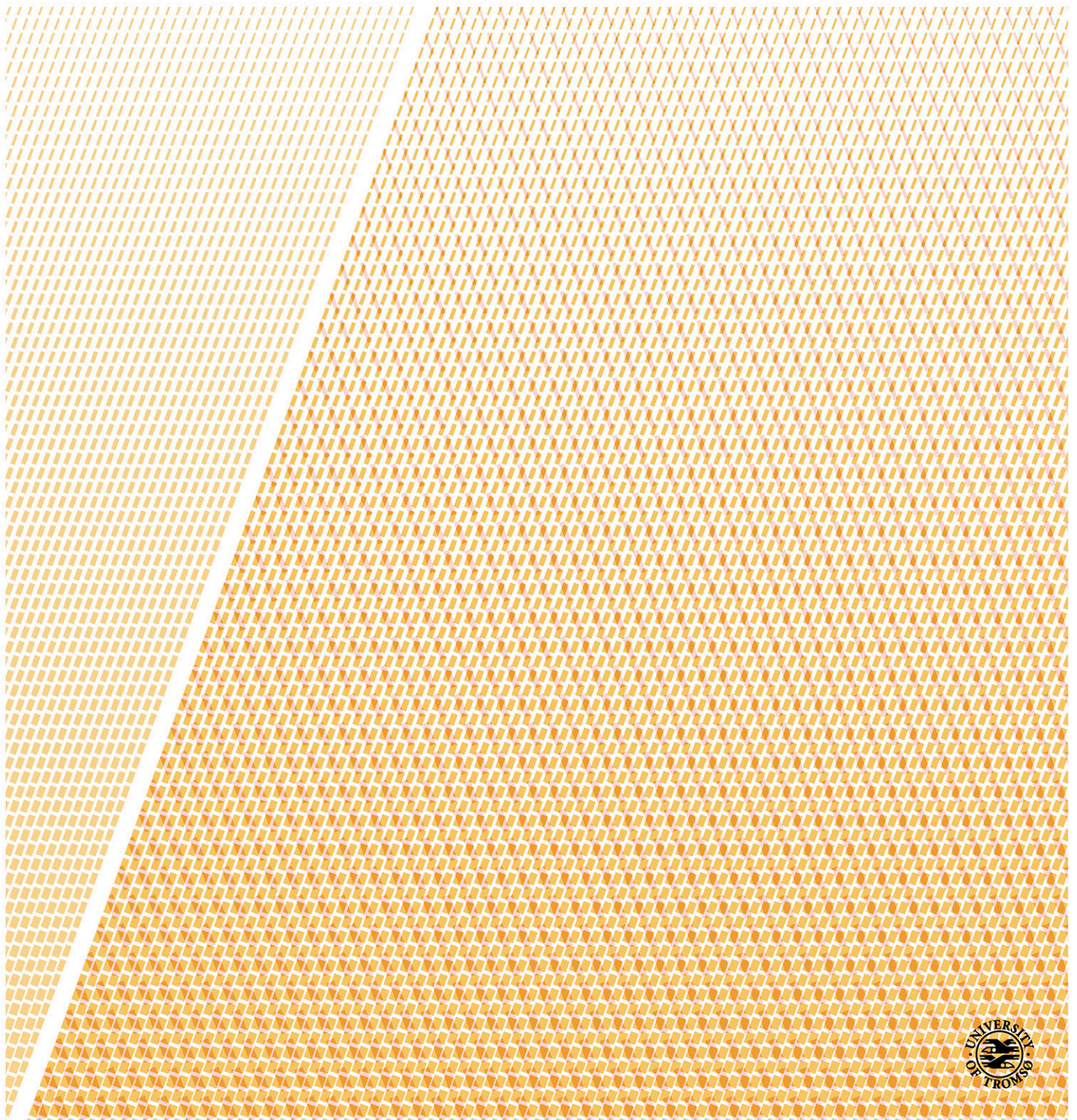


Faculty of Health Sciences

**Postoperative insulin resistance and the metabolic
and cellular responses to single-dose preoperative
oral carbohydrate supplementation**
Experimental studies in pigs

—
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2. LIST OF PAPERS

Paper I

Gjessing PF, Fuskevåg OM, Hagve M, Revhaug A, Irtun Ø. Hyperinsulinemic-euglycemic step clamping with tracer glucose infusion and labeled glucose infusate for assessment of acute insulin resistance in pigs. *Am J Physiol Endocrinol Metab* 2010; 298:1305-1312.

Paper II

Gjessing PF, Hagve M, Fuskevåg OM, Revhaug A, Irtun Ø. Single-dose preoperative carbohydrate treatment in the immediate preoperative phase diminishes development of postoperative peripheral insulin resistance. *Clin Nutr* 2014; <http://dx.doi.org/10.1016/j.clnu.2014.02.010>.

Paper III

Gjessing PF, Constantin-Teodosiu D, Hagve M, Lobo DN, Revhaug A, Irtun Ø. Preoperative CHO supplementation attenuates post-surgery insulin resistance via reduced inflammatory inhibition of the insulin-mediated restraint on muscle pyruvate dehydrogenase kinase 4 expression. Submitted for publication Feb 2014.

Paper IV

Hagve M, Gjessing PF, Fuskevåg OM, Revhaug A, Larsen TS, Irtun Ø. Skeletal muscle mitochondria exhibit decreased pyruvate oxidation capacity and increased ROS release during surgery-induced acute insulin resistance. Manuscript.

3. SELECTED ABBREVIATIONS

ADP – adenosine diphosphate

Akt1 – protein kinase B

ANT – adenine nucleotide translocase

ATP – adenosine triphosphate

ATR – carboxyatractyloside

CHO – carbohydrate

EGR – endogenous glucose release

FFA – free fatty acids

FOXO1 – forkhead transcription factor 1

GDP – guanosine diphosphate

GIR – glucose infusion rate

HEC – hyperinsulinaemic-euglycaemic clamp

H₂O₂ – hydrogen peroxide

IFM – intramyofibrillar mitochondria

IKK β – inhibitor kappa B kinase

IL-6 – interleukin-6

IRS1 – insulin receptor substrate 1

JNK1/2 – c-Jun-n-terminal kinase

mRNA – messenger ribonucleic acid

NF κ B – nuclear factor kappa B

O₂⁻ – superoxide

PC – palmitoyl-L-carnitine

PDC – pyruvate dehydrogenase complex

PDK4 – pyruvate dehydrogenase kinase 4

PDP – pyruvate dehydrogenase phosphatase

PPAR – peroxisome proliferator-activated
receptor

RF – relative fluorescence

ROS – reactive oxygen species

SOCS3 – suppressor of cytokine signalling

SS – steady-state

SSM – subsarcolemmal mitochondria

TNF α – tumor necrosis factor alpha

V_{max} – maximal respiration

V_{oligo} – leak respiration

WGD – whole-body glucose disposal

UCP – uncoupling protein

4. INTRODUCTION

Central to the development of a catabolic state after surgical trauma is the onset of a temporary loss of tissue sensitivity to circulating levels of insulin, the most powerful anabolic hormone ¹. During the last decades, development of acute insulin resistance and concomitant hyperglycaemia after surgery has been identified as an independent negative predictor of outcome ² and hospital stay ³, regardless the preoperative diabetic state of the patient. The primary site of insulin resistance after surgery is skeletal muscle in which both non-oxidative and oxidative glucose disposal becomes inhibited ^{4,5}. The degree and duration of postoperative insulin resistance is related to the extent of the surgical procedure ³ and postoperative stress-hormone concentrations, especially cortisol ⁶, but also to the systemic inflammatory response ^{6,7}. Previous research suggests that during and after surgery cross-talk between pro-inflammatory cytokines and muscle insulin signalling exists ^{8,9}. The cellular pathophysiological alterations seem to bear close resemblance to those seen in chronic insulin resistant states, such as metabolic syndrome and diabetes mellitus type 2, which are associated with chronic low-grade inflammation ¹⁰, mitochondrial dysfunction and increased production of reactive oxygen species ¹¹, but the underlying mechanisms are still not fully understood.

Development of peripheral postoperative insulin resistance can be reduced by administration of a carbohydrate rich beverage prior to surgery instead of overnight fasting. This simple preoperative intervention has been shown to improve postoperative oxidative glucose disposal ^{12,13} and shorten hospital stay after major abdominal surgery ¹⁴. Furthermore, in a recent study investigating the adherence level to the advanced recovery after surgery (ERAS) protocol in colorectal surgery patients, preoperative

carbohydrate treatment was identified as a major independent contributor to reduced risk of postoperative nausea and vomiting, pain, diarrhoea, dizziness and wound dehiscence¹⁵.

The established routine of preoperative carbohydrate supplementation consists of a large dose of 100 g in the evening and a smaller dose of 50 g up to two hours prior to surgery and is recommended (grade of recommendation A) as part of the preoperative metabolic care of most surgical patients¹⁶. However, the necessity of a preoperative evening dose has been questioned, as modern guidelines allow patients to eat until 6 hours prior to surgery^{17,18} and previous research has indicated a predominant effect of the dose given immediately before surgery¹⁹.

Lastly, the mechanisms underlying the effect of preoperative carbohydrate supplementation are still unclear, although interestingly, recent evidence suggests that modulation of the inflammatory response to surgery plays a key role in maintaining postoperative insulin sensitivity²⁰.

Thus, the present work was initiated to investigate the cellular mechanisms that underlie the development of postoperative insulin resistance and the prophylactic effect of oral carbohydrate treatment immediately prior to surgery.

5. BACKGROUND

5.1. Metabolic alterations in response to injury

In a phylogenetic perspective, metabolic changes to major injury are part of a well preserved physiologic response which purpose is to increase the chance of survival. The priority-setting of whole body substrate and nutrient distribution changes in the aftermath of physical trauma, with the potential of subsequent infection and inability to provide food. Enhanced protein breakdown in skeletal muscle is followed by redistribution of amino acids to provide for hepatic acute phase protein synthesis and gluconeogenesis, proliferation and vital functions of sanatory immunocompetent cells and enterocytes together with endothelial integrity and protein synthesis in the healing wound ^{21,22}.

Lipolysis and the overall oxidation rate of fat and ketone bodies increase as they take over as primary fuels to cover energy expenditure in the liver, kidney, heart and skeletal muscle ²³⁻²⁵. Concurrently, peripheral glucose uptake and disposal is impaired ^{24,26} and available glucose is shunted to glucose dependent cells and tissues such as red and white blood cells, neurons, wound tissue and the renal medulla.

Stress-related alterations to the homeostatic internal environment in man were anticipated already by the ancient Greek natural philosophers ²⁷. Sir David Cuthbertson was the first to describe in detail the metabolic alterations following injury during his pioneer work in the 1930`s ²⁸. Based on animal experiments and measurements on patients with accidental lower limb injuries he introduced the terms ‘ebb’ and ‘flow’ in an attempt to create a chronological description of post-injury metabolic responses. The initial ‘ebb’ phase was characterized by tissue hypoperfusion, peripheral vasoconstriction and decreased metabolic activity during the first hours after injury. Within 24 hours the

ebb phase was normally followed by a hypermetabolic state, called the ‘flow’ phase, characterized by breakdown of body tissues and increased metabolic rate. His findings of an acute reduction in metabolic rate during ‘ebb’ probably was influenced by small animal research on severe injury and haemorrhage with circulatory hypovolemia, and the increment in metabolic rate during ‘flow’ is much less than originally believed²⁹, and do not reflect the actual metabolic changes seen after modern elective surgery accompanied by multimodal stress-reducing approaches^{30,31}. However, his subdivision of two post injury phases of metabolic change on the basis of metabolic rate prevails, influences research on the field and is still quoted.

5.2. Acute insulin resistance and diabetes of injury

Acute changes in carbohydrate metabolism were first scientifically reported during the late 19th century with the observation of glycosuria following ether anaesthesia by Reynoso in 1853³². His findings were supported by the French physiologist Claude Bernards experiments in 1877³³ in which he demonstrated acute hyperglycaemia after haemorrhagic shock and later by Weddell in 1934³⁴ who reported hyperglycaemia during and after surgery. Since then the terms “stress diabetes” and “diabetes of injury” have been used to describe the phenomenon of acute trauma induced insulin resistance and hyperglycaemia, the cardinal feature of stress metabolism.

In an otherwise healthy individual exposed to major trauma, fat and amino acids are available in great abundance. On the other hand, due to the need for solution in water and electrolytes to maintain an isotonic environment³⁵, glucose is stored of relatively limited quantity as glycogen in skeletal muscle (~400 grams) and liver (~100 grams)³⁶.

The role of acute insulin resistance in the complex metabolic response to trauma seems to be multifaceted. For one thing, augmentation of hepatic glucose output and reduced utilisation of glucose in skeletal muscle increases the total glucose pool. Concurrently, the liver and musculature converts from glucose to fat oxidation, which reduces total glucose consumption. Available glucose is shunted to glucose dependent tissues through non-insulin dependent glucose uptake. In the early phase after physical trauma, acute insulin resistance thereby also reduces protein catabolism as the need for gluconeogenic precursors is reduced. In addition increased blood sugar concentration serves as a significant osmotic force which counteracts haemorrhagic hypovolemia and shock³⁷. In a phylogenetic perspective these alterations in nutrient and substrate distribution are probably meant to support survival of the organism after injury within a period of the first few hours and days, after which a state of convalescence is reached or the individual dies. On the other hand, in a modern clinical setting with sterile elective surgery, antibiotic treatment and advanced postoperative care, including fluid and nutrient substitution, it seems that these ancient adaptive physiologic responses to trauma has turned out to be negative with regard to postoperative patient recovery. Therefore, the quest for effective and risk free modulation of these deeply rooted metabolic changes has been and still is an unresolved priority.

5.3. Postoperative insulin resistance

The extent of acute insulin resistance after elective surgery is associated with the type and magnitude of operation and tissue injury^{38,39}. This state of metabolism is temporary and lasts, with large individual variation, for at least 5 days after uncomplicated open

cholecystectomy, after which insulin sensitivity normalizes with recovery of the patient⁴⁰. Depending on methodological differences, type of surgery and time of measurement, studies on sites and detailed changes in glucose metabolism during postoperative insulin resistance have been somewhat divergent. During the first postoperative day insulin resistance has been shown to be of mere peripheral origin^{12,13} or to include both hepatic and peripheral tissues, with metabolic alterations being most pronounced in skeletal muscle^{4,5,12}, whereas a shift towards hepatic insulin resistance occurs during the following days⁴¹. A general finding is that non-oxidative glucose disposal becomes severely inhibited and virtually unresponsive to exogenous insulin infusion after surgery^{4,5,12,13,41}, whereas rates of glucose oxidation are either unaltered^{13,41} or more often reduced with a concurrent increase in rates of fat oxidation^{4,5,12} and elevated plasma free fatty acids (FFA), indicating increased lipolysis with reduced fat tissue responsiveness to insulin⁴.

Until recently, elevated blood glucose after surgical trauma was accepted as a necessary part of convalescence. This perception changed however, as hyperglycaemia, the most prominent metabolic feature of insulin resistance, was shown to be severely detrimental to postoperative intensive care patients in a landmark clinical, prospective, randomized study comparing intensive insulin therapy and standard insulin treatment in mainly cardiac surgery patients^{42,43}. Further, a recent prospective study of 273 patients undergoing elective cardiac surgery has shown that for each 1 mg/kg/min decrease in glucose infusion rate during intraoperative hyperinsulinaemic-euglycaemic clamping as a measure of increasing insulin resistance, there was increased incidence of major complications and severe and minor infection independent of the patients preoperative

diabetic status ². Hyperglycaemia has also been shown to be associated with increased length of stay, in-hospital complications and mortality after non-cardiac surgery in patients without pre-existing diabetes ⁴⁴. Insulin resistance itself is suggested to be a better predictor of outcome than glycaemic control per se ⁴⁵ and despite an increasing interest and research on acute insulin resistance, the underlying mechanisms are still not fully understood.

5.4. Insulin signalling

Insulin regulation of glucose uptake is mediated via complex intracellular signalling pathways ⁴⁶ (Fig. 1). The insulin receptor consists of two intracellular β -units which are activated by trans-autophosphorylation following binding of insulin to two extracellular α -subunits. This enables the receptor to phosphorylate several downstream targets. Among these targets, the insulin receptor substrates (IRS) are crucial for the organisation and initiation of insulin's metabolic actions ⁴⁷. Once activated, IRS1 recruits to the cell membrane phosphoinositide-3-kinase (PI3K), which then catalyses the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-trisphosphate (PIP₃). The downstream proteins phosphoinositide-dependent protein kinase (PDK1) and Akt1 are also recruited to the inner surface of the plasma membrane followed by phosphorylation of Akt1 on Thr³⁰⁸ by PDK1. The additional phosphorylation on Ser⁴⁷³ by mTOR complex 2, which also is activated by PI3K, is prerequisite for full activation of Akt1 ⁴⁸. Together with signalling through an alternative pathway, the activation of Akt1 results in translocation of glucose transporter protein GLUT4 from intracellular deposit vesicles to the plasma membrane. Akt1 also inactivates glycogen

synthase kinase 3 β and thereby abrogates its restraining effect on glycogen synthase, facilitating the formation of glycogen from glucose. By inhibitory serine residue phosphorylation on forkhead transcription factor 1 (FOXO1) by Akt1, insulin reduces mRNA expression of pyruvate dehydrogenase kinase 4 (PDK4), a key inhibitor of the pyruvate dehydrogenase complex, which regulates the flux of glucose derived pyruvate into the mitochondria.

