TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Ernährungsmedizin der Technischen Universität München

Central insulin signaling as a regulator of systemic inflammation

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Vollständiger Abdruck der von der Fakultät für Medizin
der Technischen Universität München
zur Erlangung des akademischen Grades eines

Doktors der Medizin

genehmigten Dissertation.

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Prüfer der Dissertation:	1. Univ.-Prof. Dr. J.J. Hauner
	2. Priv.-Doz. Dr. M. Bajbouj

Die Dissertation wurde am 08.07.2013 bei der Technischen Universität München
eingereicht und durch die Fakultät für Medizin am 09.07.2014 angenommen.

Gewidmet meinen Eltern, Alan und Eva.

“Success is the ability to go from one failure to another with no loss of enthusiasm.”

–Sir Winston Churchill (British politician, 1874-1965)

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1 Abbreviations and Acronyms

#

α7-nAChR. α7 subunit of the nicotinic acetylcholine receptor
μL. Microliter
μU. Micro units

A

ACh. Acetylcholine
AChR. Acetylcholine receptor
ACSF. Artificial cerebrospinal fluid
ACTH. Adrenocorticotropin hormone
AP. Anterior-posterior
AP-1. Activator protein 1

B

BBB. Blood brain barrier
BCA. Bicinchoninic acid
BSA. Bovine serum albumin

C

CARS. Compensatory anti-inflammatory response syndrome
CLP. Cecal ligation and puncture
CNS. Central nervous system
COX-2. Cyclooxygenase 2
CRH. Corticotropin-releasing hormone
CRP. C-reactive protein
CXCL. Chemokine (C-X-C motif)

D

DIC. Disseminated intravascular coagulation
dL. Deciliter
DNA. Deoxyribonucleic acid
DTT. Dithiothreitol
DV. Dorsal-ventral

E

EDTA. Ethylenediaminetetraacetic acid
ELISA. Enzyme-linked immunosorbent assay

G

GAPDH. Glyceraldehyde-3-phosphate dehydrogenase

H

h. Hour
H_2O_2. Hydrogen peroxide

HFD. High-fat diet
HPA. Hypothalamic-pituitary-adrenal
HRP. Horse radish peroxidase

I

ICAM. Intercellular adhesion molecule
ICU. Intensive care unit
ICV. Intracerebroventricular
IFN. Interferon
IgG. Immunoglobulin G
IIT. Intensive insulin therapy
IKK-γ. I-kappa-B kinase subunit gamma
IL. Interleukin
iNOS. Inducible nitric oxide synthases
IP. Intraperitoneal
IPGTT. Intraperitoneal glucose tolerance test
IR. Infrared
IU. International unit
IV. Intravenous

J

JAK. Janus kinase

K

kDA. Kilodalton
kg. Kilogram

L

L. Liter
LD. Lethal dose
LDS. Lithium dodecyl sulfate
LPS. Lipopolysaccharide

M

mA. Milliampere
MBL. Mannose-binding lectin
MCP-1. monocyte chemotactic protein 1
mg. Milligram
MHC. Major histocompatibility complex
MIF. Macrophage migration inhibitory factor
ML. Medial-lateral
mM. Millimolar
mmol. Millimole
MODS. Multiple organ dysfunction syndrome
MOPS. 3-(N-morpholino) propanesulfonic acid
mU. Milli units

N

NaOH. Sodium hydroxide
NF-κB. Nuclear factor kappa-light-chain-enhancer of activated B cells
NICE-SUGAR. Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation
NIRKO. Neuronal insulin receptor knock out

NPY. Neuropeptide Y
NPY/AgRP. Neuropeptide Y/agouti-related protein

P

PAGE. Polyacrylamide gel electrophoresis
PAMP. Pathogen-associated molecular pattern
PBD. Days post-burn
PE. Polyethylene
PNS. Parasympathetic nervous system
POMC/CART. Proopiomelanocortin/cocaine- and amphetamine-regulated transcript
PVDF. Polyvinylidene fluoride

R

RCD. Regular chow diet
RNA. Ribonucleic acid
ROS. Reactive oxygen species
rpm. Revolutions per minute

S

SDS. Sodium dodecyl sulfate
SIRS. Systemic inflammatory response syndrome
SNS. Sympathetic nervous system
Stat. Signal transducer and activator of transcription

