).

Exercise/diet and insulin sensitivity

Starting from the assumption that high energy/glucose availability induces metabolic maladaptation (towards a glycolytic

phenotype) and insulin resistance, we designed a study (**Chapter 4**) during which a CHO- reduced, restricted energy diet was combined with exercise induced glycogen depletion. Exogenous glucose availability was decreased by the CHO-reduced diet and endogenous glucose availability was manipulated by applying or not applying exercise-induced glycogen depletion. The main outcome of this lifestyle intervention study was that the CHO-reduced, low energy diet succeeded in improving insulin sensitivity in obese, sedentary people. By taking into account the energy deficit produced by the exercise and compensating for it, we tried, as much as possible, to isolate the specific effects of exercise. No specific effects of the high intensity glycogen depleting exercise on insulin resistance were observed. Our study seems to suggest that low energy and/or the exogenous glucose availability are the preponderant factors in enhancing insulin sensitivity in obese, sedentary individuals. Other parameters (i.e., lipid profile, oxidative metabolism) were also improved mainly by the diet. The hypothesis that the transient reductions in muscle glycogen, leading to elevated glucose clearance, would affect insulin sensitivity positively and permanently, seems to be discarded. Exercise however, was not completely redundant. HIIT, which we chose mainly for its glycogen depleting capacity, preserved lean mass and resistin levels, and improved cardiovascular fitness. It is likely that the positive effects of HIIT on insulin sensitivity shown in previous studies (Tjonna *et al.*, 2008; Babraj *et al.*, 2009) are not due to the exercise per se but to the energy deficit induced by the exercise (Braun & Brooks, 2008).

Unfortunately, only 8 of the 19 participants in the diet and exercise study agreed to have muscle biopsies taken. This restricted the inference value of the biopsy results. However, if we consider that HIIT did not add any further benefits to insulin sensitivity and metabolism, we could pool the muscle biopsy data of the two groups, thereby increasing the sample size. Obviously, this procedure has some limitations since it assumes that the exercise did not have significant effects at cellular level. Bearing in mind this limitation we found a

slight decrease in ACC with the "low glucose availability" condition (**Appendix, Fig. A.7**). No differences were found in the protein expressions of MondoA and the other enzymatic markers, except for TXNIP which increased.

As shown in the case study (**Chapter 5**), the combination of reduced-CHO diet, HIIT, and energy restriction might be a good way to improve glucose regulation in T2DM patients, in particular those who are at an early stage of the disease. This lifestyle intervention could reduce the doses of medications that these patients would need otherwise. Although this study followed the effects of our reduced-CHO, high intensity exercise program on only one diabetic patient, we have applied it for 4 weeks instead of 2. The intervention was well tolerated and showed potential.

Limitations of the research program

With the studies presented in this thesis we aimed to advance our understanding of the causal relationship between refined-CHO/sugar consumption and the onset of insulin resistance. To investigate this relationship we adopted a multidisciplinary approach. This approach allowed us to observe psychological, behavioural, and whole body physiological parameters as well as cellular biological parameters. Because of the broadness of this approach, a strategic decision had to be made. We preferred to have an insight in the global phenomenon rather than focusing only on a single aspect. Of course, this decision implied that some aspects had to be sacrificed. Therefore the thesis could not investigate the separate effects of energy availability on the metabolism of skeletal muscle cells or the separate effects of sweet without energy supply on taste and liking.

On the one hand we decided to focus on what we believed would most likely happen in the "real world" in sedentary/lightly active people, and therefore, we assumed that high glucose availability is usually