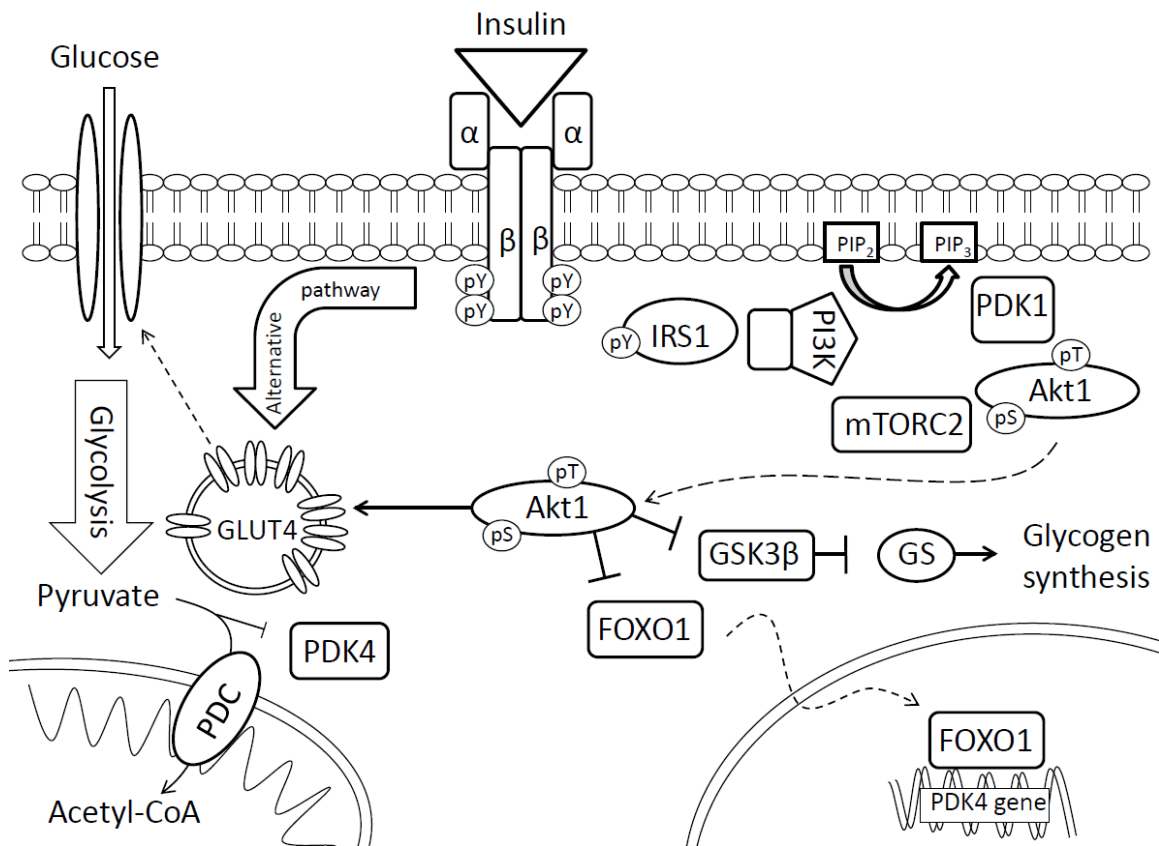


Figure 1 Insulin signalling

5.5. Counter-regulatory hormones and acute insulin resistance

The initiation and course of the metabolic stress response is orchestrated by a complex interrelated triad of nervous, endocrine and humoral systems. Perception of injury, tissue damage and fluid loss by afferent neurogenic stimuli from the site of tissue damage, and

from baro- and volume-receptors is followed by increased secretion of pituitary hormones and activation of the sympatho-adrenergic axis^{49,50}. Thus, the ‘ebb’ phase is dominated by a time limited neuroendocrine response with a classic signature of elevated levels of catecholamines, cortisol and glucagon. Because these so-called stress hormones respond to, and are capable of counteracting hypoglycaemia, they are also called counter-regulatory hormones⁵¹. The counter-regulatory hormones work in concert, with complementary and additive effects on creating and maintaining hyperglycaemia and insulin resistance during stress.

The ‘ebb’ phase is later succeeded by the hypermetabolic ‘flow’ phase, characterized by catabolism and followed by a gradual shift into an anabolic state of recovery. During the ‘flow’ phase the counter-regulatory hormones usually normalize and insulin levels elevate to above normal^{26,52}. The features of stress metabolism after injury can, in part, be mimicked by prolonged infusion of a hormone cocktail containing cortisol, glucagon and catecholamines^{53,54}. However, the plasma concentrations needed in order to induce such metabolic alterations in otherwise healthy volunteers are substantially higher than those seen in patients with a similar metabolic profile²⁴. Also, in patients undergoing elective surgery, investigations have shown discrepancy in the extent, duration, correlation and timing of the elevation of counter-regulatory hormones and the course of stress metabolism^{7,38,40,52,55}. The transient and moderate rise in stress hormones during and immediately after surgery is not synchronous with the much longer lasting postoperative insulin resistance and catabolic state. Thus, the traditional view of pure hormonal control of metabolism in the postoperative setting lacks consistency⁵⁵.

5.5.1. Glucagon

Glucagon stimulated glycogenolysis is one of the most sensitive metabolic effects of hormones on any tissue⁵⁶. The effect of glucagon alone on glucose output from the liver is transient, lasting only a couple of hours^{57,58} and does not make a major contribution to the hyperglycaemic response after surgery¹. The extent and duration of hyperglycaemia is limited due to counteracting elevation of insulin levels and an indirect increase in glucose clearance⁵⁷. However, when glucagon is infused in dogs in combination with cortisol, the increase in glucose production becomes sustained indicating synergistic and potentiating effects⁵⁷. Glucagon also has a central role in sustaining hyperglycaemia during stress hormone infusion, by reducing hepatic glucose uptake, increasing hepatic uptake of peripherally released lactate and alanine and stimulating gluconeogenesis^{59,60}. Acute actions of glucagon on free fatty acid metabolism remain controversial. Up-regulation of hormone-sensitive lipase activity and increased release of glycerol has been reported by in vitro studies on rat adipocytes⁶¹ and in vivo in humans⁶². However, no such effect of glucagon concentrations within the physiological range was seen in more recent studies in humans⁶³ or in swine⁶⁴.

5.5.2. Epinephrine and norepinephrine

Like glucagon, epinephrine has a transient, but smaller increasing effect on glucose production. When infused together with glucagon it aggravates hyperglycaemia by further increasing gluconeogenesis in addition to the glycogenolytic effects of glucagon^{57,65}. The persistent hyperglycaemia seen during infusion of epinephrine is due to its direct negative effects on peripheral glucose uptake and, at relatively high circulating

levels⁶⁶, inhibition of insulin secretion^{57,66}. In general, circulating levels of insulin are acutely suppressed by epinephrine after major injury, although this specific effect of epinephrine has a threshold⁶⁶ and insulin secretion in the immediate period following less severe trauma can vary extensively⁶⁷⁻⁷⁰. Epinephrine is a cardinal inductor of lipolysis and release of FFA and glycerol from adipose tissue into circulation by stimulating hormone-sensitive lipase. Whereas a 4-5 fold increase in circulating levels of epinephrine is required to induce hyperglycaemic responses, the lipolytic effects of epinephrine occur at lower levels⁷¹. Due to the mechanisms of the glucose-fatty acid cycle (chapter 5.6.2.), increased availability of FFA also leads to decreased glucose uptake by competitive inhibition of glucose oxidation. Norepinephrine is mainly a sympathetic neurotransmitter and its metabolic actions as a hormone, resulting from synaptic “spill over”, are similar to those of epinephrine, but are of less magnitude in dogs⁷² and require much higher circulating concentrations in humans⁷³. In swine, research indicate higher β -adrenergic lipolytic potency of norepinephrine than of epinephrine⁶⁴.

5.5.3. Cortisol

Circulating concentrations of cortisol is a strong predictor of stress induced insulin resistance after surgery⁶. An increase in blood sugar and both hepatic and peripheral insulin resistance, as indicated by increased endogenous glucose release (EGR) and decreased whole-body glucose disposal (WGD) during hyperinsulinemic clamp studies, has been demonstrated six hours after acute cortisol excess in man⁷⁴. Importantly, these metabolic effects persisted after normalization of plasma cortisol levels⁷⁴. Other

investigators have reported development of insulin resistance exclusively in peripheral tissues and preserved suppressive effects of insulin on EGR after 2 days of glucocorticoid treatment, using the glucose clamp technique⁷⁵. Cortisol induced insulin resistance can be ascribed to different post-receptor defects in the insulin signalling pathway^{76,77}, which are more pronounced in muscle, at least after several days of cortisol excess⁷⁷. More specifically, inhibition of insulin induced phosphorylation of the insulin receptor⁷⁸, decreased muscle IRS1 expression⁷⁸, inhibited phosphorylation of Akt1⁷⁹ and a decrease in translocation of GLUT4 transporters to the cell membrane^{80,81} has been demonstrated after cortisol excess for 2-12 days. Insulin stimulated glycogen production is reduced^{80,82} and a shift from glycogen synthesis to glycolysis provides normal production of lactate⁸⁰, which constitutes an important substrate for gluconeogenesis. Cortisol further maintains gluconeogenic precursor supply by increasing protein breakdown, and liberation of amino acids, especially alanine^{74,83}. Independently of glucose transport, a decrease in oxidative glucose disposal is seen both in rats⁸⁰ and in healthy volunteers⁷⁵ through increased pyruvate dehydrogenase kinase 4 (PDK4) expression⁸⁴ and/or increased plasma FFA levels and oxidation⁸⁵.

5.6. Regulation of glucose oxidation

5.6.1. The glucose-fatty acid cycle

Glucose and fatty acids comprise approximately 80% of oxidative metabolism in humans, whereby the total reciprocal utilization of these substrates to a great extent is controlled by insulin secretion⁸⁶. In order to tightly adjust adenosine triphosphate (ATP) production to the actual needs of the body, several regulatory mechanisms on different levels,

originally described by Randle ⁸⁷, co-ordinate and fine-tune the utilization of glucose and fatty acid in skeletal muscle mitochondria. Elevated levels of circulating glucose stimulate insulin secretion, which in turn suppresses the action of hormone sensitive lipase and the release of free fatty acids from adipose tissue. Further, the resulting increase in glucose oxidation reduces fatty acid oxidation through malonyl-CoA inhibition of carnitinepalmitoyl-transferase-1, which is responsible for transport of fatty acids into the mitochondria. These mechanisms arrange for unimpeded insulin-stimulated glucose disposal with less competition from FFA in times when glucose is abundant. In the fasting state, at low serum glucose and insulin concentrations, FFA levels rise in order to provide fuel for oxidative ATP production in skeletal muscle. The increase in β -oxidation of fatty acids suppresses muscle uptake and oxidative disposal of glucose by inactivating the pyruvate dehydrogenase complex (PDC) which regulates the flux of carbohydrate derived pyruvate into the mitochondria. Suppression of PDC activity is crucial in order to conserve glucose, because no metabolic pathway for conversion of Acetyl-CoA to glucose exists in mammals ⁸⁸.

5.6.2. Pyruvate dehydrogenase kinase 4

The activity of PDC is controlled by a covalent mechanism involving a competing pyruvate dehydrogenase kinase (PDK) and phosphatase (PDP) reaction cycle ⁸⁹. The resulting inter-conversion cycle determines the amount of PDC existing in non-phosphorylated (active) form ⁹⁰. The PDK protein family comprises of four isoforms (PDK1-4) ⁹¹, whilst PDP has two isoforms (PDP1-2) ⁹². Although the PDK isoform proteins are expressed in most tissues, including liver, skeletal and heart muscles, the

specific activity of PDK4 is several-fold greater than any other member of this protein family⁹¹, thereby assigning to PDK4 isoform the greatest regulatory significance. PDK4 reduces the rate of glucose oxidation by inhibition of PDC activity. Increased muscle PDK4 expression has been observed in rodent streptozotocin-^{93,94} and high-fat diet-induced insulin resistance models⁹⁵, but the causal relationship between insulin resistance and PDK4 expression and the mechanisms underlying the regulation of this gene is still a subject of investigation. FFA activated signalling via peroxisome proliferator-activated receptor (PPAR) α induces PDK4 gene expression⁹⁶, but this is not obligatory in skeletal muscle^{97,98}, where another transcription factor, forkhead transcription factor 1 (FOXO1), has been shown to play a key role^{84,99}. Further, PDK4 expression is elevated by cortisol and reduced by insulin⁹⁶. The acute and pronounced suppressive effect of insulin on skeletal muscle PDK4 expression is independent of its restraining effect on plasma FFA⁹⁸ and interestingly, the effect is impaired in acute insulin resistance induced by lipid and lactate infusion¹⁰⁰.

5.7. Regulation of PDK4 expression by cortisol and insulin

During conditions characterized by higher-than-normal circulating levels of FFAs, acute glucocorticoid excess or reduced insulin availability, it appears that up-regulation of PDK4 mRNA and protein is due to up-regulation of FOXO1 activity⁸⁴. Cortisol-induced elevation of muscle PDK4 mRNA and protein expression is modulated by complex hormone response units including enhanced binding kinetic of FOXO1 protein to its promoter on the PDK4 gene, which increases its responsiveness to the glucocorticoid receptor. On the other hand, FOXO1 is known to be regulated by the IRS1/Akt1

signalling pathway¹⁰¹ and plays a key role in the inhibition of PDK4, as insulin stimulation, in addition to dissociating the glucocorticoid receptor from the PDK4 gene, leads to phosphorylation of FOXO1, which inhibits translocation into the nucleus and binding to the promoter¹⁰² (Fig. 1).

Thus, the cellular PDK4-regulating pathways of insulin and cortisol interact at the level of FOXO1, which plays an important role in both the induction and inhibition of PDK4 gene expression.

5.8. The inflammatory response to surgery and its effects on insulin signalling

Research on links between the immune system, proinflammatory cytokines and metabolic disorders has increased tremendously during the last decades¹⁰³ and there is a growing understanding that obesity, type 2 diabetes and the metabolic syndrome are caused by chronic low-grade systemic inflammation¹⁰⁴. The proinflammatory cytokines tumor necrosis factor α (TNF α), interleukin (IL) -1 β and IL-6 in particular, have also been objects of investigation on the search for humoral mediators of insulin resistance subsequent to surgical trauma. TNF α is released into the peritoneal cavity after major abdominal surgery¹⁰⁵. Further, it has been shown that major elective abdominal surgery induces a systemic pro-inflammatory response, as seen by elevations in plasma IL-1 β and IL-6 levels^{20,106,107} lasting up to 3 days postoperatively. Especially the IL-6 response correlates well with the extent and duration of surgery¹⁰⁸ and development of postoperative insulin resistance^{6,7} and together with cortisol, IL-6 is one of the strongest predictors of acute insulin resistance after surgery⁶. The source of these inflammatory mediators, primarily acting in auto- and paracrine manners, is mainly local monocytes

and macrophages activated by tissue damage. Extensive inflammatory activity leads to spill-over of cytokines into circulation, enabling the cytokines to exert humoral effects on cells and tissues distant from the site of injury.

Pro-inflammatory cytokines activate the serine/threonine kinases inhibitor κ B kinase (IKK β) and c-Jun N-terminal kinase (JNK), which impair the downstream propagation of insulin signalling by inhibitory phosphorylation of Ser³⁰⁷ on IRS1. These alterations in insulin signalling are seen in insulin sensitive tissues in chronic insulin resistant states ¹⁰⁹ as well as in skeletal muscle during acute insulin resistance after surgery ⁸. Further, previous studies have shown rapid up-regulation of JNK expression after surgery ¹¹⁰, increased ratio of phosphorylated to total JNK in muscle after burn injury ¹¹¹ and an association between activation of JNK and IKK β and increased inhibitory Ser^{307/312} IRS1 phosphorylation in the liver immediately after trauma and haemorrhage in rodents ¹¹², and in skeletal muscle following TNF α infusion in healthy humans ¹¹³. Equally, suppressor of cytokine signalling 3 (SOCS3), which binds to IRS1 and targets it for proteasome degradation ¹¹⁴, is rapidly up-regulated in the liver following lipopolysaccharide-induced endotoxemia in rodents ¹¹⁵ and in skeletal muscle after major abdominal surgery ⁹.

The latter patient-based study did cover a broad range of genes (45 genes), which collectively provided enough evidence to support the contention that several important genes involved in inflammatory pathways, such as IL-6 and TNF α ⁹, are activated in the muscle (rectus abdominis) next to the site of surgery. In line with these observations, nuclear factor κ B (NF κ B), which is a transcription factor that enhances the nuclear inflammatory response following activation by a variety of extra cellular inflammatory

signals, is persistently up-regulated both in chronic inflammatory diseases ¹¹⁶ and in skeletal muscle after surgery ⁹.

5.9. Oxidative stress-induced insulin resistance and mitochondrial uncoupling

Mitochondrial production of ATP through oxidative phosphorylation constitutes the core of aerobic metabolism. An unavoidable consequence of this process is the formation of reactive oxygen species (ROS) by interaction of free electrons and oxygen ¹¹⁷. In both skeletal muscle and liver, mitochondria in addition to NADPH oxidase, are thought to be the most important source of superoxide ($O_2^{\bullet-}$) and subsequently hydrogen peroxide (H_2O_2) ¹¹⁸ release. These are highly reactive molecules capable of destroying cellular functional and architectural components and membranes due to direct contact or lipid peroxidation. Due to their destructive nature ROS are scavenged by an array of intrinsic defence systems in the cell, including several different forms of superoxide dismutase, catalase and glutathione peroxidase which convert $O_2^{\bullet-}$ into oxygen and H_2O_2 , which is further quickly broken down into water and oxygen by catalases ¹¹⁷. In addition, mitochondrial proton leakage can be increased by uncoupling proteins (UCP) as a feedback-mechanism to decrease ROS production, which leads to less effective mitochondria with decreased potential for energy production ¹¹⁹.

In chronic insulin resistant states, mitochondria display decreased oxidative capacity and increased ROS release and lipid-induced uncoupling, probably due to increased substrate availability resulting in lipotoxicity and alterations in the mitochondrial membrane structure and electron transport ^{118,120,121}. Aside of being a by-product of oxidative metabolism, recent evidence supports the role of ROS as a regulator

molecule and as an inductor of insulin resistance through activation of the aforementioned stress-induced serine/threonine kinases, such as IKK β and JNK^{118,122,123} or by direct oxidative modification of the insulin receptor and/or phosphotyrosine phosphatases^{124,125}, leading to impairment of insulin signalling. In addition, in vitro research models have identified a direct correlation between insulin resistance and mitochondrial O₂⁻ without consistent change in the upstream insulin signal transduction pathway^{126,127}, indicating IRS1/Akt1-independent regulatory properties of ROS on insulin sensitivity through not yet identified mechanisms.

Lipolytic activity stimulated by the hormonal changes after surgery leads to conversion of stored triglycerids into glycerol and fatty acids, although their plasma concentrations may not change markedly¹. In skeletal muscle an acutely (1 h) increased supply of palmitic acid within physiological plasma concentrations has shown to induce a rise in mitochondrial ROS production¹²⁸. In addition, hyperglycaemia per se induces mitochondrial ROS overproduction¹²⁹ and most intriguingly, in vitro and in vivo rodent studies have shown that ROS has a causal role in multiple forms of insulin resistance in skeletal muscle, adipose tissue and liver, including glucocorticoid-, TNF α - and trauma and haemorrhage-induced acute insulin resistance, which could all be reversed or prevented by antioxidant treatment and/or pharmacologic and genetic strategies that override mitochondrial O₂⁻ production^{11,127,130}. Further, glucocorticoids¹³¹ and TNF α ¹³² inhibit oxidative phosphorylation, causing ineffective and dysfunctional mitochondria, and increased levels of skeletal muscle UCP2 and UCP3 mRNA and protein expression has been shown to develop along with insulin resistance after surgical trauma in rats¹³³.