T

T2DM. Type 2 diabetes mellitus
TBS. Tris based buffered saline
TBS/T. Tris based buffered saline containing tween 20
TF. Tissue factor
TGF. Transforming growth factor
TMB. 3,3´,5,5´-tetramethylbenzidine
TNF. Tumor necrosis factor
TNF-sR. Soluble tumor necrosis factor receptor

2 Introduction

2.1 Sepsis

Sepsis is a major cause of morbidity and mortality, fatal in 20% to 50% of sufferers and the second most common cause of death in patients hospitalized in non-coronary intensive care units (ICU). Accounting for about 210,000 deaths per year, the Center for Disease Control and Prevention estimates that sepsis is the tenth highest cause of death in the United States (Martin *et al.*, 2003). What is more, according to recent reports, the incidence of sepsis is rising at a rate of 1.5% per annum despite massive investment in resources to modernize and improve care in critically ill patients (Angus *et al.*, 2001). Due to increasing life expectancy and more frequent invasive interventions, a further rise in the incidence of sepsis is expected for the future. Sepsis is defined as a systemic host response to an infection in the presence of two or more of the following conditions: hypo- or hyperthermia, tachycardia, tachypnea, leukocytopenia or leukocytosis. Clinical signs of sepsis, however, are also frequently displayed by patients with no measurable levels of bacteria in the blood, for instance in those with acute pancreatitis or trauma. To address this discrepancy the term systemic inflammatory response syndrome (SIRS), which requires no detectable presence of bacterial infection, was established at a Consensus Conference held by the Society of Critical Care Medicine and the American College of Chest Physicians in 1992 (Bone *et al.*, 1992). In addition, the terms “severe sepsis”, “septic shock” and multiple organ dysfunction syndrome (MODS), defined as altered organ function in an acutely ill patient requiring medical intervention to achieve homeostasis, have been introduced to differentiate between the progressing stages of disease (Table1).

Sepsis is currently perceived as a complex dysregulation of inflammation arising when the host is unable to successfully control infection. Microbacterial components responsible for initiating the septic process and recognized by the innate immune system are referred to as pathogen-associated molecular patterns (PAMPs) (Janeway *et al.*, 1998). The initial host and PAMP interaction is followed by widespread activation of the innate immune response, the purpose of which is to coordinate the defense mechanisms involving both humoral and cellular components. Throughout the body numerous immune cells, including monocytes, macrophages and lymphocytes as well as endothelial and epithelial cells produce the powerful pro-inflammatory cytokines and other mediators that trigger an appropriate immune response. At the same time production of acute phase proteins, such as C-reactive protein (CRP) increases, and humoral defense mechanisms such as the complement system are

activated. Activation of the complement system results in the production of the pro-inflammatory complement split product C5a, which together with other mediators further enhances cytokine and chemokine production. Phagocytic cells such as neutrophils and macrophages respond to many of these mediators by producing reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) a substance crucial for killing bacteria but unfortunately also capable of inducing tissue damage. Damage to endothelial and epithelial cells, usually an important barrier in the containment of infection and inflammation, leads to increased vascular permeability allowing further dissemination of infection (Riedemann *et al.*, 2003).

Clotting disorders are commonly associated with sepsis since pro-inflammatory cytokines are also powerful inducers of coagulation. Coagulation pathways are not only activated by endogenous signals but are also initiated by bacterial components, inducing expression of tissue factor (TF) on mononuclear and endothelial cells. Tissue factor activates a series of proteolytic cascades that result in the conversion of prothrombin to thrombin, which in turn generates fibrin from fibrinogen. In addition, normal regulatory fibrinolytic mechanisms (fibrin breakdown by plasmin) are impaired and three naturally occurring anticoagulant proteins – antithrombin, protein C and tissue factor pathway inhibitor are down-regulated. As a result there is increased production and reduced elimination of fibrin, leading to development of fibrin clots in small blood vessels, inadequate tissue perfusion and ultimately organ failure (Cohen, 2002). Moreover, 30-50% of patients with sepsis suffer from the more severe clinical clotting disorder, disseminated intravascular coagulation (DIC) (Levi *et al.*, 1999). In the course of DIC, coagulation inhibitors and clotting factors are consumed and thrombocytopenia, attributed to the entrapment and consumption of platelets with the development of multiple clots, occurs. Decreased inhibitor levels cause further clotting and a negative feedback loop develops with increased coagulation leading to even more clotting, ultimately terminating in the uncontrolled bleeding observed in patients with DIC.