accompanied by high energy availability. On the other hand, a limited amount of funding has contributed to restricting our research.
A euenergetic diet group in the soft drink and in the diet exercise intervention studies (**Chapters 3and 4**) would have provided additional valuable information. However, this information is not completely unknown. Many studies have investigated the effects of high- or low-CHO diets and high-, moderate-, and low-intensity exercise in euenergetic conditions (Bisschop *et al.*, 2002; Black *et al.*, 2005; Noakes *et al.*, 2006; Holtz *et al.*, 2008; Hansen *et al.*, 2009; Sacks *et al.*, 2009; Foster *et al.*, 2010). These studies seem to agree that "a calorie is a calorie" (Westman *et al.*, 2007), thus for the same energy deficit, a similar weight loss is to be expected regardless the macronutrient availability or the exercise intensity. For insulin sensitivity the picture is more complex, that is, energy deficit does improve insulin sensitivity (Black *et al.*, 2005), but the diet macronutrient composition interacts with this improvement. In fact, Sacks *et al.* (2009) in their randomised two year trial found that high-CHO energy restricted diet could not reduce fasting serum insulin. Moreover, Noakes *et al.* (2006) clearly demonstrated that an isocaloric, very low CHO diet lowers fasting insulin concentrations more than other isocaloric diets. Several studies also agree on the fact that low-CHO availability reduces cardiovascular risk, by increasing HDL levels (Noakes *et al.*, 2006; Sacks *et al.*, 2009; Foster *et al.*, 2010). Finally, it is important to note that, as Holtz *et al.* (2008) have demonstrated, macronutrient availability (i.e., CHO deficit) in the meal following exercise alters glucose and fat metabolism regardless of the energy balance. Holtz's study makes an important point, that is, "consuming carbohydrate after exercise benefits athletes, but may be detrimental to sedentary, overweight individuals, who do not have the same metabolic machinery (e.g., high mitochondrial density, oxidative enzymes, etc) and who exhibit impaired insulin action compared with their lean counterparts" (Holtz *et al.*, 2008). Their conclusions might be applicable to our diet and exercise intervention study where we could

not separate the effects of CHO restriction from the effects of energy restriction on insulin sensitivity.

Potential areas for future research

The studies presented in this thesis open up new areas of research. Referring to the *in vitro* studies, the next step could be to study the role of TXNIP in the high glucose availability-induced onset of insulin resistance. ROS has been identified as a direct cause of insulin resistance (Paolisso *et al.*, 1994). Indeed, plasma hydroperoxide (an oxidative stress marker) levels are higher in T2DM patients compared to non-diabetic controls (Nourooz-Zadeh *et al.*, 1997). Moreover, obese individuals have augmented ROS levels (Vincent & Taylor, 2006). As we have seen previously, TXNIP's negative regulation of the thiol-reducing system makes cells more susceptible to oxidative stress by ROS (Nordberg & Arner, 2001) and it modulates redox status and ROS-mediated signalling to regulate metabolism and other processes (Muoio, 2007). For these reasons a pharmaceutical inhibition of TXNIP might have positive effects on the insulin function in T2DM patients. TXNIP-induced ROS formation blockade could be achieved via N-acetylcysteine (NAC) therapy. In cancer research, N-acetylcysteine (NAC) has been used to block TXNIP-induced ROS formation [e.g. SK-MEL-28 melanoma, (Cheng *et al.*, 2004)]. NAC is the acetylated variant of the amino acid L-cysteine. It is an excellent source of sulfhydryl groups, it stimulates glutathione synthesis, and it acts directly as a free radical scavenger (Kelly, 1998). NAC is traditionally used for treatment of congestive and obstructive lung diseases and against paracetamol intoxication. There is a great deal of *in vitro* evidence showing that NAC increases insulin sensitivity in pancreatic beta-cells (Kaneto *et al.*, 1999) as well as in skeletal muscle cells (Haber *et al.*, 2003). Finally, *in vivo* oral administration of NAC improved insulin sensitivity in women with polycystic ovary syndrome (Fulghesu *et al.*, 2002; Masha *et al.*, 2009).

Further development of the research presented here could lead to a better understanding of the effects of chronic soft drink consumption on the glucose sensing systems and the endocannabinoid system, and the possible role of exercise in protecting against the negative effects of soft drink consumption. As described previously, endocannabinoid receptors (CB1 and 2) are present in many tissues, including the brain, liver, skeletal muscle, adipose tissue, and gastrointestinal tract (Engeli & Jordan, 2006). The endocannabinoid system is implicated in the central and peripheral regulation of energy expenditure (Matias & Di Marzo, 2006). Chronic consumption of soft drinks and therefore high glucose availability alters insulin response also via varying the central and peripheral function of the endocannabinoid system. Indeed, the endocannabinoid system has been shown to be upregulated in obesity (Engeli *et al.*, 2005) and CB1 agonists have a positive effect on reducing body weight in obese individuals (Engeli, 2008). For these reasons it is important to investigate the effects of high glucose availability on the endocannabinoid system.