These findings collectively indicate that increased ROS levels constitute an important pathophysiological factor in the development of both acute and chronic insulin resistance.

5.10. From preoperative 'nil-per-mouth' to preoperative oral carbohydrate loading

The tradition of preoperative fasting originates from the era when general anaesthesia was introduced in the mid-19th century. In 1848, less than a year after the discovery of its anaesthetic effects, a 15 year old girl suffered the first registered anaesthesia death after chloroform inhalation for removal of a toe-nail ¹³⁴. Although the cause of her death was not clear, and later became extensively debated, the autopsy revealed a stomach distended with food and signs of pulmonary aspiration ¹³⁵. Subsequently, preoperative fasting in order to avoid anaesthesia-related vomiting and pulmonary acid aspiration was proposed only a few years after the very first implementation of general anaesthesia ¹³⁶. The dogma of preoperative 'nil-per-mouth' after midnight was upheld until the early 1980s, when it was challenged by several clinical trials. Based on the data from these trials, new national guidelines were approved and implemented in Norway in 1994, followed by several other countries, including the US, the UK, Denmark and Sweden, allowing a light breakfast and preoperative oral liquids until 6 and 2 h before general anaesthesia, respectively ^{18,137}.

About the same time, the first investigations into preoperative carbohydrate supplementation to reduce postoperative insulin resistance was performed and the authors showed that insulin sensitivity decreased significantly less after elective open cholecystectomy in patients who received an overnight infusion of 5 mg/kg/min glucose compared to traditional fasting. It was speculated that enzymatic changes favouring

gluconeogenesis and active preoperative carbohydrate preservation could play a role for this finding, because no differences in hormone concentrations between the groups were seen ^{55,138}. Complete maintenance of insulin sensitivity postoperatively was later achieved in patients undergoing total hip replacement when a prolonged hyperinsulinaemic-euglycaemic clamp initiated 3 h preoperatively and continued throughout surgery was used to induce continuous perioperative hyperinsulinaemia ¹³⁹. The patients in the control group exhibited a catabolic hormone profile, in contrast to the insulin treated patients who had significantly lower circulating concentrations of cortisol, glucagon and insulin-like growth factor binding protein, in addition to lower free fatty acids and fat oxidation rates. It was concluded that the principal mechanisms of action of the clamp treatment were minimization of the endocrine stress response and the change of the preoperative metabolic setting from a fasted to an absorptive state ¹³⁹.

A CHO rich beverage was then developed and tested safe for ingestion up to 2 h prior to commencement of surgery ¹⁴⁰ and administration of a large dose of 800 ml (100 g CHO) in the evening before surgery and a smaller dose of 400 ml (50 g CHO) immediately prior to surgery has since become established routine ¹⁶. This regimen of preoperative oral CHO loading has been shown to reduce development of peripheral insulin resistance on the first postoperative day after colorectal surgery compared to overnight fasted patients, without any effect on hepatic insulin sensitivity or counter-regulatory hormones ¹². Based on indirect calorimetry data, the authors concluded that the improved insulin sensitivity was due to better maintenance of glucose oxidation ¹². The same results were found when the effect of the beverage on development of insulin resistance immediately after total hip replacement surgery was examined ¹³. Additional

analyses revealed a plausible relation between increased rates of glucose oxidation and decreased pre- and intraoperative plasma levels of FFA and fat oxidation rates.

Interestingly, the effect persisted into the postoperative period, and was further enhanced during exogenous insulin infusion, when plasma levels of FFA were again equal to those in fasted patients ¹³. However, as the authors pointed out, it could not be proven whether the effect of the carbohydrate drink was specific in blunting surgery-induced insulin resistance or if the mechanism underlying the improvement in insulin sensitivity was of general character independent of the surgical trauma.

It has later been shown that whole-body insulin sensitivity is increased in non-operated healthy volunteers three hours after ingestion of the morning dose, but is unaffected by the CHO load given in the previous evening ¹⁹. This indicates a predominant role of the CHO load administered in the immediate preoperative phase and that the effect of the CHO drink on insulin sensitivity could be of general character such as facilitated glucose disposal, also known as the Staub-Traugott effect (see next section). In addition, this finding and the fact that modern fasting guidelines allow patients to eat until 6 h prior to surgery ¹⁴¹ puts a question mark to the relevance of the evening CHO dose and the extent to which it contributes to reduce postoperative insulin resistance.

Recently, a significant reduction in circulating concentrations of IL-6, one of the strongest predictors of postoperative insulin resistance ⁶, was demonstrated together with attenuated development of insulin resistance in CHO loaded patients until the 3rd day after major abdominal surgery ²⁰. Further, in patients scheduled for laparoscopic cholecystectomy, preoperative administration of a carbohydrate-based oral nutritional supplement containing glutamine and antioxidants has been shown to reduce

intraoperative plasma FFA concentrations and skeletal muscle metallothionein 1A mRNA expression, an indicator of oxidative stress ¹⁴². The same study also showed a reduction in muscle PDK4 gene- and protein expressions compared to preoperatively fasted patients indicating reversal of starvation-induced changes in PDK4 ¹⁴³, although it was not possible to attribute these effects to a specific constituent of the drink.

Thus, the mechanisms by which preoperative CHO intake inhibits development of postoperative insulin resistance and to which extent each of the CHO loads administered as part of the established clinical routine contributes to postoperatively enhanced insulin sensitivity is still poorly understood.

5.11. Facilitated glucose disposal / Staub-Traugott effect

Ingestion of 15 – 50 g of glucose is followed by an increase in peripheral insulin action and enhanced glucose disposal within ~3 h in non-operated healthy volunteers ^{19,144}.

Based on these findings, it has been suggested that triggering of facilitated glucose disposal (also known as the Staub-Traugott effect) and maintenance of this effect into the postoperative phase could explain the improvement in insulin sensitivity seen after surgery in preoperatively CHO loaded patients ¹⁴⁵. The Staub-Traugott effect was discovered almost a century ago ¹⁴⁶⁻¹⁴⁸, is characterized by improved glucose tolerance to sequential glucose loading and constitutes a physiologic factor in day-to-day glucose homeostasis. Research on the physiological mechanisms underlying the Staub-Traugott effect has been largely equivocal and the potential contribution to the prophylactic effect of preoperative CHO loading is therefore difficult to determine. The causes of the Staub-Traugott effect are suspected to be multifactorial and may even gradually come into play

with increasing demands on glucose homeostasis ¹⁴⁹, including enhanced glucose potentiation of insulin secretion by incretins and increased suppression of hepatic glucose release ¹⁵⁰, decreased hepatic insulin clearance ¹⁴⁹, suppression of free fatty acids ¹⁵¹ or enhancement of peripheral insulin sensitivity ^{19,144}.

6. AIMS OF THE STUDIES

Despite the last decade's increasing interest and research, the mechanisms underlying the development of postoperative insulin resistance and the prophylactic effect of preoperative carbohydrate treatment remain enigmatic. However, pathophysiological similarities between chronic and acute insulin resistant states exist. On this basis, the present thesis has the following aims:

Paper I:

To establish hyperinsulinaemic-euglycaemic step clamping with tracer glucose infusion and labelled glucose infusate for assessment of acute insulin resistance in pigs and to evaluate whether the premises for utilization of the technique would be altered by surgical instrumentation needed in order to combine this technique with invasive investigative methods.

Paper II:

To test the hypothesis that a single dose of preoperative oral CHO treatment 2 h prior to surgery is sufficient to maintain insulin sensitivity in the immediate postoperative phase. We further wanted to investigate the basis of such an effect and whether it is limited to pigs exposed to surgical trauma or if it is of general character, such as the Staub-Traugott effect.

Paper III:

To test the hypothesis that preoperative oral CHO supplementation reduces the magnitude of surgery-mediated inflammatory responses and impairment of insulin signal

transduction in skeletal muscle, thereby ameliorating insulin inhibition of muscle PDK4 expression in the immediate postoperative phase.

Paper IV:

To assess the change in mitochondrial functions with special focus on pyruvate oxidation capacity, levels of ROS release and uncoupling in skeletal muscle and liver mitochondria during a state of acute insulin resistance on the 2nd day after major abdominal surgery.

7. MATERIALS AND METHODS

7.1. Ethical approval

The study protocols were approved by the committee of the Norwegian Experimental Animal Board and all experiments were conducted in compliance with the institutional animal care guidelines and the National Institute of Health's (NIH) Guide for the Care and Use of Laboratory Animals [Department of Health and Human Services Publication no. (NIH) 85-23, revised 1985].

7.2. Animals, anaesthesia and instrumentation

Locally bred Yorkshire/Landrace hybrid pigs (~30 kg) were stalled and acclimatized in the animal research facilities for a week before experiments. Animals were submitted to a 12:12-hr light-dark cycle, a standardized diet and ad libitum access to water. The experiments were commenced between 6 and 7 a.m. Following sedation by intramuscular injection of 15 mg/kg ketamine, 1 mg/kg midazolam and 1 mg atropine and mask inhalation of 4% isoflurane in 100% O₂ the pigs were orotracheally intubated and gas anaesthesia was continued throughout the experiments at a minimal alveolar concentration of 0.8-1.5% mixed with 40-60% oxygen (Paper I-III) or with nitrous oxide and oxygen (40/60%) (Paper IV). Deep anaesthesia was induced through an i.v. bolus of 0.01 mg/kg fentanyl and maintained with i.v. infusion of 0.02 mg/kg/h fentanyl and 0.3 mg/kg/h midazolam. Respiration and minimal alveolar isoflurane concentration were monitored and anaesthesia was adjusted according to blood gas analysis and snout reflex tests. The general experimental setup and instrumentation is described in detail in paper 1

and is shown in figure 2. Paper specific surgical procedures are described in 7.6. and shown in figure 3 A-E.

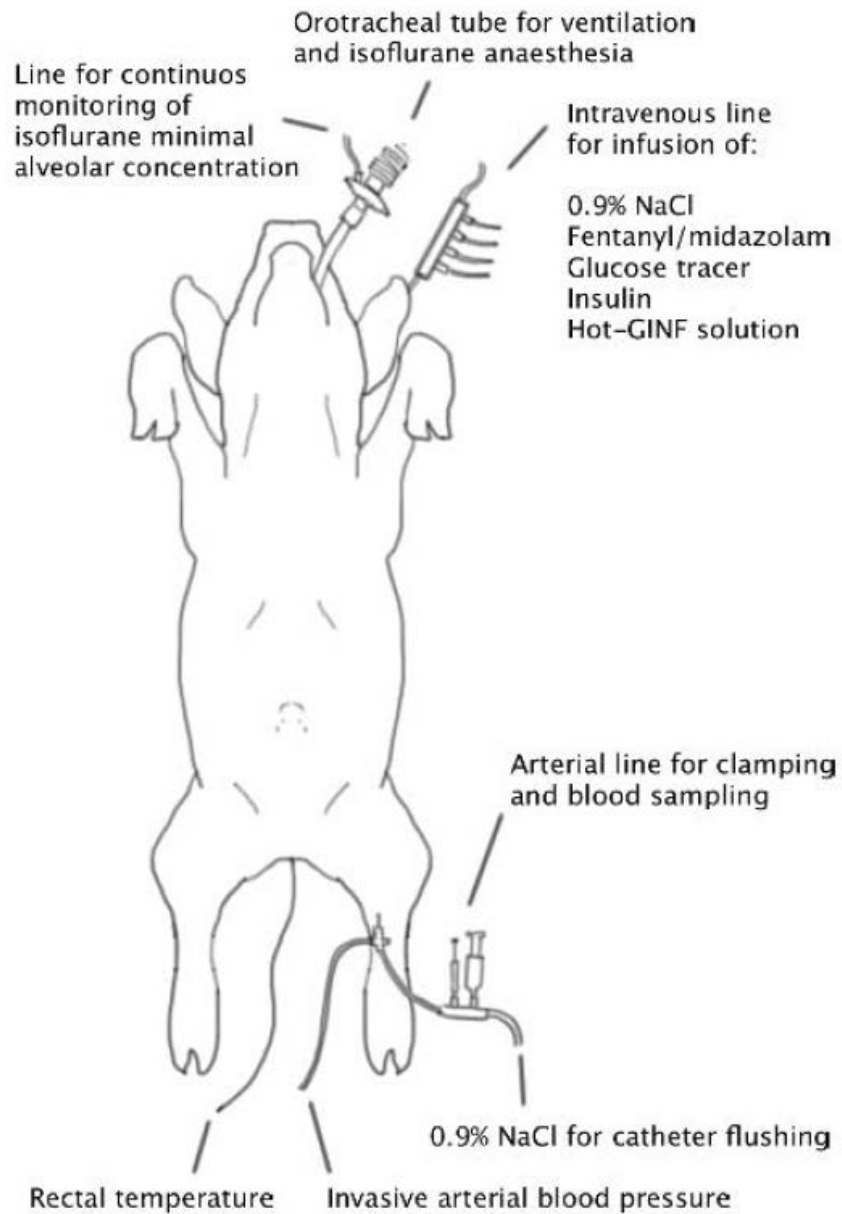
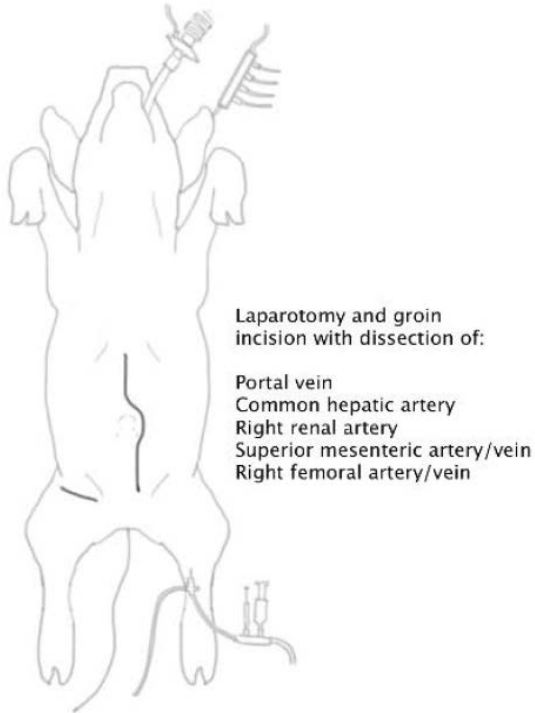


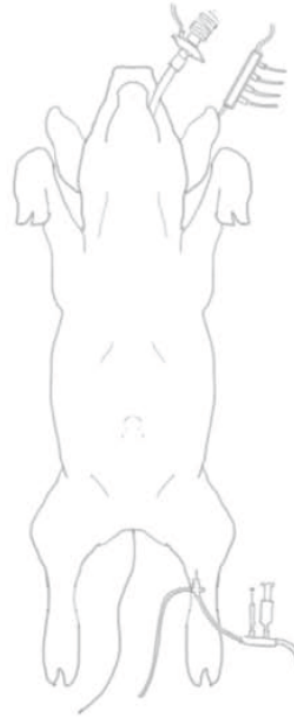
Figure 2 General experimental setup and instrumentation

Figure 3 Paper specific procedures

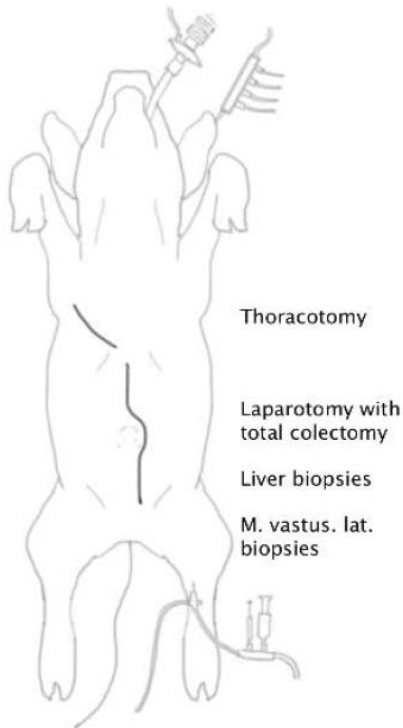
A Paper I: Substudy A



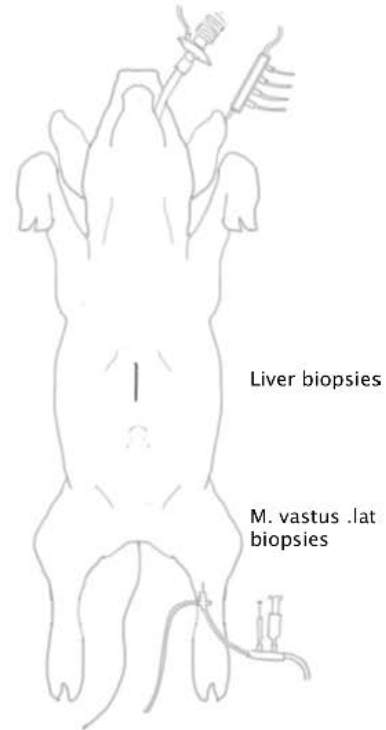
B Paper I: Substudy B



C Paper II/III: Surgery groups /Paper I: substudy C



D Paper II: Control groups

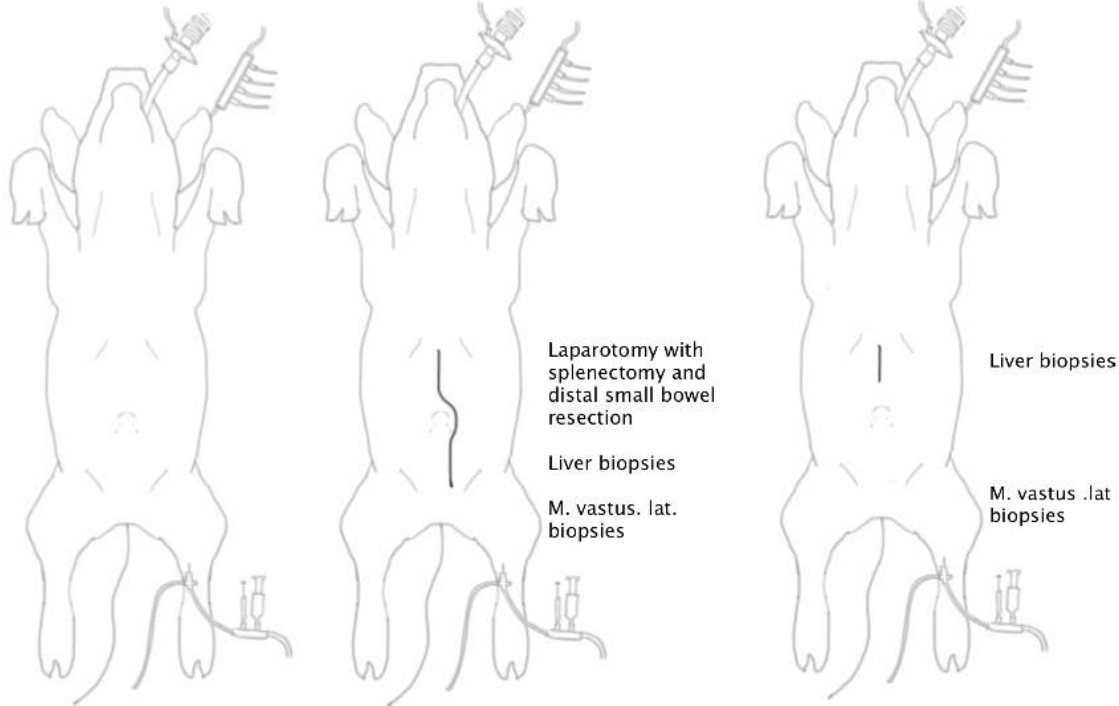


E Paper IV:

Experimental day 1

Experimental day 2

Experimental day 3



7.3. Assessment of acute insulin resistance

. The “gold standard” for assessment of insulin sensitivity, hyperinsulinaemic-euglycaemic clamping (HEC) was first introduced in 1979 by DeFronzo et al ¹⁵² and is based on infusion of insulin at a constant rate, while simultaneous infusion of glucose is titrated to euglycaemia. Aside from non-insulin dependent glucose uptake and residual endogenous release of glucose, the steady-state (SS) glucose infusion rate (GIR) equals the amount of glucose utilized in peripheral insulin sensitive tissues and thus, HEC gives a picture of whole body insulin sensitivity. The major advantage over other methods of insulin sensitivity measurement is that the investigator is in control of both insulin and glucose levels. In order to separate changes in hepatic from peripheral insulin sensitivity, i.e. changes in rates of EGR from rates of WGD, HEC can be combined with tracer dilution methodology ¹⁵³. Prior to HEC, basal EGR and WGD are calculated during

infusion of glucose tracer. The tracer infusion is then continued during the subsequent HEC and glucose kinetics are calculated based on non-SS equations and the SS GIR necessary to maintain euglycaemia. Further development of this method has shown that maintenance of constant tracer enrichment during clamping minimizes dependency on non-SS equations and generates more accurate data. A frequently used procedure is labelling of the glucose infusate at an atom percent enrichment approximately equal to that measured during basal SS condition, so-called hot-GINF HEC. If a stable glucose tracer is used, knowledge of the approximate level of basal SS tracer enrichment is prerequisite for correct labelling because immediate analysis during the procedure is not feasible.