The ultimate cause of death in sepsis patients is commonly multiple organ dysfunction syndrome MODS. The pathogenesis of organ dysfunction is multifactorial and only partly understood to date; tissue hypoperfusion and hypoxia being the dominant factors. A further contributing factor is impaired peripheral vascular tone caused by vasoactive substances such as histamine and prostanoids. In the presence of these substances peripheral resistance falls, causing hypotension, which together with the impaired clotting mentioned above and micro vascular occlusion leads to hypoperfusion of tissue, inadequate oxygenation and ultimately organ failure.

Table 1: Clinical definition of sepsis. SIRS = systemic inflammatory response syndrome $PaCO_2$ = arterial partial pressure of carbon dioxide; MODS = multiple organ dysfunction syndrome.

Stage of disease	Clinical signs
SIRS	Temperature >38.3°C or <36°C Heart rate >90 beats/min Respiratory rate >20 breaths/min or $PaCO_2$ <32 mmHg White blood cell count >12 °- 109/l or <4 °- 109/l, or >10% immature band forms
Sepsis	Systemic response to infection, manifested by two or more of the conditions listed under SIRS. (SIRS + evidence of infection)
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion, or hypotension including lactic acidosis, oliguria, or acute alteration in mental state
Septic shock	Sepsis-induced hypotension (e.g. systolic blood pressure <90 mmHg or a reduction of >40 mmHg from base line) despite adequate fluid replacement, associated with the presence of perfusion abnormalities that may include lactic acidosis, oliguria, or an acute alteration in mental state. Vasopressor- or inotropic-treated patients may not be hypotensive at the time of measurement
MODS	The presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention

2.2 Diabetes of injury

Before critical illness ultimately leads to death, patients typically progress through several clinical states: they often develop acute disturbance of the body's closely controlled homeostasis due to an intense counterregulatory hormone and cytokine response. Stress induced alteration of glucose metabolism is one example; commonly referred to as 'stress diabetes' or 'critical illness diabetes' (McCowen *et al.*, 2001) (Marik *et al.*, 2004).

In healthy, non-diabetic individuals increased blood glucose concentrations stimulate the pancreatic β-cells to release insulin, which in turn mediates peripheral glucose disposal and suppresses hepatic gluconeogenesis in order to maintain blood glucose homeostasis. Skeletal muscle is the principal site of peripheral insulin-mediated glucose uptake. In the muscle, glucose is subsequently directed to either glycogen formation (an important storage pathway) or glycolysis, where it is metabolized in the citric acid cycle, resulting in energy production via oxidative phosphorylation. Glucose is stored as glycogen in the liver or converted to fatty acids for storage as triglycerides in adipose tissue. A decline in blood glucose concentration as observed in the postabsorptive state leads to release of glucagon from pancreatic α-cells as well as increased levels of counterregulatory hormones such as catecholamines, cortisol and growth hormone. Together, these hormones increase blood glucose levels (thus maintaining normoglycemia) by stimulating hepatic glycogenolysis and gluconeogenesis while inhibiting peripheral insulin-mediated glucose disposal.

Under pathological conditions such as critical illness there are significant changes in energy substrate metabolism (Mizock, 2001). Release of the classic counterregulatory hormones

glucagon, epinephrine and cortisol opposes the normal action of insulin, leading to increased adipose tissue lipolysis, levels of circulating free fatty acids and skeletal muscle proteolysis. The resulting increase in gluconeogenic substrates such as glycerol, alanine and lactate contributes to enhanced hepatic glucose production, despite hyperinsulinemia. Together with the hormone-mediated rise in hepatic glycogenolysis, this ultimately leads to hyperglycemia. Finally, peripheral insulin resistance, characterized by impaired insulin-mediated glucose uptake despite normal or increased insulin concentrations, further promotes elevated blood glucose (Carlson, 2003). In summary, hyperglycemia in acute critical illness results from a combination of increased hepatic glucose production and decreased peripheral glucose utilization.