Finally, a randomised clinical trial in T2DM patients to test the long term effects of the lifestyle intervention employed in **Chapter 4** should be conducted. The data we show in **Chapter 4** and **5** suggest that this lifestyle intervention could have several benefits and that if the energy deficit produced by the high-intensity exercise is not compensated, even greater insulin sensitivity is to be expected.

Conclusions

The human passion for sweet tasting food/drinks comes from ancestral survival mechanisms, which must have evolved when absorbable CHO and sugar were hard to obtain. However, nowadays, in industrialised countries, sweet sugary food/drinks are widely available and thus human's natural attraction to sweet becomes a problem. This thesis confirmed that people have a positive attitude towards sweet. Furthermore, we observed that overweight/obese people have a stronger

attraction to sweet than lean individuals. This stronger subconscious attitude towards sweet in the overweight/obese population is perhaps due to changes in the perception of sweet taste intensity, which are likely caused by continuous sweet food/drinks consumption (e.g., soft drinks). Indeed, we found that one month of soft drink consumption was enough to change taste and liking for sweet in otherwise sporadic soft drink consumers. Large amounts of sugary food/drinks consumption, when not accompanied with adequate physical activity, produces high exogenous glucose availability, which we have shown that this glucose abundance changes the metabolism of the skeletal muscle tissue. Seemingly, high energy/glucose availability promotes cellular adaptations in the skeletal myofibres, which shift the metabolic phenotype from oxidative/insulin sensitive to more glycolytic/insulin resistant. We demonstrated that the oversupply of glucose increases the risk of T2DM by reducing insulin sensitivity and disrupting insulin cell signalling. Glucose sensing proteins, such as MondoA, seem to play a major role in these adaptations. In order to reduce the risk of T2DM in obese sedentary individuals, exogenous glucose and energy restriction are two valuable and effective methods. A short term exercise-induced glycogen depletion training program does not appear tofurther improve insulin sensitivity if the exercise-induced energy deficit is compensated by the diet. However, high intensity exercise is an effective tool for preserving lean mass and improving cardiovascular fitness in a short period. Finally, glucose availability plays a major role in the metabolism of skeletal muscle tissue and can be associated with insulin function.

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APPENDIX

Western blots of oxidative markers

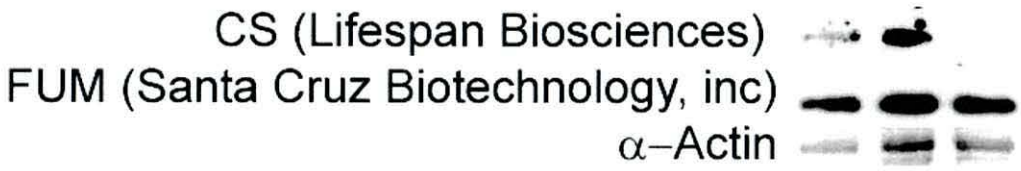

Figure A.1 Western blots rappresenting Citrate Synthase (CS) protein expression in human primary myotubes exposed to high 15 mM D-glucose. CS was blotted using IgG_1 mouse monoclonal primary antibody (LS-C35936) purchased from Lifespan Biosciences, Interchim, France. The primary antibody was probed overnight (1:250) and the membrane was incubated in pico luminol reagent solution for 5 minutes, then it was exposed to X-ray film for 10 minutes. The other blots show fumarate hydratase (FUM) protein expression in the same samples, FUM was blotted by using IgG_{2b} mouse monoclonal antibody (SC-100743), purchased from Santa Cruz Biotechnology, Inc. USA. The primary antibody was probed for 4 hours (1:2,000) and the membrane was incubated in pico luminol reagent solution for less than 5 minutes, then it was exposed to X-ray film for 10-15 s.