The liver and peripheral tissues generally exhibit different degrees of insulin sensitivity¹⁵⁴ and insulin resistance can be caused by unresponsiveness or insensitivity to the hormone, or a combination of both¹⁵⁵. Therefore, hot-GINF HEC performed at stepwise increasing insulin infusion rates, so-called step clamping, gives more detailed information about sites, degrees and specific features of insulin resistance than a clamp performed at a single insulin infusion rate.

7.4. Measurement of tracer enrichment

Serum concentrations of the stable glucose tracer D-6,6-²H₂-glucose used in combination with HEC for the measurements of insulin sensitivity in the present experiments were analyzed by LC/MS/MS using Waters Acquity™ UPLC system (Waters, Milford, MA, USA) with an autosampler and a binary solvent delivery system interfaced to Waters Micromass® Quattro Premier™ XE benchtop tandem quadrupole mass spectrometer

(Waters, Manchester, UK) (technique described in detail in paper 1). For quantitative analysis, the following MRM transitions were used: m/z 179→89 and m/z 179→119 for glucose, m/z 181→89, m/z 181→91 and m/z 181→121 for D-[6,6-²H₂]glucose and MRM transition m/z 185→92 and m/z 185→123 for the internal standard D-[¹³C₆]glucose. Isotopic enrichment was calculated as tracer-to-tracee ratio after subtracting the isotopic enrichment of a background serum sample. An aliquot of the D-[6,6-²H₂]glucose infusate was analysed for the isotope concentration to calculate the actual infusion rate for each infusion experiment.

7.5. Calculation of glucose kinetics

Total glucose appearance rate (Ra) and WGD were calculated using modified versions of Steele's equations^{156,157}

$$Ra = \frac{F}{E(t)} - \frac{pV \times [C(t)/(1 + E(t))] \times (dE(t)/dt)}{E(t)} \quad \text{and} \quad WGD = Ra - pV \times \frac{dC}{dt}$$

where F is the tracer infusion rate, E is the tracer enrichment, p is the pool fraction taken as 0.65¹⁵⁶, V is the distribution volume of glucose taken as 230 ml/kg¹⁵⁸ and C is the glucose concentration. F consisted of the continuous tracer infusion alone prior to clamping, as opposed to during clamping where F was the sum of the continuous tracer infusion and tracer infused with the labelled glucose infusate during the last 40 min of each clamp. A linear curve was fit to the glucose concentration and tracer enrichment raw data by linear regression in order to minimize analytical variation and improve accuracy of the calculations. EGR was calculated by subtracting GIR from the calculated Ra.

7.6. Study design

7.6.1. Paper I

Substudy A: The glucose tracer was given as a 150-min long primed (6 mg/kg), continuous (0.12 mg/kg/min) infusion. The priming dose and continuous infusions rate were calculated from the decline in serum enrichment after a glucose tracer bolus in unpublished pilot experiments. Glucose kinetics were calculated based on tracer enrichment measured during the last 30 min of infusion. Circulating concentrations of hormones were measured 10 min before and 30 min prior to the end of tracer infusion. The procedure was repeated following surgical instrumentation after 4 days, consisting of a median laparotomy and groin incision followed by dissection of the portal vein, common hepatic artery, right renal artery and vein, superior mesenteric artery and vein, and right femoral artery and vein (Figs. 3A and 4).

Substudy B: Primed, continuous tracer infusion (basal period) was commenced 150 min prior to and continued throughout three consecutive clamp periods. Three 120-min long clamps were performed at stepwise increasing insulin infusion rates (0.2, 0.8, and 1.6 mU/kg/min) while glucose (200 mg/ml) labelled with tracer at 2.1 atom percent enrichment, according to the measured level of basal SS enrichment from substudy A was titrated to euglycaemia at 4.5 mmol/l. Arterial tracer enrichment was measured every 10th min during the last 30 min of the basal and successive clamp periods. Serum insulin was measured 30 min prior to the end of the basal period and every 30th min during the last hour of the successive clamp periods. Circulating hormone and FFA concentrations were measured 30 min prior to the end of the basal and clamp periods. After a period of 4 days,

the procedure was repeated with three different insulin infusion rates (0.4, 1.2, and 2.0 mU/kg/min) (Figs. 3B and 4).

Substudy C. A three-step clamp with insulin infusion rates at 0.4, 1.2, and 2.0 mU/kg/min was performed after major surgical trauma consisting of a right-sided thoracotomy, midline laparotomy, total colectomy, and closure of the incisions (Figs. 3C and 4).

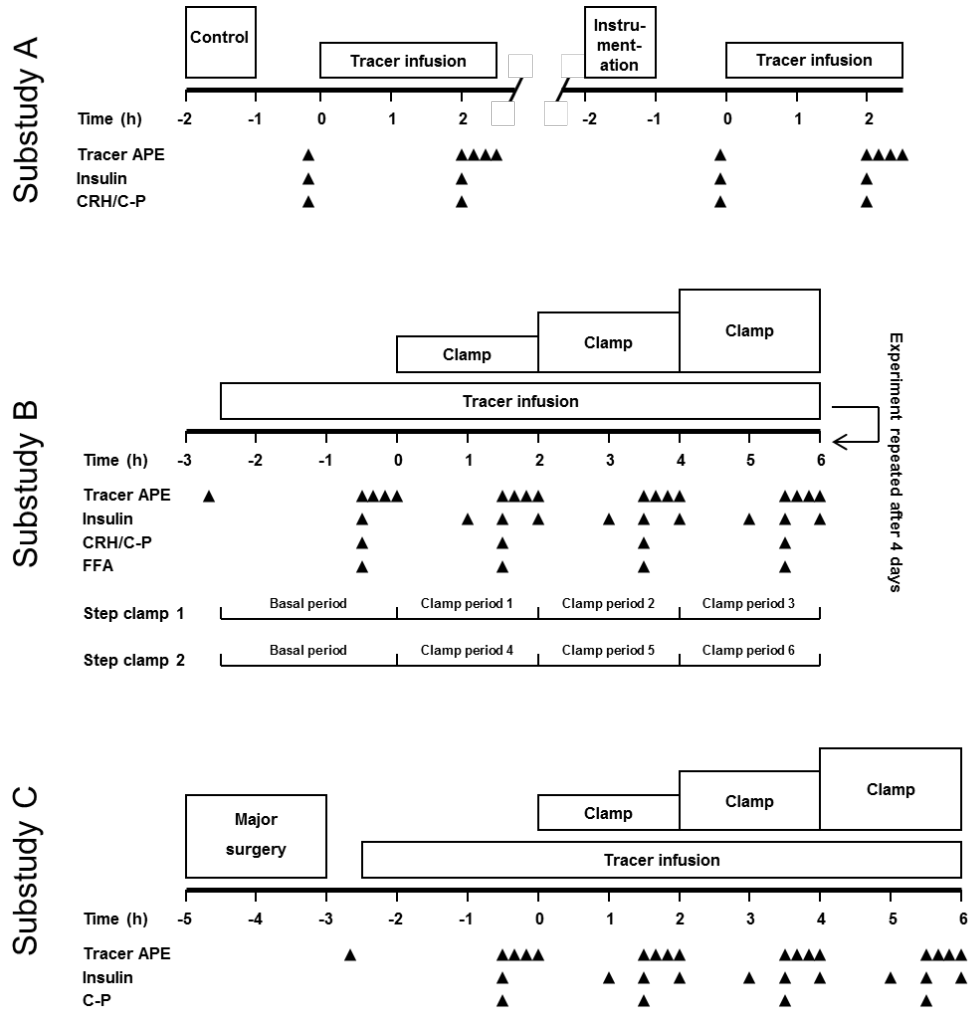


Figure 4 Study design paper I

7.6.2. Paper II

Pilot: In order to ensure adequacy of the CHO treatment dose in regard to postprandial hyperglycaemic and hyperinsulinaemic response, four pigs with an indwelling single-

lumen tunnelled central venous catheter received 200 ml of the carbohydrate beverage (12.6 g/100 ml carbohydrate, 79 % polysaccharides, 260 mOsm/kg, Nutricia preOp, Nutricia, Zoetermeer, The Netherlands). Repeated blood samples were collected from 10 min before to two hours after complete oral administration.

Main study: 32 pigs randomized to two surgery groups (surgery as described in paper I) and two non-operated time-matched control groups (each $n = 8$) received an oral morning dose of 200 ml containing 25 g carbohydrate (CHO/surgery and CHO/control) or were fasted overnight (fasting/surgery and fasting/control). The fasting/surgery group was used in paper I and II. Circulating concentrations of glucose, hormones and FFA were measured serially throughout the experiments and repeated hind limb muscle and liver biopsies were collected for measurement of glycogen content (paper II) and molecular analyses (paper III) (Figs. 3C and 5). Animals in the two non-operated time-matched control groups were treated identically except for the surgical trauma (Figs. 3D and 5).

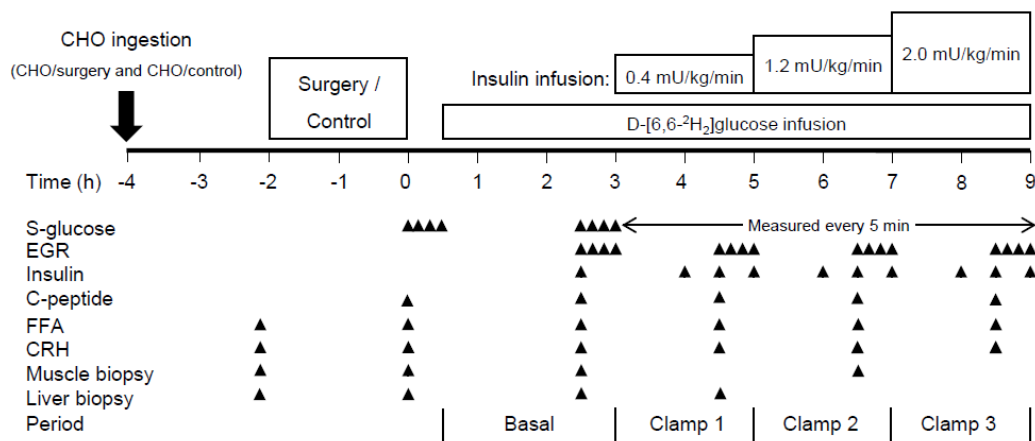


Figure 5 Study design main study paper II / paper III

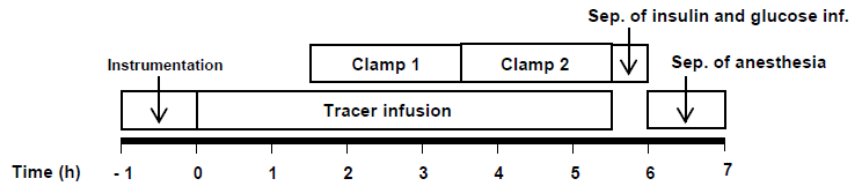
7.6.3. Paper III

Open muscle biopsies sampled at commencement of and immediately after surgery, and during the end of the second hyperinsulinaemic-euglycaemic clamp in the fasting/surgery and CHO/surgery groups in paper II (Figs. 3C and 5) were analysed by fast qRT-PCR and IR-Western blotting. The author performing the analyses (DC-T) was blinded to the treatment allocations.

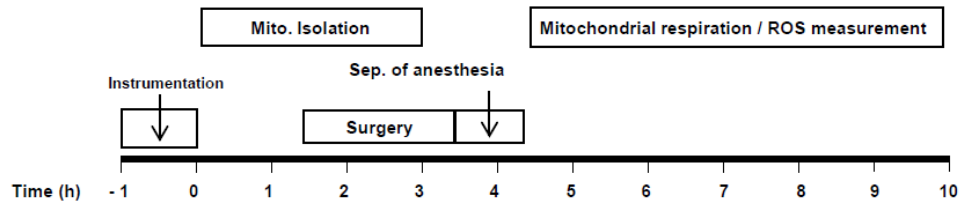
7.6.4. Paper IV

Eight pigs were examined in three consecutive experiments. On experimental day one the animals were anesthetized and preoperative levels of basal glucose turnover and peripheral and hepatic insulin sensitivity were assessed by tracer infusion followed by two-step hotGINF HEC. After three days (experimental day 2), biopsies from liver and skeletal muscle were sampled during anaesthesia for isolation of mitochondria followed by a midline laparotomy and resection of the spleen and 1.5 m of the distal small bowel with primary anastomosis 1 m proximal to the ileocecal junction. Animals were brought back to the animal facilities for surveillance and postoperative care. On the 2nd postoperative day (experimental day 3), animals were re-anesthetized, followed by sampling of blood and biopsies and measurement of postoperative of insulin sensitivity (Figs. 3E and 6).

EXP DAY 1



EXP DAY 2



EXP DAY 3

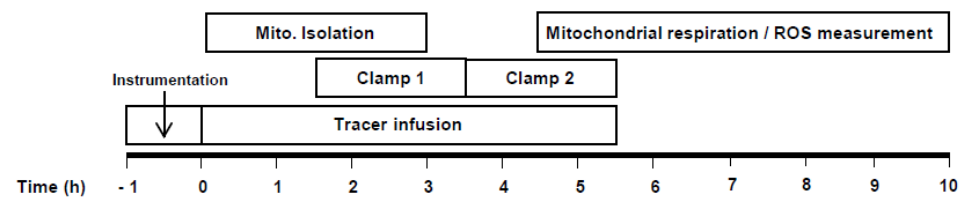


Figure 6 Study design paper IV

7.7. Real-time PCR measurements

Total RNA was isolated from frozen wet muscle (~30 mg) using Tri Reagent (Sigma Aldrich), according to the manufacturer's protocol. Total RNA quantification, first-strand cDNA synthesis and real-time PCR protocols were carried out as previously described¹⁵⁹. PDK4 Taqman primer/probe sets were obtained from Applied Biosystems (Foster City, CA, USA). The housekeeping gene hydroxymethylbilane synthase (HMBS) was used as an internal control. Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. The preoperative values were used as calibrator with a value of 1 within each group.

7.8. Protein extraction and Western blotting measurements

Cytosolic and nuclear proteins were extracted from approximately 30 mg frozen wet muscle tissue using a modified method by Blough¹⁶⁰. Muscle samples were lysed in the presence of phosphatase and protease inhibitors and protein content was quantitated using a Bradford assay. Protein lysates were run on a 4-12% Bis-Tris acrylamide gel (Invitrogen, UK) for 2 hrs at constant voltage (200 V) and transferred to a polyvinylidenedifluoride membrane (PVDF) overnight at constant 100 mA, in ice-cold buffers (4°C) as described by Constantin et al¹⁵⁹.

7.9. Measurement of mitochondrial respiratory activity and uncoupling

Measurement of oxygen consumption in isolated (isolation procedures described in detail in paper IV) skeletal muscle subsarcolemmal (SSM) and intramyofibrillar (IFM) and liver mitochondria was performed by high resolution respirometry, using an oxygraph (Oxygraph 2k, Oroboros Instruments, Austria) with a closed 2 mL chamber at 30 °C. Respiration was performed in respiration medium (pH 7.4) containing (all in mmol/L): KCl 100, MOPS 50, EGTA 1, KH₂PO₄ 5, and BSA 1 mg/mL. Mitochondria were added to the medium to give a final concentration of ~0.1-0.3 mg/mL. Respiration was measured with 5 mM malate + either 2.5 mM glutamate, 10 mM pyruvate or 2.5 μM palmitoyl-L-carnitine in separate runs. ADP (0.02 mmol/L) was added to achieve V_{max}. Mitochondrial leak oxygen consumption (V_{oligo}) was recorded after depletion of ADP and addition of 4 μg/mL oligomycin. To estimate mitochondrial proton leakage through ANT and UCP 2/3, 25 μmol/L carboxyatractyloside (ATR) and 500 μmol/L guanosine-diphosphate (GDP) were added and V_{ATR+GDP} was recorded after addition of oligomycin.

To estimate the postoperative change in the effect of ATR+GDP, pre- and postoperative $\Delta V_{\text{uncoupling}}$ calculated as $V_{\text{oligo}} - V_{\text{ATR+GDP}}$ were compared.