Figure 1 Factors promoting gluconeogenesis in critical illness. Counterregulatory hormones oppose the restraining effect of insulin on lipolysis and proteolysis, thus increasing substrate for gluconeogenesis. Despite hyperinsulinemia, hepatic insulin resistance leads to continued glucose production in the face of hyperglycemia. This process occurs under positive control of counterregulatory hormones, in particular glucagon and epinephrine. (McCowen *et al.*, 2001)

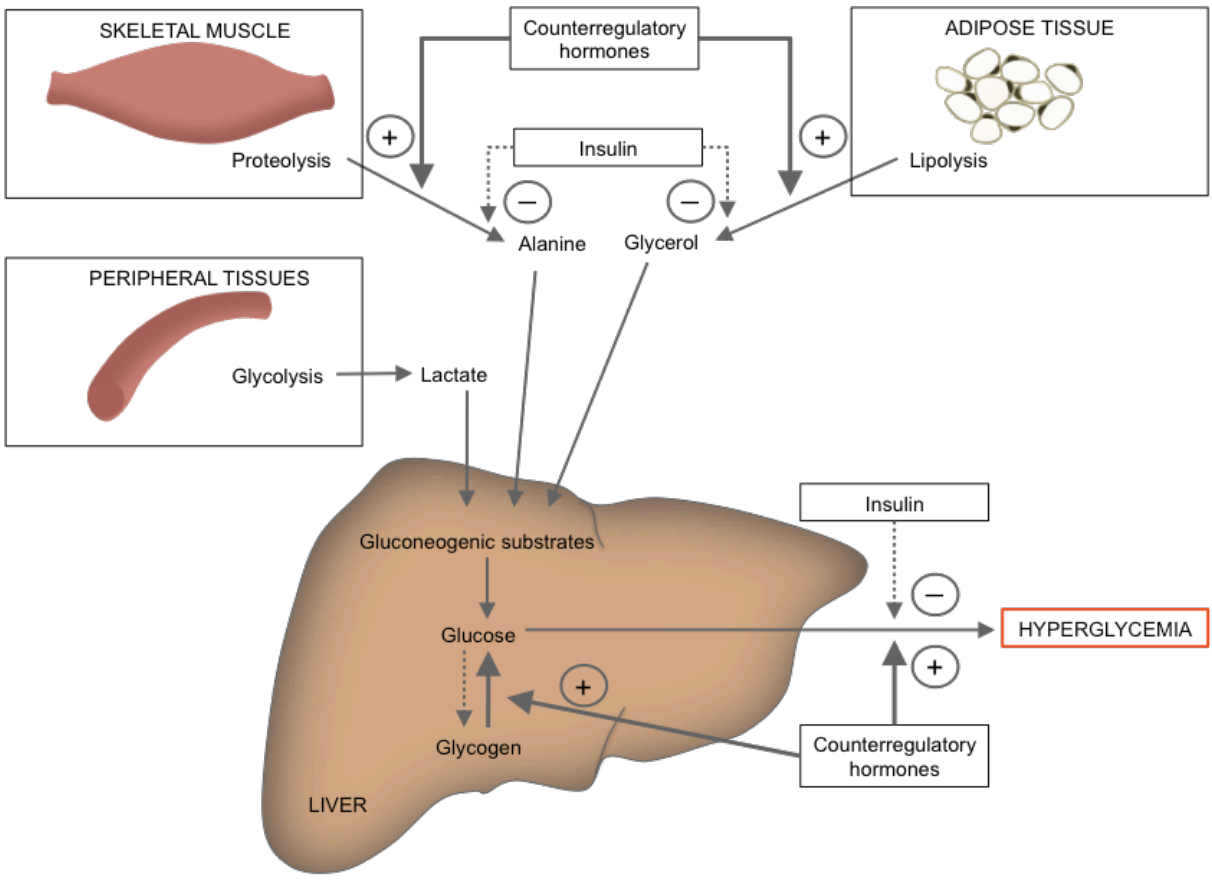


Table 2: Principal actions of counterregulatory hormones and cytokines in mediating stress hyperglycemia.