Confocal immunofluorescence of primary human myotubes

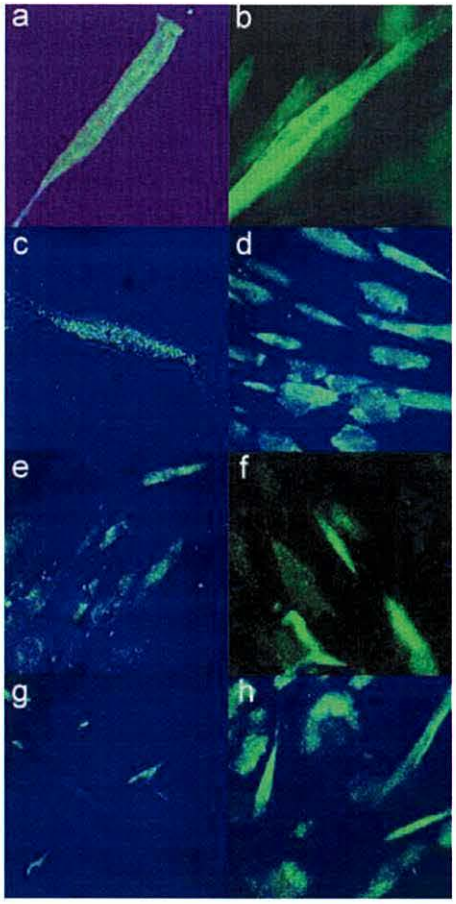

Figure A.2 Confocal immunofluorescence of myotubes, stained for phosphorylated or total GSK-3; a) P-GSK-3, High-Glucose/High-Insulin (15 mM D-glucose, 10 µg/mL of insulin); b) total GSK-3, High-Glucose/High-Insulin; c) P-GSK-3, High-Glucose/No insulin; d) total GSK-3, High-Glucose/No insulin; e) P-GSK-3, Normal-Glucose/High-Insulin (5 mM D-glucose, 10 µg/mL of insulin); f) total GSK-3, Normal-Glucose/High-Insulin; g) P-GSK-3, Normal-Glucose/No insulin; h) total GSK-3, Normal-Glucose/No insulin.

Western blots referred to in Table 3.3

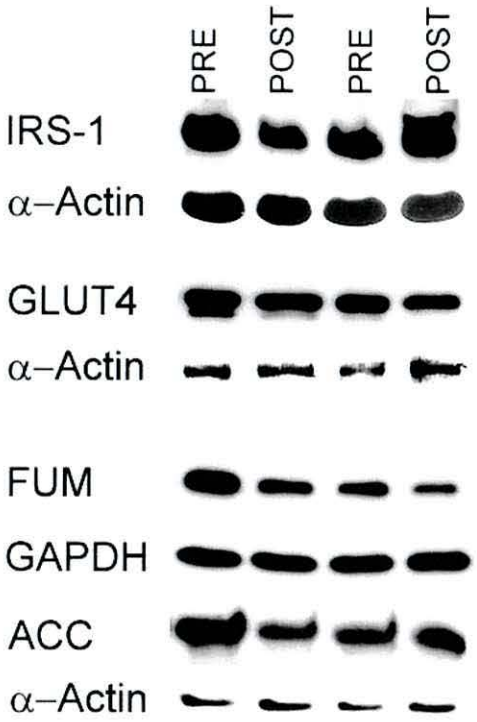

Figure A.3 Western blot analysis of IRS-1, GLUT4, FUM, GAPDH and ACC from skeletal muscle biopsy samples before and after one month of soft drink supplementation (see **Table 3**, **Chapter 3**).