7.10. Measurement of mitochondrial ROS release

H_2O_2 was estimated fluorometrically by oxidation of Amplex Red (non-fluorescent) to resofurin (fluorescent), as described by Schönfeldt et al.¹⁶¹ with modifications. Briefly, isolated mitochondria were added (~0.3 mg/mL) to a respiration buffer containing malate + glutamate or palmitoyl-L-carnitine (PC) and oligomycin (as in respiration measurements). For estimation of H_2O_2 release, 50 $\mu\text{mol/L}$ Amplex Red and 2 IU/mL of horseradish peroxidase were added to the suspension, allowing Amplex Red oxidation by H_2O_2 . Superoxide ($\text{O}_2^{\cdot-}$) release was estimated fluorometrically as described by Johnson-Caldwell et al.¹⁶². Briefly, the same mitochondria suspension as for H_2O_2 release was prepared and 5 $\mu\text{mol/L}$ MitoSOX^{red} was added. All samples were then added to a 96 well microplate and $\text{O}_2^{\cdot-}$ and H_2O_2 were measured simultaneously in separate wells. Release of $\text{O}_2^{\cdot-}$ was monitored at ex 510 nm/em 580 nm and release of H_2O_2 was monitored at ex 571 nm/em 585 nm at 30 °C, using a spectrofluorometer (Spectramax Gemini EM). The production of H_2O_2 was calculated as the increase in relative fluorescence (RF)/min over a time period of 30 min and $\text{O}_2^{\cdot-}$ release was estimated as RF after 15 min incubation.

8. SUMMARY OF RESULTS

8.1. Paper I

In paper I, we established insulin dose-response curves for hepatic and peripheral insulin sensitivity (Substudy B) (Fig. 8) and verified the applicability of this technique for assessment of acute insulin resistance in anesthetized pigs (Substudy C) by performing a series of studies with 3-step HEC in combination with glucose tracer infusion and labelled glucose infusates (Fig. 7).

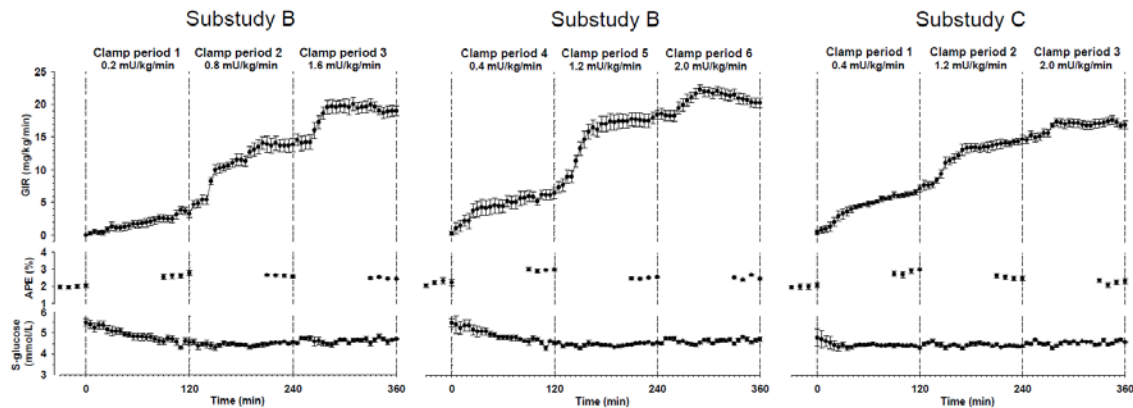


Figure 7 Step-clamp timelines during step hot-GINF HEC procedures (n=8). GIR, glucose infusion rate; APE, glucose tracer atom percent enrichment; S-glucose, serum glucose. Data are means \pm SE.

We further found that instrumentation and dissection needed for combination of the technique with other investigative methods such as regional blood flow measurements and sampling did not elicit alterations in the hormonal milieu (Table 1) or glucose homeostasis that would change the premises for correct labelling of the glucose infusates (Table 1). Higher serum insulin concentrations, probably caused by decreased metabolic insulin clearance rate were found after surgery (Fig. 8), which should be kept in mind when utilizing the described method for measurement of acute insulin resistance in porcine research models.

Figure 8 Calculated endogenous glucose release (EGR) and whole-body glucose disposal (WGD) from both step hot-GINF HEC procedures in substudy B (anaesthesia only; $n=8$) and the step hot-GINF HEC performed after major surgery (surgery; $n=8$) plotted vs. serum insulin (S-insulin) levels. Data are means \pm SE

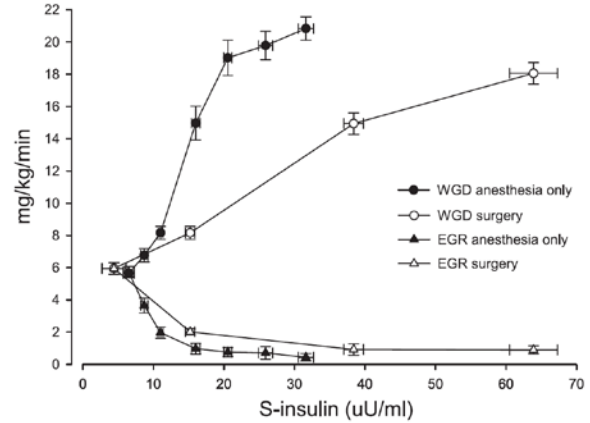


Figure 9 Relative changes in EGR and WGD from basal at low (clamp 2 in substudy B vs. clamp 1 in substudy C) and high (clamp 6 in substudy B vs. clamp 2 in substudy C) serum insulin ($n=8$). Data are means \pm SE. * $P<0.01$; ** $P=0.001$; *** $P<0.001$ vs. anaesthesia only.

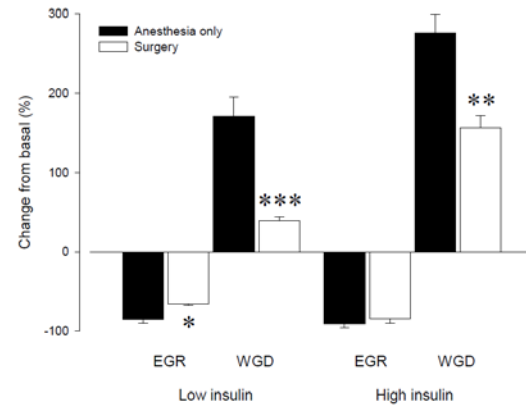


Table 1 Substudy A: circulating hormone concentrations

	Control	Instrumentation
Insulin, $\mu\text{U/ml}$	7.5 ± 0.7	7.7 ± 0.6
C-peptide, ng/ml	0.13 ± 0.05	0.17 ± 0.05
Cortisol, nmol/l	57.5 ± 20.32	59.0 ± 12.7
Glucagon, pmol/l	16.4 ± 1.2	16.1 ± 1.7
Epinephrine, nmol/l	0.56 ± 0.13	0.47 ± 0.10
Norepinephrine, nmol/l	0.47 ± 0.08	0.33 ± 0.04

Data are means \pm SE. No significant difference noted between control and instrumentation.

Table 2 Substudy A: tracer enrichment and glucose kinetics

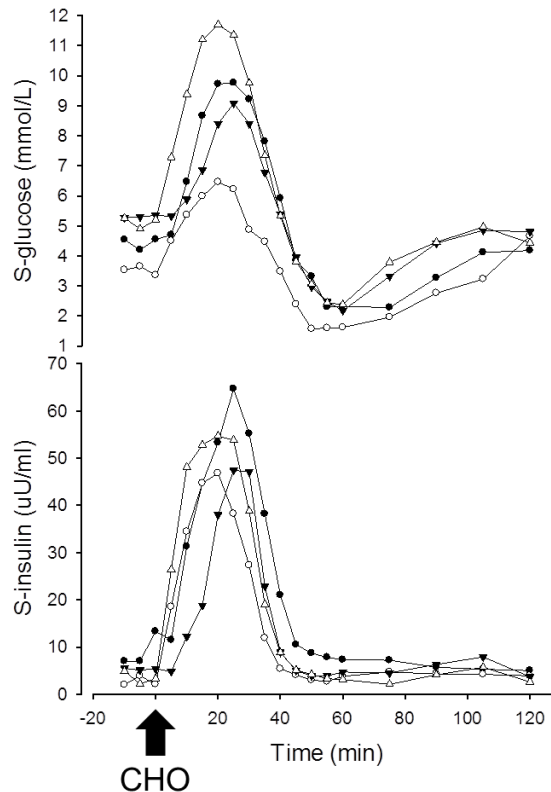
	Control	Instrumentation
Tracer APE, %	2.11 ± 0.05	2.19 ± 0.09
CV Tracer APE, %	5.4 ± 0.4	7.3 ± 1.4
WGD, $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$	5.65 ± 0.15	5.55 ± 0.30
EGR, $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$	5.62 ± 0.16	5.45 ± 0.30
S-glucose, mmol/l	4.66 ± 0.19	4.54 ± 0.25
CV S-glucose, %	4.7 ± 0.4	5.6 ± 1.4

Data are means \pm SE. APE, atom %enrichment; CV, coefficient of variation; WGD, whole body glucose disposal; EGR, endogenous glucose release; S-glucose, serum glucose. Control, anaesthesia only; instrumentation, surgical instrumentation. No significant difference noted between control and instrumentation.

8.2. Paper II

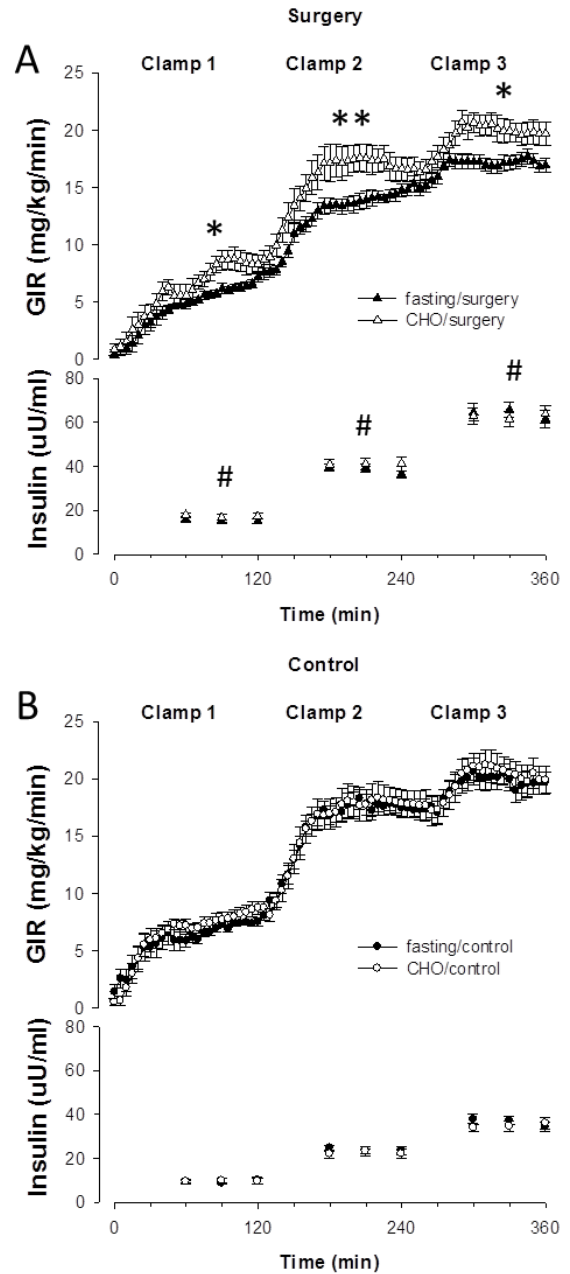
In paper II, the pilot study revealed that a dose of ~ 0.85 g CHO pr kg bodyweight, which is equivalent to the standard morning dose given to humans, induces hyperglycaemia and a concomitant uniform rise in serum insulin similar to the levels seen in human studies^{140,163} (Fig. 10).

Figure 10 Serum glucose and insulin in four separate pigs plotted against time after administration of an oral carbohydrate (CHO) load of 25 g (arrow).



In the main study, the same single CHO dose was then shown to increase the GIR needed to maintain euglycaemia during the last 40 min of each step-clamp in the CHO/surgery compared with the fasting/surgery group (Fig. 11A), indicating increased postoperative whole-body insulin sensitivity after preoperative CHO supplementation. The non-operated control groups exhibited equal whole-body insulin sensitivity (Fig. 11B), indicating that the effect of CHO is limited to surgically stressed animals.

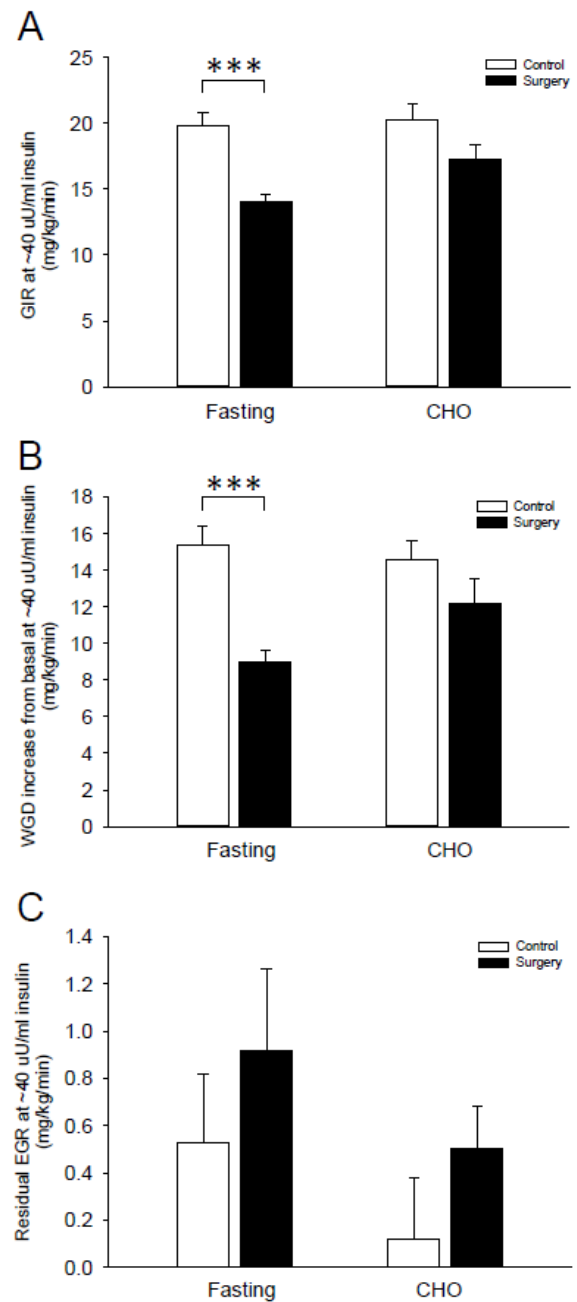
Figure 11 Glucose infusion rate (GIR) and serum insulin during three consecutive hyperinsulinaemic-euglycaemic clamps at stepwise increasing insulin infusion rates (0.4, 1.2 and 2.0 mU/kg/min) plotted against time in fasted and CHO treated operated (A) and non-operated control animals (B). Each group $n=8$. Data are means \pm SE. *Significantly different from fasting surgery, one symbol $P<0.05$; two symbols $P<0.02$ (Student's independent t-test). #Significantly different from corresponding control group, $P<0.001$ (Student's independent t-test). The fasting/surgery group is the same group shown in figure 7.



Due to different insulin serum concentrations during clamping (Fig. 11), insulin sensitivity in the fasting and CHO treated groups was compared at $\sim 40 \mu\text{U/ml}$ (i.e. clamp 2 in the surgery groups vs. clamp 3 in control groups). A 29% lower SS GIR was seen after surgery in fasted pigs whereas the decline in CHO treated pigs did not reach statistical significance (-15% , $P=0.088$) (Fig. 12A). Compared to their non-operated controls, surgery induced a 41% decline in insulin-stimulated WGD at $\sim 40 \mu\text{U/ml}$ serum

insulin in fasted pigs. The postoperative decline was reduced to a mere 16% in CHO treated pigs ($P=0.180$) (Fig. 12B). No effect of CHO treatment was seen on hepatic insulin sensitivity (Fig. 12C) or glycogen content in liver and muscle after surgery.

Figure 12 Postoperative glucose infusion rate (GIR) (A) and insulin stimulated whole-body glucose disposal (WGD) (B) during clamping at $\sim 40 \mu\text{U/ml}$ s-insulin in fasted and carbohydrate treated animals (CHO) compared to their respective control groups. Each group $n=8$. Data are means \pm SE. ***Significant difference by Student's independent t-test, $P<0.001$.



Preoperatively fasted and CHO supplemented pigs displayed an equal increase in serum cortisol concentrations during the experiments compared to their respective non-operated controls. Glucagon was increased in CHO/surgery compared to CHO/control during the low insulin clamp (Fig. 13).

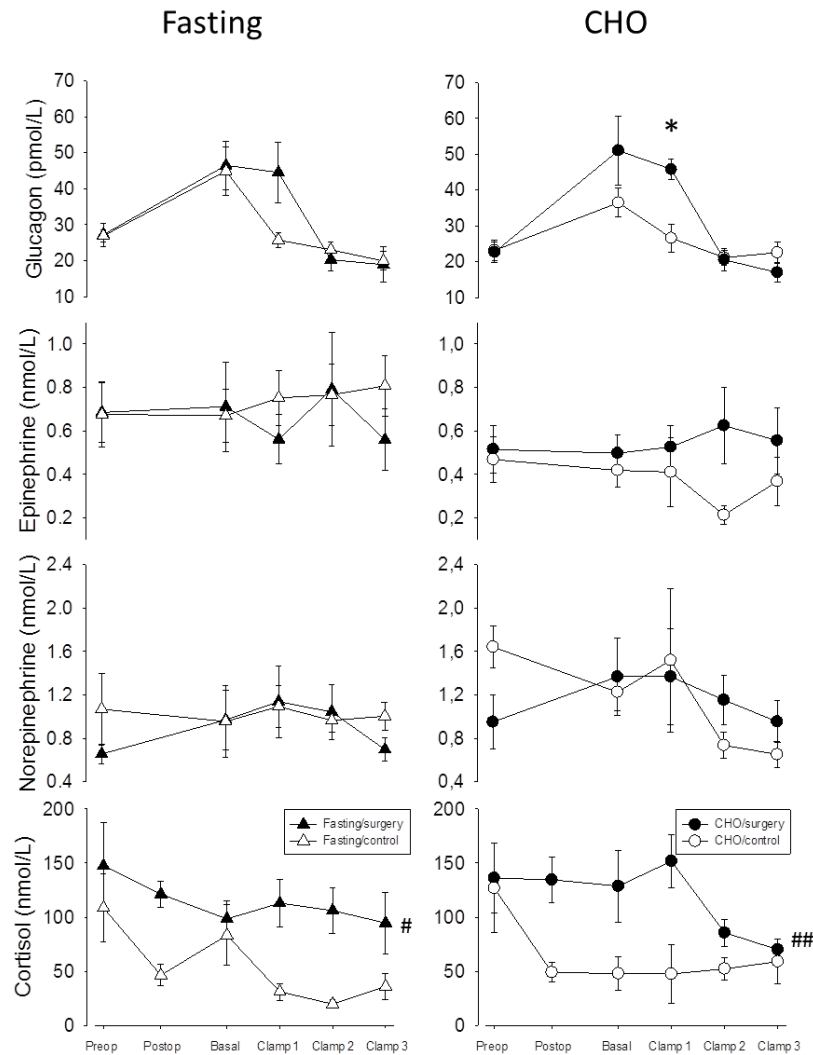


Figure 13 Counter-regulatory hormone profiles during the experiments. Each group $n=8$. Data are means \pm SE. *Significant difference from preoperative, $P<0.02$; #Significant difference by group, one symbol $P<0.02$, two symbols $P=0.01$ (two-way (time and group) repeated measures ANOVA).