Hormone	Mechanism
Glucocorticoids	Skeletal muscle insulin resistance Increased gluconeogenesis Increased lipolysis
Glucagon	Increased hepatic glycogenolysis Increased gluconeogenesis
Epinephrine	Increased gluconeogenesis Increased skeletal and hepatic glycogenolysis Increased lipolysis Skeletal muscle insulin resistance Direct suppression of insulin secretion
Norepinephrine	Increased lipolysis Increased gluconeogenesis
Tumor Necrosis Factor alpha	Skeletal muscle insulin resistance Hepatic insulin resistance

2.3 Critical illness and type 2 diabetes mellitus

In a recent publication it was estimated that the prevalence (defined as the total number of cases of a disease in a population at a given time) of diabetes for all age-groups worldwide was 2.8% in 2000 and will rise to 4.4% in 2030. The total number of diabetics is expected to rise from 171 million in 2000 to 366 million in 2030. (Wild *et al.*, 2004). Type 2 diabetes mellitus (T2DM) is a metabolic disease with a complex pathophysiology characterized by insulin resistance, decreased pancreatic insulin secretion, altered lipid metabolism and hyperglycemia. Combined, these pathologies cause well-documented, detrimental chronic effects including cardiovascular, renal, neurologic and organ damage as well as immunologic organ system dysregulation. As a consequence, critically ill patients with T2DM have a poorer prognosis than non-diabetic patients.

T2DM was established to be an independent risk factor related to significant morbidity and mortality after cardiac surgery. In patients undergoing cardiac surgery, with and without cardiopulmonary bypass, T2DM was identified as an independent predictor for several postoperative outcome variables all associated with higher postoperative morbidity and prolonged hospital stay (Bucerius *et al.*, 2003). Another study investigating the incidence of pre-existing diseases or risk factors and their effects in critically ill trauma patients concluded that amongst other factors, T2DM is predictive of outcome in critically ill trauma patients (Bochicchio *et al.*, 2006).

There is evidence that pre-existing T2DM is not only a risk factor in critically ill surgical patients but is also implicated with regard to susceptibility to infection. In a study investigating

the effect of pre-existing diabetes on the host immune response, acute organ function and mortality in patients hospitalized with community-acquired pneumonia, patients diagnosed with T2DM had a higher risk of acute kidney injury during hospitalization and were more likely to die of cardiovascular and kidney disease (Yende *et al.*). Not only for pneumonia but also for other infection related diseases, T2DM and hyperglycemia were shown to be strong and independent risk factors for hospitalization and outcome. For example among patients hospitalized for urinary tract infection, diabetics had an increased risk of death 28 days after admission compared with non-diabetic patients (Benfield *et al.*, 2007). It has also been shown that T2DM is an independent risk factor for ICU nosocomial bloodstream infections, which have a severe impact on the outcome of critically ill patients and attributable mortality of up to 35% (Pittet *et al.*, 1994). Diabetic patients were shown to have a 5.9-fold probability of developing blood stream infections severe enough to require management in an intensive care unit and generally associated with a high mortality rate (Laupland *et al.*, 2004; Damas *et al.*, 2008) although this increased mortality rate could not be confirmed in subsequent studies (Michalia *et al.*, 2009)

Considering the pathophysiology and the detrimental effects of T2DM described above, it is not difficult to imagine a plausible association of T2DM with increased ICU mortality. Critically ill diabetics are less resilient to metabolic and other stress factors, simply because of pre-existing organ dysfunction. In addition, chronic macro- and micro-vascular disorders associated with T2DM are likely to increase the heterogeneity of blood flow and tissue metabolism, both detrimental in the setting of acute sepsis. All in all there is overwhelming evidence indicating increased risks for patients with T2DM who are critically ill. Taken together with the finding that patients with pathological conditions often develop changes in glucose metabolism comparable to those associated with T2DM, one could speculate that a vicious circle is established with critical illness inducing diabetes and diabetes worsening critical illness.