Amplification curves for TXNIP and 18s

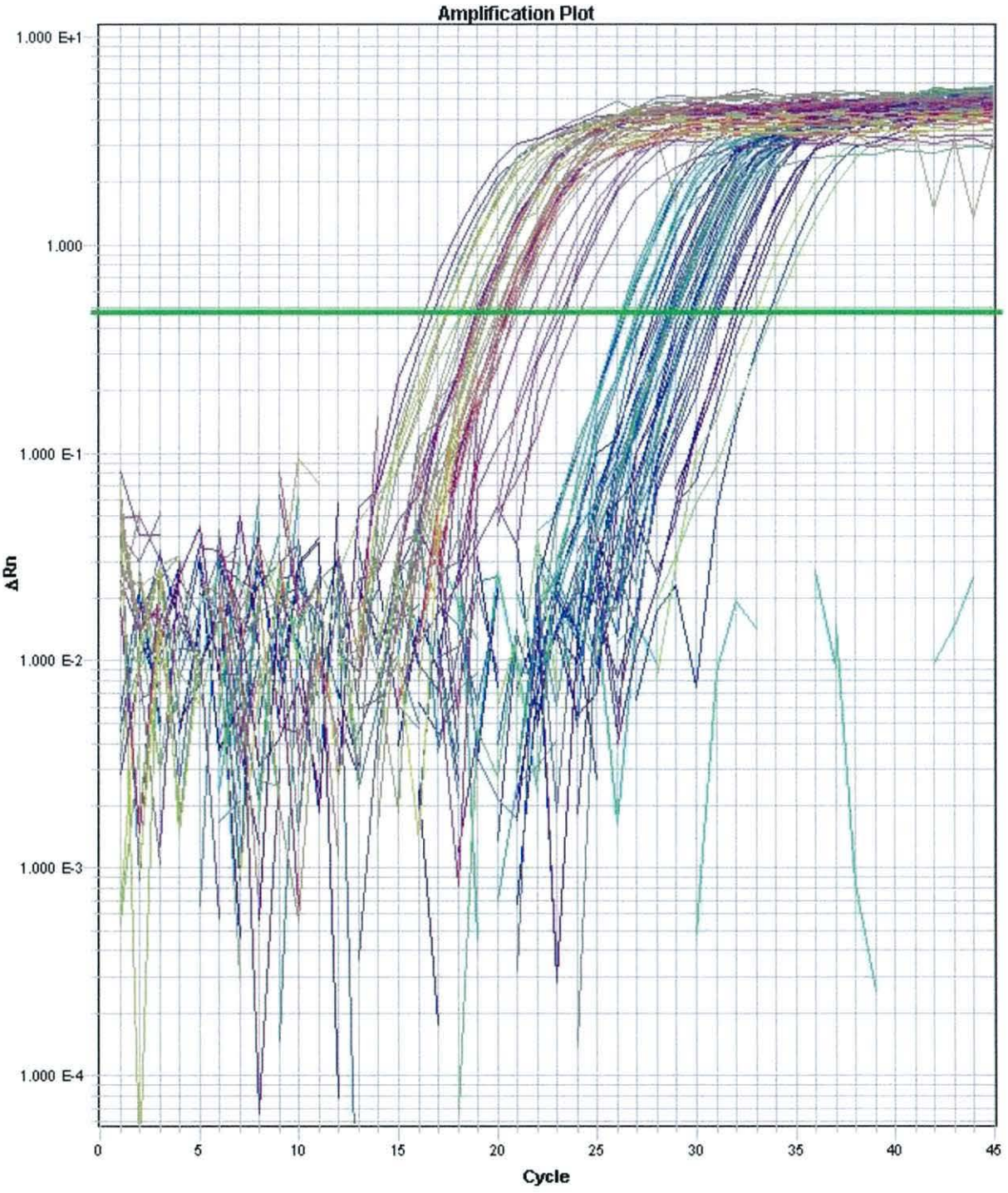

Figure A.4 TXNIP (darker colours) and 18s (lighter colours) amplification curves visualised during real-time PCR.

Examples of Myosin Heavy Chain isofroms related to Chapters 3 and 4.

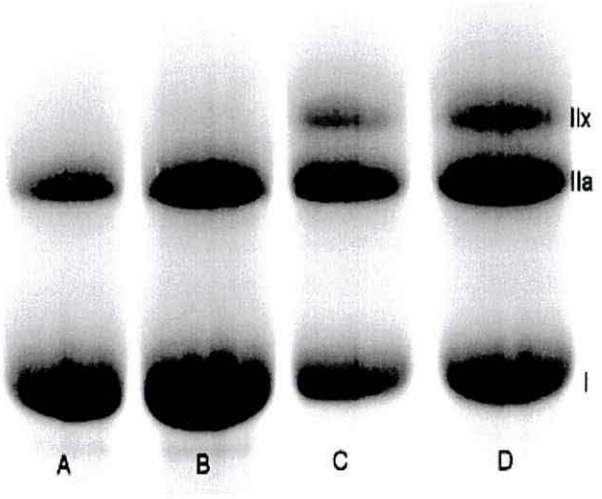

Figure A.5 Myosin Heavy Chain isoforms (bands IIx, IIa, and I) distribution. Lanes A and B are from a normal weight, physically lightly acrive participant, who took part in the studies presented in **Chapter 3**. Lanes C and D belong to a obese, sedentary participant who took part in the study presented in **Chapter 4**.