A strong negative correlation between mean cortisol and insulin stimulated WGD was seen in fasted (Fig. 14A), but not in CHO treated animals (Fig. 14B).

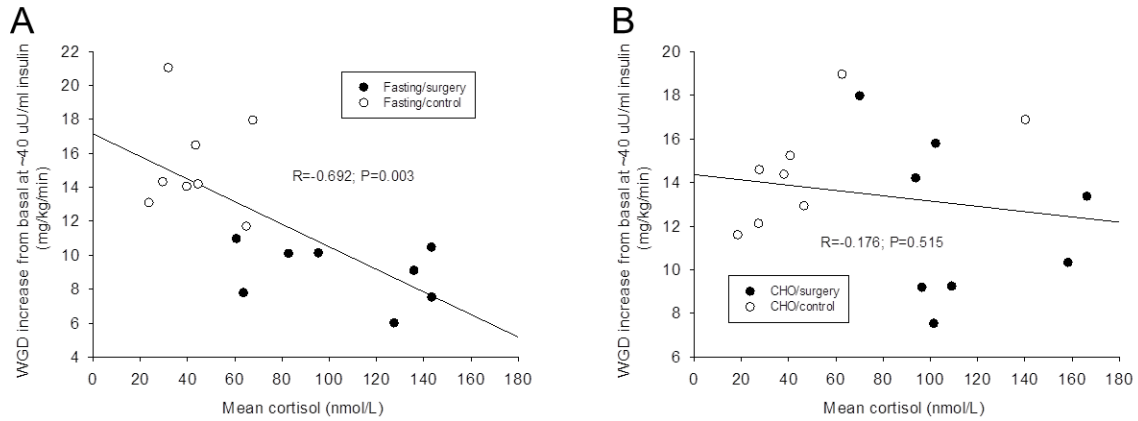


Figure 14 Correlation between mean cortisol after surgery/control and the insulin stimulated increase in whole-body glucose disposal (i.e. peripheral insulin sensitivity) in fasted (A) and carbohydrate treated animals (B). Pearson's correlation was used ($n = 16$).

FFA concentrations were reduced in the CHO/surgery group compared to the fasting/surgery group both perioperatively and during the step-clamp procedures. Compared with the fasting/control group there was a similar, but statistically non-significant ($P=0.09$) trend towards lower perioperative FFA in the CHO/control group, whereas no difference was seen during the step-clamps (Fig. 15).

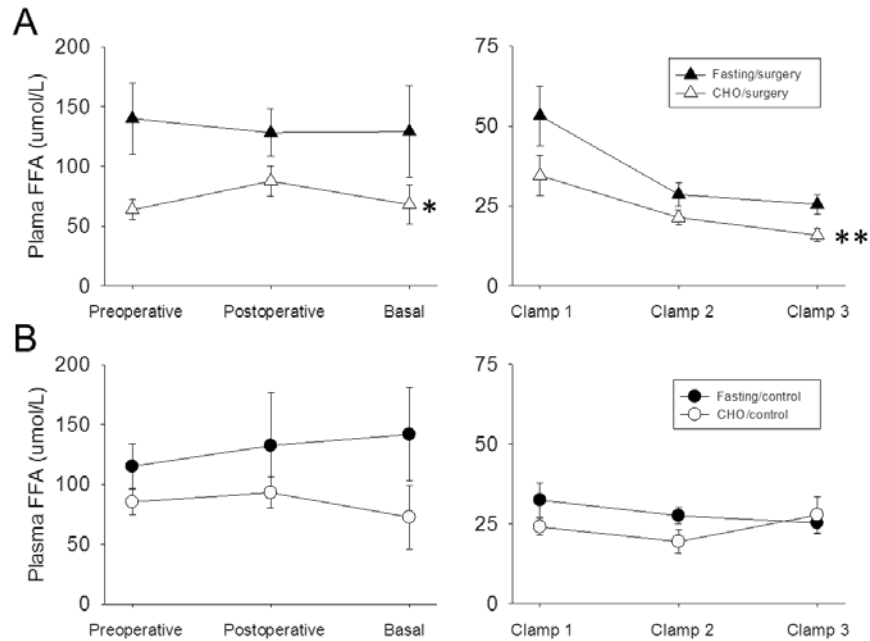


Figure 15 Free fatty acid time course perioperatively (left) and during three step clamping (right) in the surgery (A) and control (B) groups. Each group $n=8$. Data are means \pm SE. *Significantly different by group, one symbol $P<0.05$, Two symbols $P<0.02$ (two-way (time and group) repeated measures ANOVA)

8.3. Paper III

In paper III, we showed that skeletal muscle inflammatory responses to surgical trauma, as measured by the expression of IKK β (Fig. 16A), SOCS3 (Fig. 16B) and ratio of phosphorylated to total JNK2 (Fig. 16C) were significantly lower in the CHO group than in the fasted group during the postoperative measurement of insulin. Further, the ratio of phosphorylated to total NF κ B protein decreased in the CHO group during clamping indicating reduced inflammatory activity, whereas no difference was seen in the fasting group (Fig. 16D).

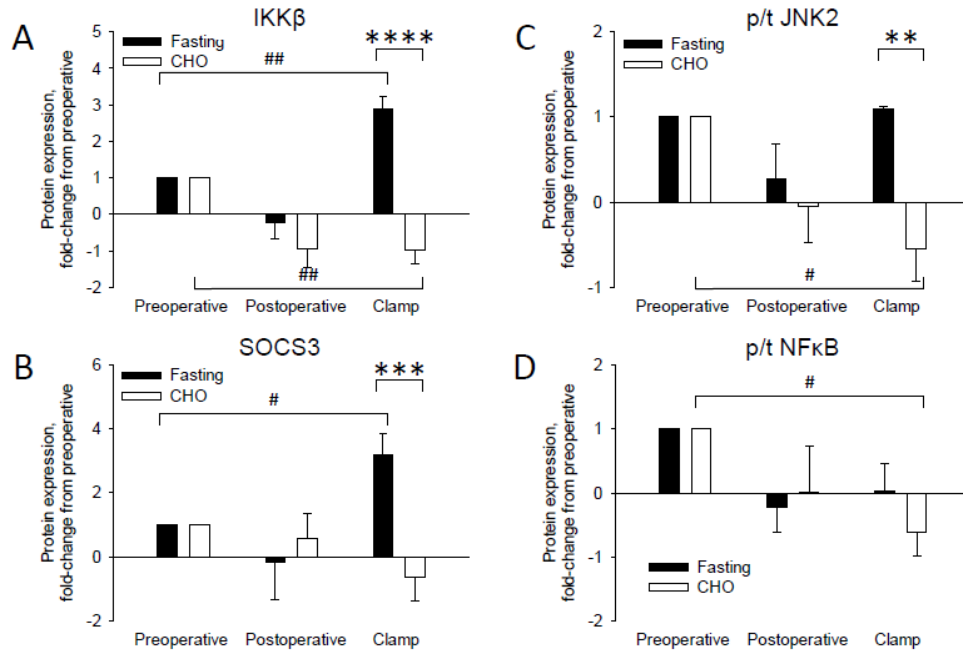
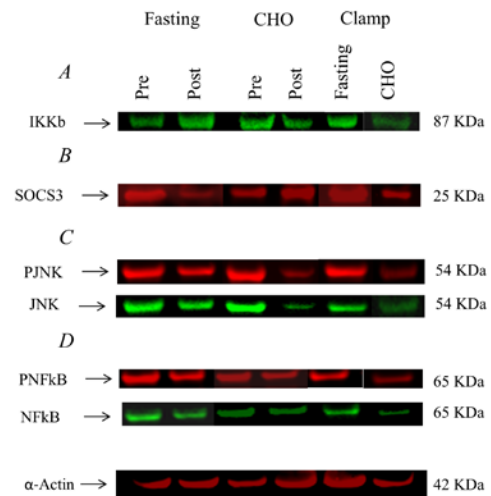


Figure 16 Mean inhibitor κ B kinase (IKK β), suppressor of cytokine signaling 3 (SOCS3) and ratio of phosphorylated to total c-Jun N-terminal kinase 2 (JNK2) and nuclear factor kappa B (NF κ B) protein expression presented as fold-change from preoperative values. Each group $n=8$. Data are means \pm SE. *Significant difference by Mann Whitney U-test, #Significant difference by Wilcoxon t-test; one symbol, $P<0.05$; two symbols; $P<0.02$, three symbols; $P<0.01$, four symbols; $P<0.001$.

Figure 17 Typical Western blots depicting IKK β , SOCS3, JNK2 and NF κ B protein bands with their corresponding molecular weights.



Further, the inhibitory Ser³⁰⁷ phosphorylated IRS1 ratio was 2.4-fold reduced during clamping in the CHO group, whereas no significant change was seen in the fasting group

(Fig. 18A). A significant decrease in the ratio of inactivated FOXO1 to total FOXO1 was seen during clamping in the fasted group (-2.3-fold, $P=0.05$), but not in the CHO group (-1.1-fold, $P>0.05$; Fig. 18B), demonstrating inhibitory action of insulin on FOXO1 in both groups, albeit to a greater extent in the preoperatively CHO treated animals.

Figure 18 Mean ratio of Ser³⁰⁷ phosphorylated to total insulin receptor substrate 1 (IRS1, Fig. A) and Ser²⁵⁶ phosphorylated to total forkhead transcription factor 1 (FOXO1, Fig. B) presented as fold-change from preoperative values. Each group $n=8$. Data are means \pm SE. *Significant difference by Wilcoxon t-test, $P<0.02$.

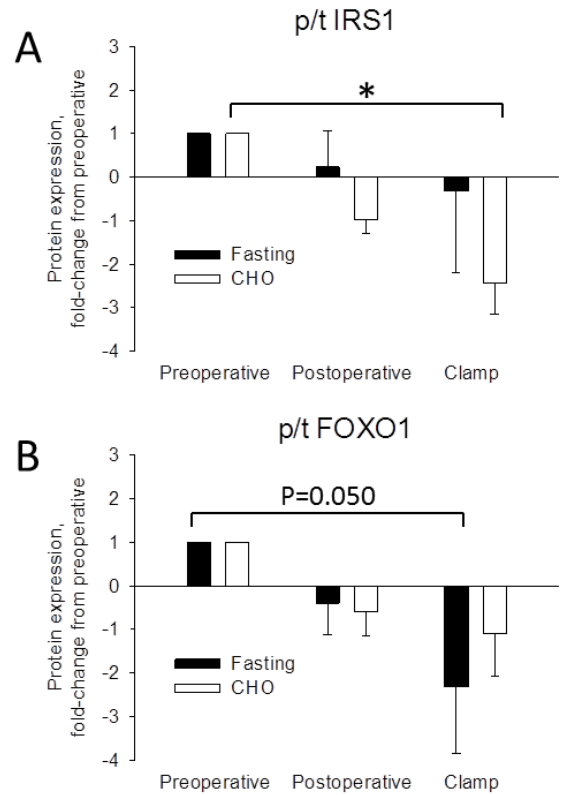
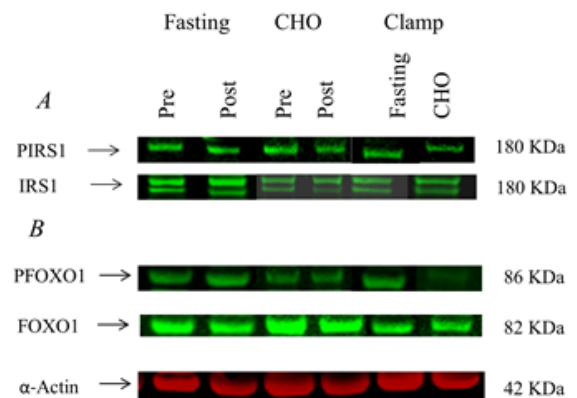
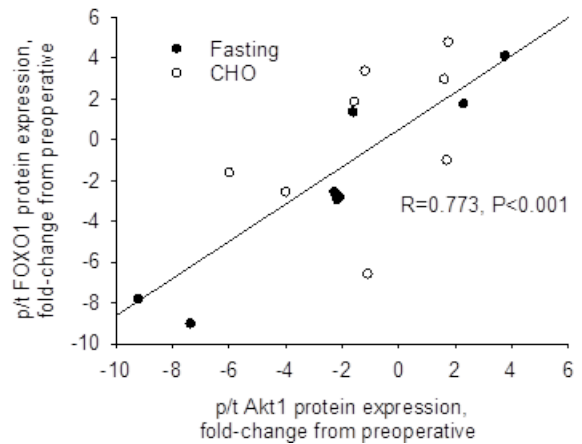


Figure 19 Typical Western blots depicting IRS1 and FOXO1 protein bands with their corresponding molecular weights.



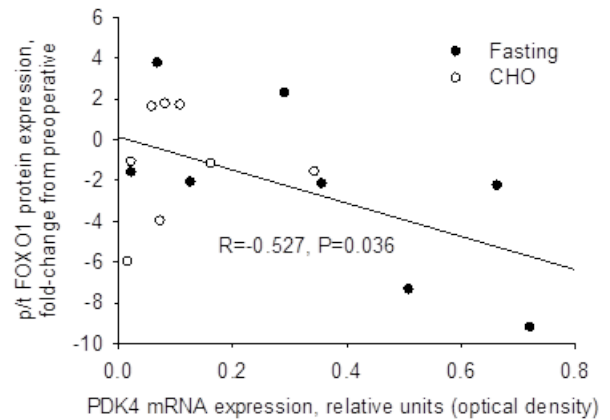
The restraining action of insulin on FOXO1 activity, i.e. ratio pFOXO1/tFOXO1 was confirmed by a strong correlation between the fold-change in phosphorylated to total Akt1 (i.e. activation) protein to fold-change phosphorylated to total FOXO1 (i.e. inactivation) protein from preoperative during insulin stimulation (Fig. 20).

Figure 20 The Pearson's correlation between fold-change from preoperative levels in Ser⁴⁷³ phosphorylated to total Akt1 and Ser²⁵⁶ phosphorylated to total forkhead transcription factor 1 (FOXO1) in skeletal muscle during hyperinsulinaemic-euglycaemic clamping ($n=16$).



The fold-change in ratio of phosphorylated to total FOXO1 during the clamp was significantly inversely correlated to the levels of PDK4 mRNA expression (Fig. 21).

Figure 21 The Pearson's correlation between pyruvate dehydrogenase kinase 4 (PDK4) gene expression and fold-change from preoperative levels in Ser²⁵⁶ phosphorylated to total forkhead transcription factor 1 (FOXO1) in skeletal muscle during hyperinsulinaemic-euglycaemic clamping ($n=16$).



Following surgery a similar ~20-fold increase in muscle PDK4 mRNA expression was seen in both groups (Fig. 22A), whereas during the 4 hours of hyperinsulinaemic-euglycaemic clamping the inhibitory effect of insulin on muscle PDK4 mRNA expression in the CHO group was almost twice as great than in the fasting group compared to the postoperative values (Fig. 22B). Consequently, PDK4 protein expression measured during the clamp was almost 5-fold lower in the CHO-loaded group than in the fasted group when compared with the preoperative values (Fig. 22C).

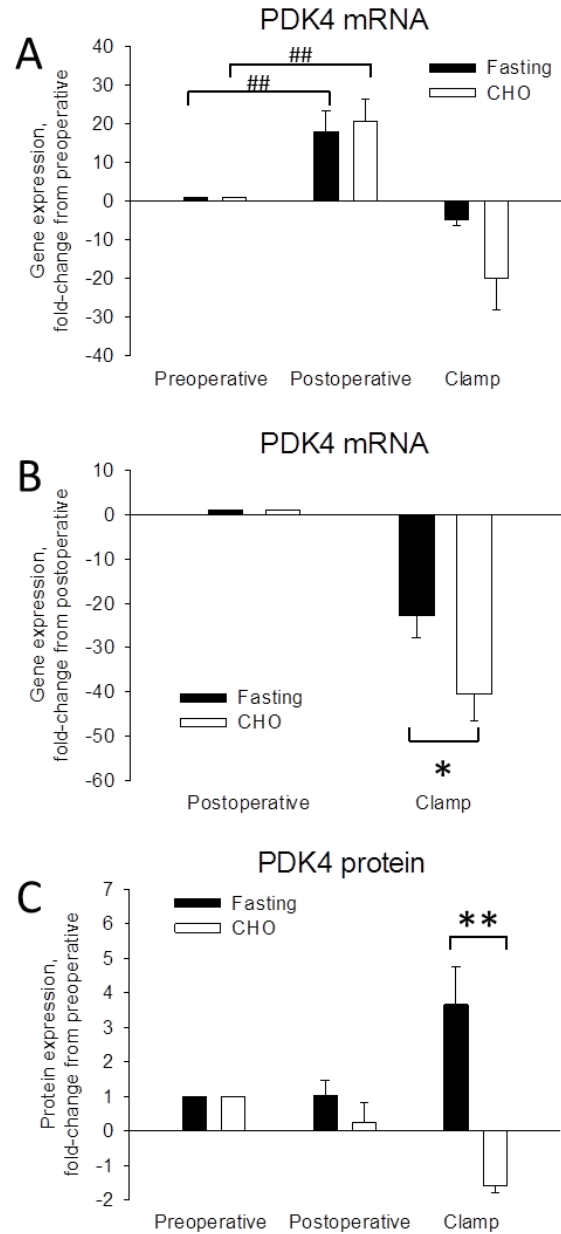


Figure 22 Mean pyruvate dehydrogenase kinase 4 (PDK4) gene expression reported as fold-change from pre- and post-operative values and expression PDK4 protein reported as fold-change from preoperative. Each group $n=8$. Data are means \pm SE. *Significant difference by Mann Whitney U-test, #Significant difference by Wilcoxon t-test, one symbol $P<0.05$; two symbols $P<0.01$.