2.4 Intensive insulin therapy

The hyperglycemic condition observed in the context of critical illness has, until recently, been thought of as an adaptive and beneficial stress response. Blood glucose levels up to 200 mg/dL (11.1 mmol/L) were seen as a reasonable target for glycemic control in critically ill patients (Boord *et al.*, 2001). The elevated glucose levels observed after injury were believed to be a mechanism by which the energy requirements of organs that rely on glucose as a source of energy, including the brain, the immune system, blood cells and wound tissue would be satisfied (Mizock, 1995). Taking this into account, a blood glucose concentration of 160 to 200 mg/dL (8.9 to 11.1 mmol/L) was recommended by most clinicians, maximizing glucose uptake but avoiding hyperosmolarity since hyperglycemia-induced osmotic diuresis

and fluid shifts were also feared. The persistent hyperglycemia experienced by critically ill patients is now, however, thought to be secondary to the development of insulin resistance and, therefore, may not be beneficial or adaptive. A review article investigating post-myocardial infarct outcomes in patients with and without diabetes found a consistent relationship between hyperglycemia, increased mortality and extensive myocardial damage, regardless of T2DM status (Capes *et al.*, 2000). A landmark study by van den Berghe et al. (van den Berghe *et al.*, 2001) conducted in 1548 critically ill adult patients in a surgical ICU, demonstrated reduced mortality when stress induced hyperglycemia was controlled with intensive insulin therapy (IIT). The study group received IIT with insulin infusion if the blood glucose concentration was >110 mg/dL (>6.1 mmol/L) and levels were adjusted to maintain normoglycemia between 80-110 mg/dL (4.4-6.1 mmol/L). The control patients were managed conventionally with continuous insulin infusion, started only if the blood glucose concentration was >215 mg/dL (>11.9 mmol/L) and adjusted to maintain a blood glucose concentration between 180 and 200 mg/dL (10.0-11.1 mmol/L). The results showed that use of IIT significantly reduced morbidity and mortality (4.6% vs. 8.0%). The largest reduction in mortality was seen for deaths due to multiple-organ failure with sepsis in patients hospitalized for >5 days. Further studies have revealed that the beneficial effects of IIT are also measurable in medical or mixed medical and surgical ICUs and IIT reduces the risks associated with numerous complications common in the ICU: e.g. fewer infections, less acute renal failure and liver dysfunction and shortening of the duration of hospitalization (Krinsley, 2006; Van den Berghe *et al.*, 2006). To further investigate these early findings derived either from single centers or small-scale studies conducted at several centers, a large, multicenter, randomized trial, titled Normoglycemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation (NICE-SUGAR) was designed (Finfer *et al.*, 2009). The results of this trial demonstrate only small changes in mortality with IIT. The NICE-SUGAR patients, however, required less insulin to maintain approximately the same blood glucose values observed in the landmark study, suggesting that the initial level of insulin resistance was lower in NICE-SUGAR. Meta-analysis of 26 studies (including NICE-SUGAR) suggests that IIT might be beneficial to patients admitted to surgical ICUs (Griesdale *et al.*, 2009). Irrespective of whether or not a beneficial effect was observed, the fact that patients receiving IIT had an increased risk of hypoglycemic events compared to controls was shown in most studies (Table 3).

Table 3: Risk of hypoglycemic events in clinical trials comparing IIT and conventional glycemic control.

Study	Patients, No.	Patient Type	No. hypoglycemic events / total no. patients		Possible Harm
			IIT	Control	
(van den Berghe et al., 2001)	1548	Surgical	39/765 (5%)	6/783 (0,8%)	Increased risk of **hypoglycemia**; high insulin dose is statistical positive risk factor for mortality
(van den Berghe et al., 2006)	1200	Medical	111/595 (18,6%)	19/605 (3,1%)	Increased risk of **hypoglycemia**
(Brunkhorst *et al.*, 2008)	537	Sepsis/ septic shock	42/247 (17%)	12/290 (4,1%)	Trial was stopped early for safety reasons. Increased risk of serious adverse events related to **hypoglycemia**
GLUCONTROL (Preiser *et al.*, 2009)	1078	Medical/ surgical	44/536 (8,2%)	13/542 (2,4%)	Study was prematurely stopped and therefore underpowered. IIT group with increased incidence of **hypoglycemia**
NICE-SUGAR (Finfer et al., 2009)	6030	Medical/ surgical	206/3016 (6,8%)	15/3014 (0,5%)	Higher risk of severe **hypoglycemia** in the IIT group.