OGTT curves referring to Chapter 4.

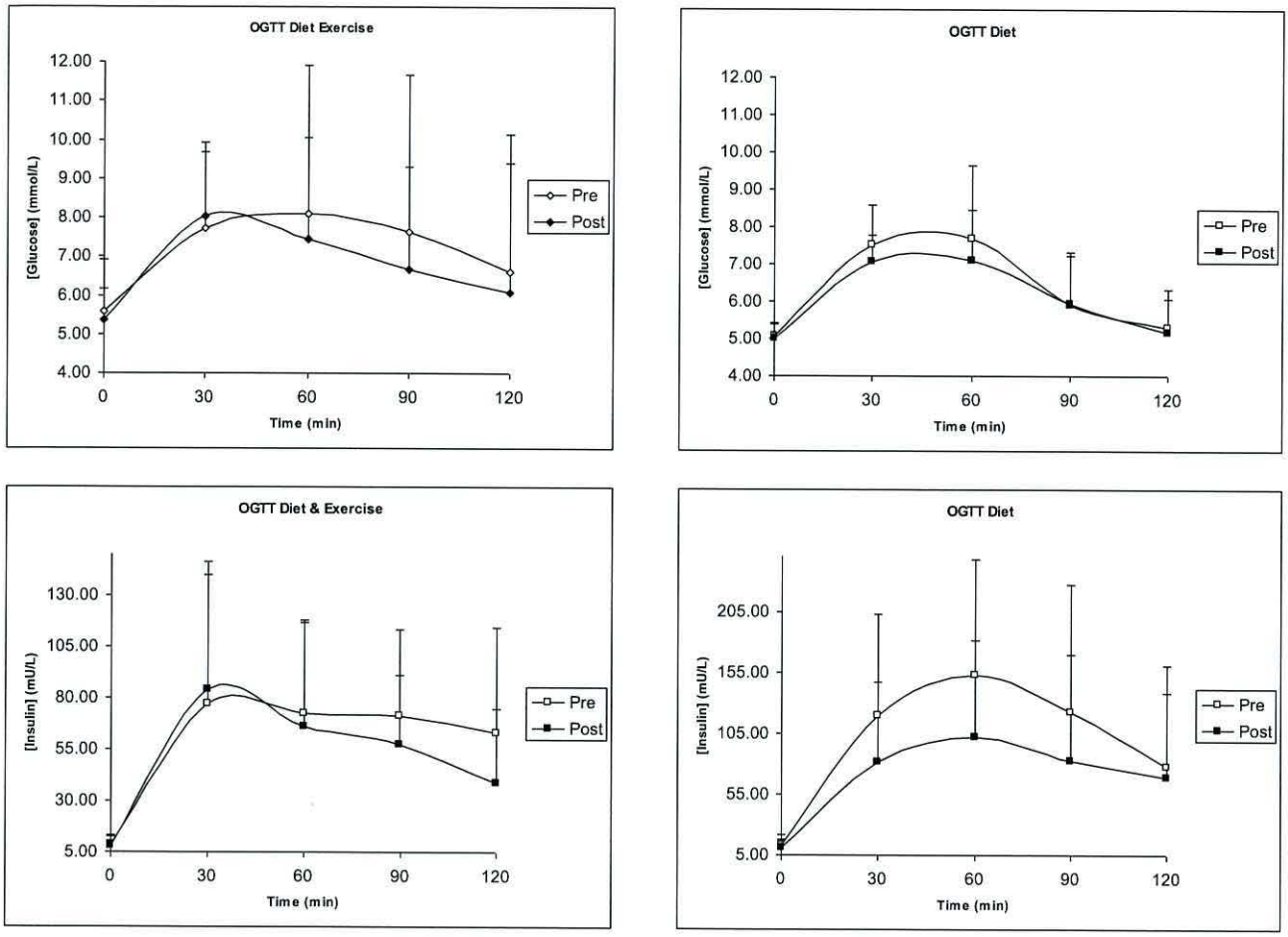

Figure A.6 Plasma glucose and insulin curves subsequent to an Oral Glucose Tolerance Test (OGTT) before and after two weeks of HIIT and restricted-CHO diet (DE) or only restricted-CHO diet (D). Data are presented as mean ± SD.