The rate of insulin stimulated whole-body glucose disposal was negatively correlated to both the level of muscle PDK4 protein expression and the ratio of phosphorylated to total NFκB (Figs. 23A and B, respectively). Furthermore, a strong positive relationship between PDK4 and p/tNFκB protein expression was observed during clamping (Fig. 23C).

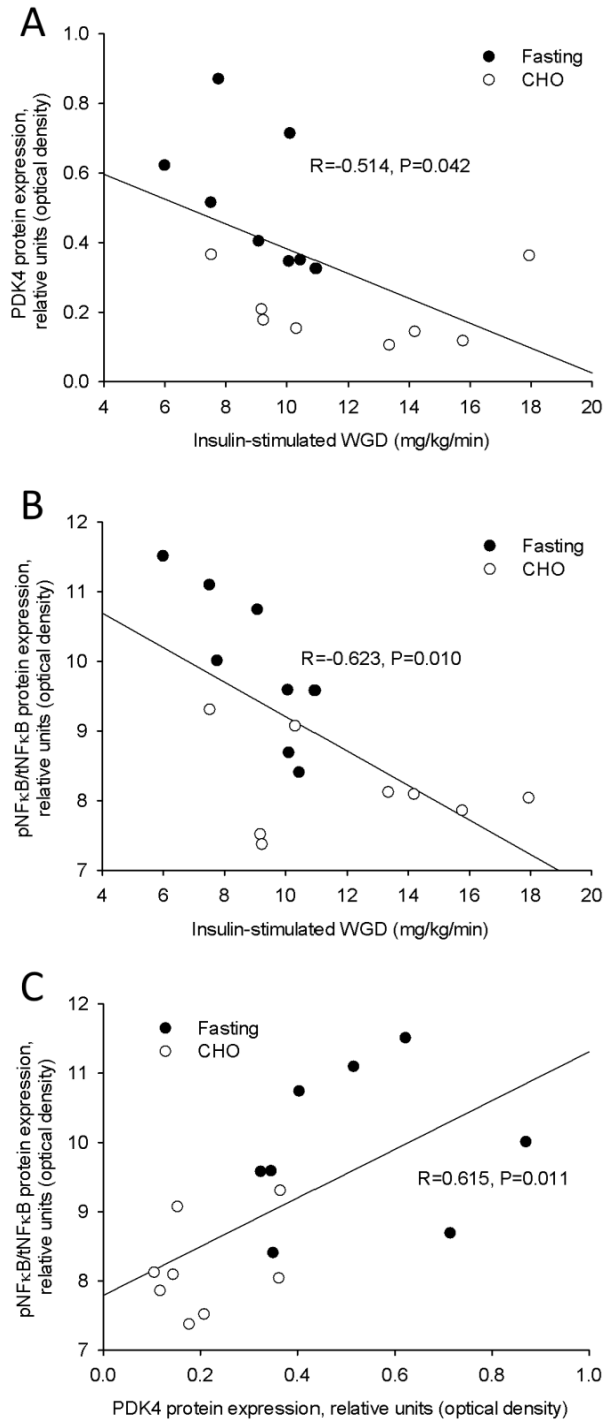


Figure 23 Correlation between pyruvate dehydrogenase kinase 4 (PDK4) protein expression (A) and ratio of Ser⁵³⁶ phosphorylated to total NFκB (B) and whole-body glucose disposal rate during hyperinsulinaemic-euglycaemic clamping. Correlation between PDK4 and p/t NFκB during clamping is shown in C. Pearson's correlation was used ($n=16$).

8.4. Paper IV

As in the immediate postoperative phase studied in paper II, a significant decrease in SS GIR at $\sim 40 \mu\text{U/ml}$ serum insulin was measured on the 2nd day after surgery when compared to preoperative (11.19 ± 0.94 vs. 17.31 ± 1.53

mg/kg/min , $P < 0.001$). Significant postoperative reductions in WGD was seen during both low and high insulin clamps, whereas EGR remained unchanged, indicating isolated development of peripheral insulin resistance (Fig. 24).

Relative hyperglycaemia was seen on the 2nd postoperative day ($5.68 \pm 0.25 \text{ mmol/L}$ vs. 4.52 ± 0.42 ; $P < 0.05$) and plasma FFA concentrations in conscious animals were also higher than before surgery, although this increase did not reach statistical significance (325 ± 48 vs. $236 \pm 36 \mu\text{mol/L}$, $P = 0.08$). Arterial plasma FFA concentrations were unchanged during the basal period and were equally suppressed during low and high insulin clamping before and after surgery. During pyruvate-induced respiration, there was a postoperative reduction in V_{max} of 61% in IFM and 40% in SSM with a modest 28% reduction of V_{oligo} in SSM, but not IFM, whereas no postoperative change was seen during respiration with glutamate as substrate. V_{max} for palmitoyl-L-carnitine was

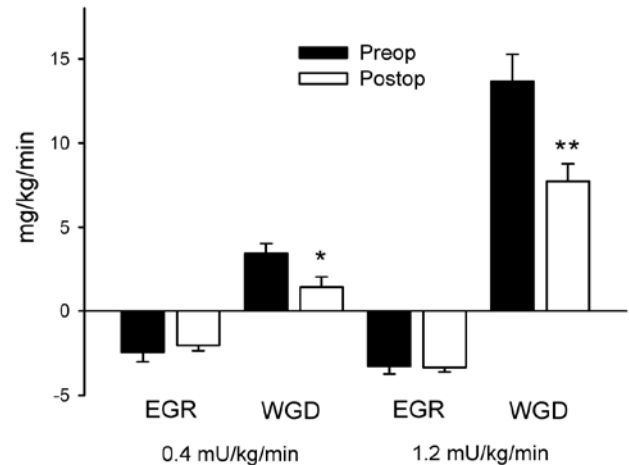
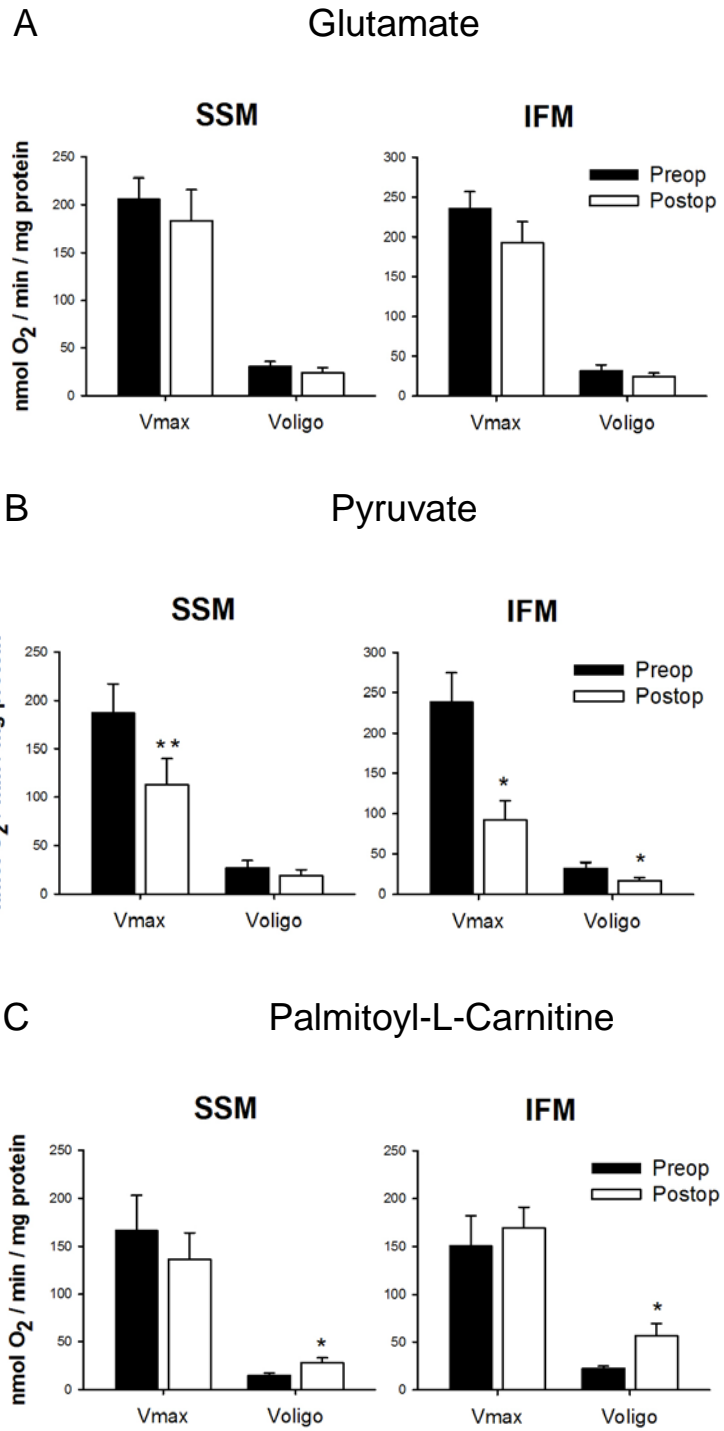


Figure 24 Changes in EGR and WGD from basal during low (0.4 mU/mg/kg) and high (1.2 mU/mg/kg) insulin clamps. Data are mean \pm SE ($n=8$). *Significantly different from preoperative, one symbol $P < 0.01$; two symbols $P < 0.002$ (Student's dependent t-test).

unchanged in SM mitochondria, while V_{oligo} was increased in both SSM (1.9-fold) and IFM (2.5-fold) (Fig. 25).

Figure 25 ADP-stimulated respiration (V_{max}) and leak respiration (V_{oligo}) in intramyofibrillar (IFM) and subsarcolemmal mitochondria (SSM). Data are mean \pm SE from mitochondrial preparations from 7 pigs. *Significantly different from preoperative, one symbol $P < 0.05$; two symbols $P < 0.001$ (Student's dependent t-test).



Postoperatively, significant increases in the rates of H₂O₂ release from SSM (~2.3-fold), IFM (~2.5-fold) and liver mitochondria (~2.3-fold) were observed using glutamate as respiratory substrate (Fig. 26).

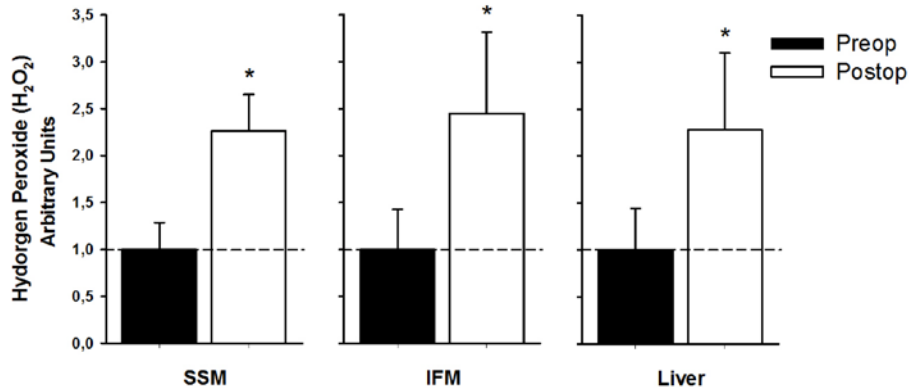


Figure 26 Hydrogen peroxide (H₂O₂) production reported as fold-change from preoperative values in intermyofibrillar (IFM), subsarcolemmal (SSM) and liver mitochondria supplied with glutamate and malate as respiratory substrates. Data are mean ± SE of mitochondrial preparations from 7 pigs. **P*<0.05 vs. preoperative (Student's dependent t-test).

When palmitoyl-L-carnitine was used as respiration substrate, O₂^{•-} release increased significantly after addition of GDP+ATR from both IFM and SSM, but not from liver mitochondria (Fig. 27).

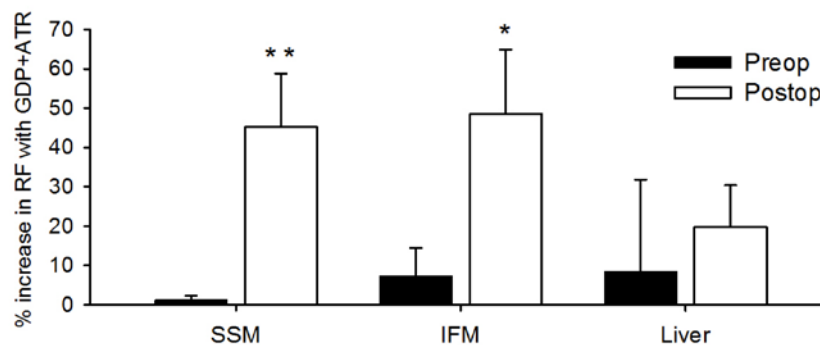


Figure 27 Pre- and postoperative % increase in relative fluorescence (RF) when superoxide (O₂^{•-}) release was estimated during V_{oligo} palmitoyl-L-carnitine respiration after addition of GDP and ATR. Data are mean ± SE of mitochondrial preparations from 7 pigs. Significantly different vs. preoperative: **P*<0.05, ***P*<0.01 (Student's dependent t-test).

With PC as respiration substrate, $\Delta V_{\text{uncoupling}}$ after addition of GDP+ATR was slightly higher in the postoperative phase in both IFM (3.04 ± 0.52 vs. 7.31 ± 1.33 nmol O_2 /min/mg protein, $P < 0.05$) and SSM (1.52 ± 0.82 vs. 3.31 ± 0.62 nmol O_2 /min/mg protein, $P = 0.05$).

9. DISCUSSION

Various hyperinsulinaemic clamps, with or without glucose tracer infusion, have previously been applied in porcine experiments to examine different metabolic effects of insulin and only in a limited number of studies tracer enrichment was kept constant by either increased infusion rates during clamping or addition of tracer to the glucose infusate¹⁶⁴⁻¹⁶⁶. Different species exhibit different basal glucose kinetics, insulin clearance rates and sensitivity^{167,168} and of special importance, specific tracer glucose infusion rates are necessary to achieve adequate tracer enrichment levels. Owing to these issues and the relatively small amount and limited comparability of existing data, we sought to establish and test the step hot-GINF HEC technique and the possibility for combination with invasive investigative procedures before implementation in the following porcine studies on postoperative insulin resistance.

In paper 1, substudy A, surgical instrumentation as described in 7.6.1. did not significantly alter circulating counter-regulatory hormone concentrations probably due to suppressive effects of anaesthesia on their release and/or that the surgical trauma was too limited to induce such a release. Further, basal insulin and C-peptide concentrations, glucose kinetics and glucose tracer atom percent enrichment were unaltered and thus, instrumentation needed for combination with invasive techniques such as organ-specific flowmetry and arterio-venous balance measurements can be accomplished without changing the labelling of the glucose infusates. Despite a relatively high maximal insulin infusion rate at 2.0 mU/kg/min in substudy B, relatively low serum insulin concentrations were achieved, probably due to the high rate of insulin clearance in pigs¹⁶⁸. However, this insulin concentration was sufficient to almost completely suppress EGR and elicit

peak metabolic efficiency in peripheral tissues, as demonstrated by the sigmoid shaped dose-response curve for WGD. Further, exogenous infusion of insulin completely diminished endogenous insulin secretion, demonstrated by C-peptide below detectable levels. Somewhat surprisingly, the insulin infusion rates selected on the basis of the dose-response curves established in non-operated pigs in substudy B, resulted in substantially higher insulin serum concentrations during step-clamping in the operated pigs in substudy C. Because C-peptide levels were below detection level, the increase in insulin was probably caused by a decrease in insulin clearance. Metabolic insulin clearance during physiological hyperinsulinaemia is inseparably linked to insulin action, since binding to the insulin receptor constitutes the first step of degradation¹⁶⁹ and substantially decreased insulin clearance has been demonstrated in patients suffering from type 2 diabetes mellitus, obesity, and other conditions characterized by severe target cell insulin resistance^{170,171}. The liver represents the primary site of insulin clearance from endogenous release to the portal circulation by first-pass removal, whereas the kidneys remove the majority of insulin from the systemic circulation¹⁶⁹. Thus, as an alternative explanation, fluid shifts and relative hypovolemia with altered perfusion of these organs could have led to higher insulin levels postoperatively. However, the fact that continuous monitoring of heart rate and blood pressure as well as diuresis was not altered after surgery (data not shown) weighs against this explanation. An increase in circulating insulin similar to our study has been demonstrated during clamping in pigs with endotoxin-induced systemic inflammation without major effects on cardiovascular parameters¹⁷². Further, metabolic insulin clearance has been reported to be increased in patients with multiple injuries²⁶, unchanged after severe scald injury in rats¹⁷³, and

decreased during hypovolemic shock in primates ¹⁷⁴, indicating the possibility for both species and trauma variations.

In paper II, the postoperative decline in overall (i.e. GIR) and peripheral insulin sensitivity (i.e. insulin-stimulated WGD) was reduced to statistically non-significant levels by the single dose of CHO compared to fasting pigs. Importantly, the dose administered is similar to the amount given to patients and led to hyperglycaemia and a concomitant uniform rise in serum insulin corresponding to the levels seen after intake of low and rapid starch meals in pigs ¹⁷⁵ and in human studies on preoperative CHO loading ^{140,163}. Although a further effect of adding the evening CHO dose therefore seems unlikely it cannot, however be concluded that the evening dose is inefficient or unnecessary because our study did not include a group receiving a single evening dose or both doses of CHO.

Because the postoperative decline in insulin-stimulated WGD was reduced, whereas EGR was not significantly affected by CHO, and no differences in liver and muscle glycogen content were found after surgery or during clamping, the improvement in postoperative insulin sensitivity in the CHO loaded group must have been caused by a higher level of oxidative glucose disposal in peripheral tissues. Skeletal muscle disposes ~80% of glucose during euglycaemic insulin stimulation ¹⁷⁶ and thus, the metabolic efficiency for glucose oxidation of skeletal muscle mitochondria after surgery must have been improved by preoperative CHO supplementation, which is in line with previous human studies ^{12,13}. At least in part, this probably resulted from the reduction in peri- and postoperative plasma FFA levels and an attenuated metabolic competition from FFA on glucose oxidation through actions of the glucose-fatty acid cycle ¹⁷⁷. In support of this

contention, mitochondrial oxidative metabolism of glucose during hyperinsulinaemic-euglycaemic clamping is highly sensitive to the prevailing levels of FFA at the commencement of insulin stimulation ¹⁷⁸. The fact that insulin sensitivity is markedly increased in fat cells after glucose ingestion due to increased insulin binding affinity ¹⁷⁹ could further explain the apparent reinforced antilipolytic effect of insulin infusion during clamping in the CHO group resulting in lower FFA.