Although it has been demonstrated in clinical intervention trials that IIT reduces morbidity and mortality in critically ill patients and is now routinely used in many ICUs, it has been difficult to differentiate the actual origin of the beneficial effect of this therapy to date. There is evidence that the beneficial effects of IIT derives from metabolic regulatory mechanisms, such as those preventing the deleterious effects of hyperglycemia and improving anabolic regulation of proteins and lipids. Recent advances, however, have underlined the importance of inflammatory and immune responses in the prognosis of critically ill patients. More and more researchers now believe that the beneficial effects of IIT are due to glycemia-independent effects of insulin in regulating inflammatory and immune responses, which act on the receptors of immune and immune-related cells to improve their functions and attenuate inflammation.

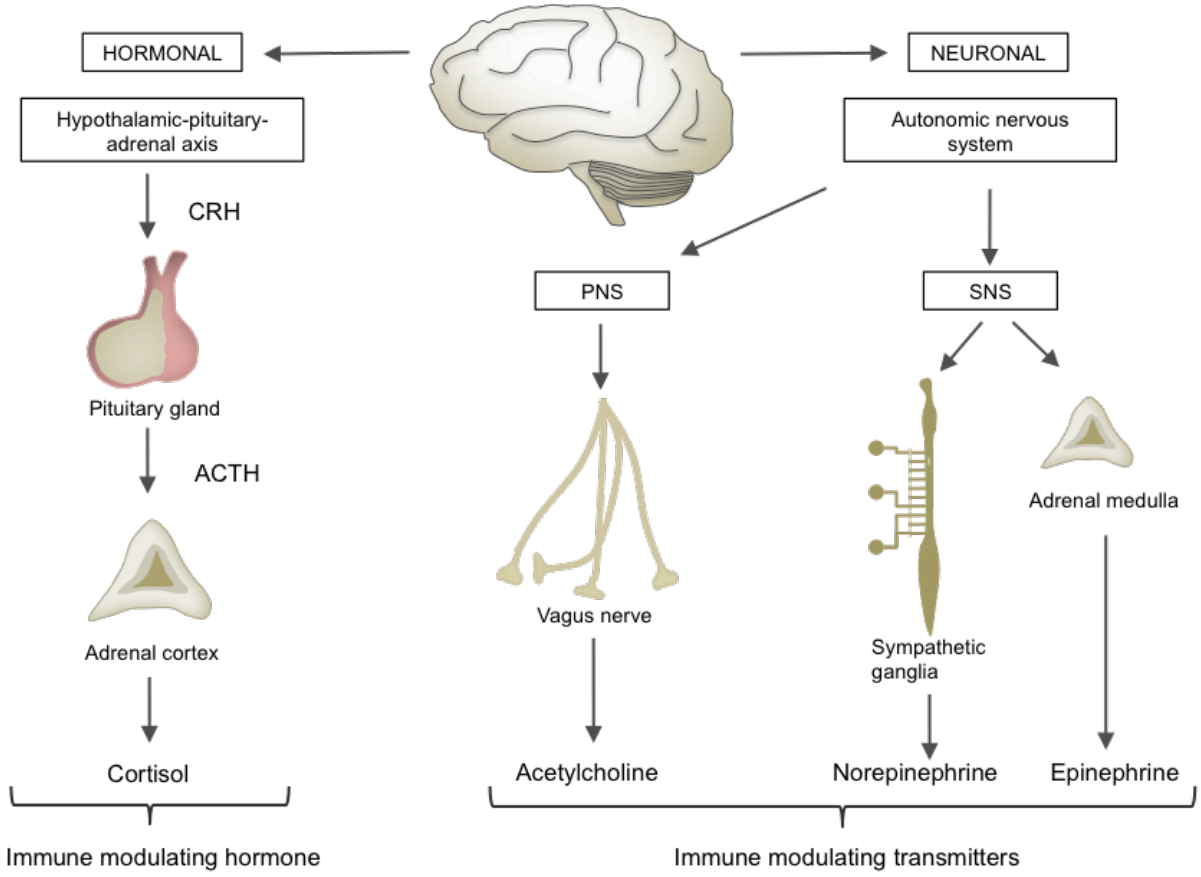
Critical illness induces imbalanced expression of inflammatory mediators such as cytokines, complement, platelet activating factor and others, and this could be improved with insulin signaling. IIT can significantly decrease pro-inflammatory cytokine levels while increasing the expression of anti-inflammatory cytokines so as to maintain the inflammatory reaction balance. It has been shown in endotoxemic rats that Insulin modulates the inflammatory response by decreasing the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, and Tumor