Western blots outcomes and pictures referring to Chaper 4.

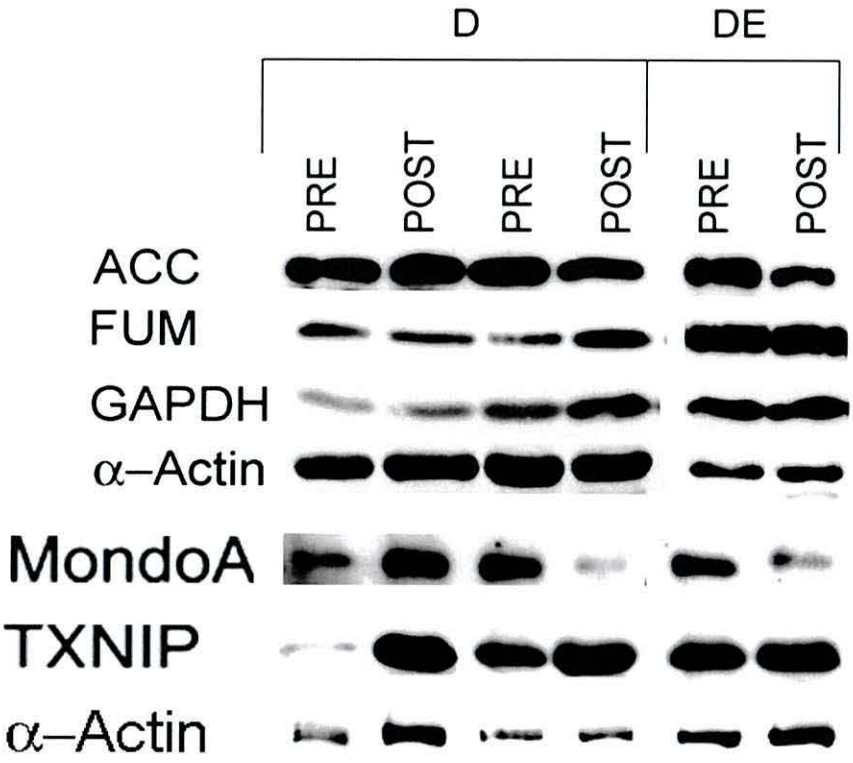

Figure A.7 Once the muscle biopsy data of the two groups (DE n = 3 and, D n = 5) were pooled ACC showed a slight decrease (n.s P = 0.297, with a large effect size, η_p^2 = 0.477) from 6.16 ± 9.08 to 2.93 ± 3.21 AU. However, FUM and GAPDH content did not change with the interventions (pre FUM 4.29 ± 5.58, post 2.84 ± 2.98 AU; pre GAPDH 3.59 ± 3.16, post 3.33 ± 3.03 AU). Pooled MondoA protein levels did not change (Pre: 1.03 ± 0.59 AU vs. Post: 0.97 ± 0.58 AU, t(7)=0.281. P=0.787, η_p^2 = 0.011), whereas TXNIP increased (Pre: 0.79 ± 0.39 AU vs. Post: 1.21 ± 0.44 AU, t(7)=-2.425, P=0.046, η_p^2 = 0.457).

CHO-restricted diet participant's instructions

Food	amount	check
Breakfast		
Golden delicious apples	1 apple	
Snack		
Finest white peach yoghurt	150 g	
Lunch		
Warburtons wholemeal bread	2 slices	
Lurpak spreadable butter	10 g	
Crunchy peanut butter	2 tablespoons	
Minestrone soup	415 g	
Snack		
Warburtons wholemeal bread	1 slice	
Olive oil mayonnaise	2 tablespoons	
Tuna steaks in oil	100 g	
Dinner		
Chicken fried rice	300 g	
Farmhouse mixed vegetables	200 g	
Olive oil mayonnaise	2 tablespoons	
Milk	2 tablespoons	
Tea		

Figure A.8 Example of a day of CHO-reduced diet adopted in the study described in **Chapter 4**.