Cortisol, the cardinal driving force of acute insulin resistance and catabolism, was increased after surgery independently of preoperative CHO supplementation. However, the strong negative correlation between cortisol and insulin-mediated glucose disposal in the fasted animals was lessened by CHO administration, indicating that hyperinsulinaemia in response to CHO administration immediately prior to surgery antagonizes the acute restraining actions of cortisol on glucose oxidation in skeletal muscle through regulation of PDK4 expression (Section 5.7.). As previously described, muscle PDK4 expression in humans was reduced by a preoperative oral nutritional supplement drink ¹⁴² and the authors speculated that this was related to a reduction in plasma FFA concentrations, possibly through subsequent reduction in PPAR α -mediated PDK4 expression ¹⁸⁰, because no effect on FOXO1 activity was seen. Of note, PPAR activity was not measured in paper III. However, the suppressive effect of insulin on PDK4 mRNA expression in skeletal muscle is independent of its restraining effect on plasma FFA ⁹⁸, which weighs against the role of PPAR signalling.

Intriguingly, preoperative CHO treatment has recently been shown to significantly reduce circulating IL-6 levels until the 3rd postoperative day after major abdominal surgery ²⁰, and multivariate analysis led to the hypothesis that modulation of the

inflammatory response to surgery was of key importance to the attenuation of postoperative insulin resistance. In support of this contention physiological levels of insulin exhibits potent and fast inhibition of NFκB in mononuclear cells ¹⁸¹ and reduces the expression of pro-inflammatory cytokines in endotoxemic human macrophages ¹⁸². Further, subcutaneous administration of insulin in a dosage with minimal or no effect on glucose levels has been shown to attenuate the hepatic and systemic inflammatory response in a dose dependent manner ¹⁸³ and increase the anti-inflammatory response to endotoxemia ¹⁸⁴ and thermal trauma in rodents ¹⁸³. In pigs, plasma TNFα and IL-6 concentrations in response to lipopolysaccharide infusion-induced systemic inflammation are reduced if the lipopolysaccharide infusion is preceded by a low-dose hyperinsulinaemic-euglycaemic clamp ¹⁷². Thus, the cause of the blunted systemic inflammatory response in this human study ²⁰ was probably not directly related to the CHO load itself, but rather was a result of anti-inflammatory properties of the concurrent preoperative hyperinsulinaemia.

On this basis, in paper III we utilized biopsies harvested during the experiments in the surgery groups in paper II to test the hypothesis that CHO attenuates postoperative insulin resistance via reduction of the inflammatory response and inhibition of insulin signal transduction in skeletal muscle, thereby improving the restraining effects of insulin on FOXO1-mediated PDK4 expression. The primary finding in paper III was the ~5-fold lower expression of PDK4 protein in the CHO group than in the fasted group (Fig. 22C) during clamping, which could have accounted for the improvement in postoperative peripheral glucose disposal in this group. Although CHO administration did not significantly attenuate the increase in PDK4 gene expression after surgery compared with

the fasting group, it did lead to improved insulin-mediated regulation of PDK4 mRNA during clamping. The reduced ratio of inhibitory Ser³⁰⁷ phosphorylated to total IRS1 (Fig. 18A) was probably an important underlying factor to the improvement in the downstream IRS1 signalling in the CHO group during the clamp as reflected by the lower fall of the phosphorylated (inactive) to total ratio of FOXO1 and by stronger inhibitory drive of insulin through the IRS1-Akt1-FOXO1 pathway on PDK4 mRNA and protein expression compared with the fasted group (Figs. 18, 20 and 22). The apparent improvement in postoperative insulin signalling in the CHO group could be explained by the lower expression of muscle IKK β , JNK2 and SOCS3, all known for their ability to negatively interact with IRS1¹⁸⁵. The notion that local expression of inflammatory mediators may contribute to activation of the cellular mechanisms that induce acute insulin resistance in skeletal muscle after surgery and that preoperative CHO supplementation modulates this inflammatory response is further supported by the observation that NF κ B activity was reduced in the CHO loaded group when compared with the preoperative levels, and correlated significantly inversely with peripheral insulin sensitivity during clamping and directly with PDK4 protein expression. Further, the fact that no difference in peripheral insulin sensitivity during clamping was seen between the CHO treated and fasted control groups in paper II weighs against the previously suggested role of facilitated glucose disposal (Staub-Traugott effect)¹⁴⁵ and supports the notion that the improvement in glucose disposal after preoperative CHO administration is prophylactic by nature and limited to surgically stressed individuals. The proposed underlying mechanism of the effect of CHO administration on postoperative peripheral insulin sensitivity is shown in figure 28.

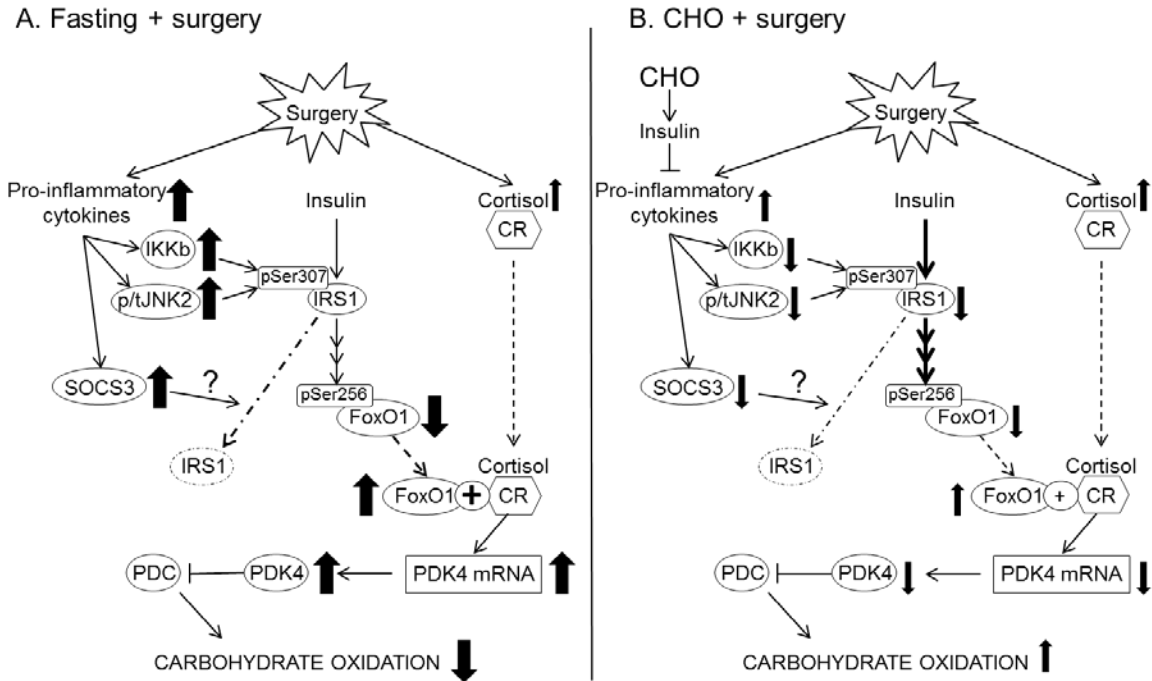


Figure 28 Proposed mechanism underlying the prophylactic effect of preoperative carbohydrate loading (CHO) on inflammatory interference with insulin regulation of pyruvate dehydrogenase kinase 4 (PDK4) expression in muscle after surgery.

A: PDK4 expression in muscle is increased after surgery both by a cortisol-induced increase in transcription and reduced insulin-mediated inhibitory phosphorylation (pSer²⁵⁶) of forkhead transcription factor 1 (FOXO1), which increases the PDK4 gene responsiveness to glucocorticoids. Insulin action is inhibited by the serine/threonine kinases inhibitor κ B kinase (IKK β) and c-Jun N-terminal kinase (JNK) which are activated by cytokines after surgery, resulting in inhibitory phosphorylation (pSer³⁰⁷) of insulin receptor substrate 1 (IRS1) and impaired downstream propagation of insulin signalling. IKK β and JNK also induce expression of suppressor of cytokine signalling 3 (SOCS3) which targets IRS1 for degradation.

B: CHO improves insulin-mediated inhibitory phosphorylation of FoxO1 and regulation of PDK4 expression in muscle after surgery by reducing postoperative IKK β , JNK and inhibitory phosphorylation of IRS1. We propose that these effects occur due to anti-inflammatory properties of hyperinsulinaemia following CHO administration in the immediate preoperative phase. \uparrow/\uparrow and \downarrow/\downarrow arrows indicate upregulation/increased activity and downregulation/decreased activity, while arrow thickness indicates the magnitude of the effect.

In paper IV, we found that on the 2nd day after major open abdominal surgery (albeit somewhat different than in paper II/III), postoperative insulin resistance was still located in peripheral tissues (i.e. skeletal muscle) and developed along with relative hyperglycaemia, a trend toward higher circulating FFA concentrations and a severe impairment in the capacity of pyruvate oxidation (i.e. V_{\max}) in skeletal muscle mitochondria. When glutamate was used as an alternative complex I substrate, which is thought to yield fairly identical respiration conditions as pyruvate ¹⁸⁶, but is transported across the mitochondrial membrane by the electroneutral glutamate/OH exchanger and is oxidized by glutamate dehydrogenase to feed the tricarboxylic acid cycle, the postoperative maximal oxidative capacity was unchanged. The same observation was made for the V_{\max} for palmitoyl-L-carnitine (PC), which feeds the electron transport chain through β -oxidation. This strongly indicates that the reduction in pyruvate oxidation is due to a reduced capacity of PDC rather than overall impairment in the mitochondrial tricarboxylic acid cycle and/or electron transport chain and further supports the involvement of PDK4, as suggested by the findings in paper III. Importantly, PDK4 up-regulation is expected to affect pyruvate oxidation in isolated mitochondria, as the protein is located within the mitochondrial matrix. However, our results are weakened by the lack of PDK4 expression and/or PDC enzyme activity measurements in paper IV. Increased FFA availability could also lead to a reduction of pyruvate flux into the mitochondria by actions of the glucose-fatty acid cycle. In this regard the current increase in plasma FFA after surgery, although not statistically significant, could have contributed to our findings.

To our knowledge, we are the first to assess liver and skeletal muscle

mitochondrial ROS release in a surgery-induced acute insulin resistant setting. This was done by direct measurement of ROS species during respiration with glutamate, a substrate which, in an experimental context, is known to induce fairly low, but physiological levels of ROS¹⁸⁷. Our results indicate that the potential for ROS release from the electron transport chain complexes in skeletal muscle and liver mitochondria is increased after surgical trauma, which could play a role in the development of postoperative insulin resistance (Section 5.9.). In contrary to what we expected, such an increase was not evident when the rate of O₂^{•-} release was measured. However, this can be attributed to sufficient scavenging of O₂^{•-} by superoxide dismutase and similar scavengers, with a subsequent increase in H₂O₂ levels.

To estimate the level of uncoupling, leak oxygen consumption was measured by blocking the ATP synthase and inhibition of the most essential mitochondrial uncouplers uncoupling protein 2/3 and adenine nucleotide translocase¹⁸⁸. While no changes were observed with glutamate or pyruvate, there was significantly increased leak oxygen consumption in skeletal muscle mitochondria respiring on PC, indicating FFA-dependent activation of uncoupling. This finding is supported by evidence that the presence of FFA derivatives is necessary for complete induction of uncoupling through a synergistic co-activation with ROS species^{189,190}. Surprisingly, the postoperative increase in V_{oligo} was only slightly lowered when exposing mitochondria to ATR and GDP. However, the severe increase in the levels of O₂^{•-} release after addition of GDP and ATR indicates that uncoupling was activated leading to higher rates of ROS elimination after surgery. Why this was not reflected by a reversal of mitochondrial leak respiration to preoperative levels when ATR and GDP was added is not clear to us, as we would have expected any

increase in V_{oligo} to be almost completely eliminated by uncoupling inhibitors. Possible reasons for this could either be a lack of specificity of ATR and GDP¹⁹¹⁻¹⁹³ and thereby only partial inhibition of uncoupling, or increased leak respiration due to proton leakage through mitochondrial membrane proteins other than UCPs and ANT.

Collectively, the results in paper IV show that alterations in physiological properties of mitochondria in insulin sensitive tissues, similar to those previously demonstrated in chronic insulin resistant states develop along with surgery-induced acute insulin resistance in skeletal muscle, although the causal relationship between these changes and the decline in insulin sensitivity remains to be determined.

10. GENERAL CONCLUDING REMARKS AND FUTURE PERSPECTIVES

A few concluding remarks on the research model are warranted when interpreting the results of our studies. The healthy, young, growing pigs studied in this experimental work may not represent a perfect imitation of the mostly adult and sometimes multimorbid human patient, although the use of swine as a model for biomedical research is considered as close to human as possible due to the nutritional, metabolic and physiological resemblance. However, it should be noted that whereas de novo fatty acid synthesis is mainly located in the human liver, glucose and other carbon precursors are incorporated readily into fatty acids in swine adipose tissue, which is the major site for de novo synthesis in pigs⁶⁴.

FFA concentrations during clamping were considerably higher in paper I than paper II. A causal explanation cannot be found in the plasma levels of catecholamines, which were equal in the two studies. However, differences in blood sample handling, amount of added lipase inhibitor to the sample vials, in the performance of FFA analyses, or colorimetric assay kit and microplate reader variability cannot be excluded.

Higher cortisol levels were seen in the two control groups in paper II than in the surgically untouched pigs in substudy B, paper I. This could be explained by surgical stress related to the mini-laparotomy and serial muscle and liver biopsy sampling in paper II.

Since the animal experiments were performed during continuous anaesthesia one should take note of the fact that several anaesthetic agents possess the ability of modifying the hormonal and metabolic responses to surgery, including those currently used. A reduction in cortisol levels and blood glucose during and immediate after surgery

can be achieved by addition of fentanyl to the anaesthetic regime¹⁹⁴. The effect is also seen in pigs¹⁹⁵, is dose dependent¹⁹⁶ and, importantly, requires administration of fentanyl prior to the onset of surgery¹⁹⁷. Induction of anaesthesia with midazolam followed by continuous infusion during upper abdominal surgery has been shown to delay the increase in circulating cortisol and glucose, and to reduce intraoperative levels of insulin¹⁹⁸. In addition midazolam has been shown to attenuate the perioperative increase in plasma epinephrine levels¹⁹⁹. Inhalational agents, including isoflurane, impair insulin secretion and glucose utilization following intravenous glucose administration^{200,201} and has been shown to increase blood glucose shortly after induction of anaesthesia²⁰². In contrast to the effects of fentanyl and midazolam, the stress hormone response to surgery is not significantly affected by anaesthesia with isoflurane^{203,204}. Furthermore, several anaesthetic agents have suppressive effects on mitochondrial electron flow and oxidative phosphorylation capacity¹¹⁷.

The apparent anti-inflammatory response in muscle tissue to preoperative CHO supplementation in our study could, at least in part, have been counteracted by pro-inflammatory effects of glucose ingestion in mononuclear cells, as previously reported in healthy humans²⁰⁵. Interestingly, addition of an insulinotropic amino acid and protein mixture to a CHO beverage increases the plasma insulin response by up to 100% without increasing hyperglycaemia²⁰⁶ and it has been shown that patients receiving preoperative CHO in combination with milk whey protein exhibit reduced acute phase response after moderate sized surgery²⁰⁷. Furthermore, the prophylactic effect of preoperative CHO loading on postoperative insulin resistance in colorectal surgery patients was improved by early postoperative intake of a polymeric nutritional supplement containing fat,

protein and carbohydrates²⁰⁸. In this regard, and with the results of the current thesis in mind, nutrient activation of the so-called anti-inflammatory neuro-immune pathway, a “hard-wired” neural system constituted by the afferent and efferent arc of the vagus nerve²⁰⁹, represents a possible link between perioperative feeding and modulation of the systemic inflammatory response to surgical trauma. Efferent vagus nerve activity mediates acetylcholine release in organs containing tissue macrophages belonging to the reticuloendothelial system, such as the liver, spleen, heart and gastrointestinal tract²⁰⁹. Acetylcholine inhibits transcription and release of pro-inflammatory cytokines from these macrophages through activation of the nicotinic acetylcholine receptor subunit $\alpha 7$, which is thought to decrease the set point of the innate immune response relative to a given injury stimulus²¹⁰. Most intriguingly, recent murine and human experiments have shown that gastrointestinal hormones, including cholecystinin and glucagon-like peptide 1 secreted from enteroendocrine cells upon absorption of macronutrients trigger the anti-inflammatory pathway through afferent vagus signalling, leading to reduced systemic inflammatory response to endotoxaemia and surgical bowel manipulation²¹¹⁻²¹³.

Thus, this thesis introduces an experimental model which can be extended with further interventions and modified with additional metabolic tracers, inter-organ blood sampling and flow measurements to investigate the metabolic and cellular effects of more complex formulated oral perioperative supplements eventually leading to new clinical alternatives on the quest to optimize perioperative nutritional care.

11. MAIN CONCLUSIONS

1. Step hot-GINF HEC is a feasible technique and seems well suited for porcine research models on acute insulin resistance, but attention must be paid to alterations in circulating insulin. Combination with invasive research techniques requiring extensive surgical instrumentation can be accomplished without the premises for utilization of the technique being altered.
2. Single-dose preoperative oral carbohydrate supplementation two hours prior to surgery is sufficient to reduce development of peripheral insulin resistance and maintain oxidative glucose disposal immediately after major surgery in pigs.
3. The postoperative improvement in glucose disposal is prophylactic by nature and seems to be limited to surgically stressed animals by antilipolytic effects and antagonist properties of preoperative hyperinsulinaemia on the suppressant actions of cortisol on carbohydrate oxidation.
4. Oral carbohydrate administration in the immediate preoperative phase reduces the skeletal muscle inflammatory response to surgical trauma, the interference of inflammatory mediators with insulin downstream signal transduction, and improves the ability of insulin to suppress FOXO1-mediated PDK4 expression in skeletal muscle after surgery. This could, at least in part, explain the prophylactic effect of carbohydrate treatment on development of postoperative peripheral insulin resistance.

5. In line with the molecular data, isolated impairment in skeletal muscle mitochondrial pyruvate oxidation capacity develops along with peripheral insulin resistance in the postoperative phase after major abdominal surgery.

6. Mitochondrial ROS release is increased in both skeletal muscle and liver after surgery, whereas mitochondria from skeletal muscle exhibit increased fatty acid-induced uncoupling. The causal relationship between these changes and the decline in insulin sensitivity remains to be determined.

12. ERRATA

Paper I:

- The expression “insulin unresponsiveness” should replace “insulin insensitivity” and vice versa throughout the paper.
- Table 4: FFA unit should be $\mu\text{mol/L}$.

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14. PAPERS I-IV

Paper 1

Paper 2

Paper 3

Paper 4