necrosis factor (TNF)-α and increasing serum levels of the anti-inflammatory cytokines IL-2, IL-4, and IL-10 independently of the glucose or electrolyte levels (Jeschke *et al.*, 2004). Furthermore, a recent publication demonstrated that continuous administration of small amounts of insulin in a non-hyperglycemic mouse model of endotoxemia led to decreased inflammation. Insulin reduced plasma levels of IL-6, TNF-α, monocyte chemotactic protein 1 (MCP-1) and decreased global mortality without affecting blood glucose levels (Kidd *et al.*, 2008). In patients with severe trauma, IIT significantly reduced the pro-inflammatory mediators TNF-α, IL-6 and CRP, when compared to control (Zhao *et al.*, 2005). After burn trauma in rats, insulin down-regulated expression of IL-1β at 1, 5 and 7 days post-burn (PBD), IL-6 (1 PBD), macrophage migration inhibitory factor (MIF) (5 and 7 PBD) and TNF-α (1 and 2 PBD). At the same time, IIT also up-regulated expression of IL-10 (2, 5 and 7 PBD), IL-2 and IL-4 (5 and 7 PBD) (Jeschke *et al.*, 2002). The same research group observed similar effects on pro- and anti-inflammatory cytokine production in pediatric patients with severe burns, where insulin decreased IL-1β and TNF-α concentrations at 30 PBD and increased IL-10 at 15 PBD when compared with controls (Jeschke *et al.*, 2004). Taken together, these results suggest that insulin treatment of critically ill rodents and humans results in reduced production of pro-inflammatory cytokines (IL-1β IL-6, TNF-α, CRP, MCP-1, MIF, etc.) and induces anti-inflammatory mediators (IL-2, IL-4 and IL-10), thus attenuating systemic inflammatory processes.

2.5 Central regulation of immunity

It has been widely acknowledged that the central nervous system (CNS) communicates via many pathways with the peripheral immune system and vice versa. This form of communication includes hormonal and neuronal mechanisms by which the brain controls immune function, and in reverse, allows the immune system to communicate with the brain via cytokines. The two major pathways by which the CNS controls immune functions are the hormonal response represented by the hypothalamic-pituitary-adrenal (HPA) axis and the neuronal response from the autonomic nervous system through release of norepinephrine (noradrenalin) from sympathetic and acetylcholine (ACh) from parasympathetic nerves (Figure 2).

Figure 2 Pathways by which the CNS modulates peripheral immune functions. CRH = Corticotropin-releasing hormone, ACTH = Adrenocorticotropic hormone, PNS = Parasympathetic nervous system, SNS = Sympathetic nervous system.



2.5.1 Hypothalamic-pituitary-adrenal axis

The HPA axis is the primary hormonal system activated as a physiological response to stress. On stimulation, corticotropin-releasing hormone (CRH) is secreted into the hypophyseal portal blood supply by neurons of the basal hypothalamus. CRH is carried to the pituitary gland where it then activates the anterior part to release adrenocorticotropin hormone (ACTH), which in turn stimulates the expression and release of glucocorticoids from the cortices of the adrenal glands. Glucocorticoids regulate the immune system on a cellular and molecular level via a wide variety of mechanisms. For example, they act on the immune system by both suppressing and stimulating a large number of pro-inflammatory or anti-inflammatory mediators. Examples of pro-inflammatory cytokines that are broadly down-regulated by glucocorticoids include TNF-α, IL-1, IL-6, IL-12 and interferon (IFN)-γ (DeRijk *et al.*, 1997). Similarly, secretion of many chemokines is strongly suppressed. In contrast, anti-inflammatory cytokines such as IL-10 and TGF-β are up-regulated by glucocorticoids. (Franchimont *et al.*, 1999; Elenkov *et al.*, 2002)

Glucocorticoids inhibit the synthesis of multiple inflammatory proteins through repression of the genes that encode them. The glucocorticoid receptor is located in the cytosol and upon ligand binding dissociates from a protein complex, dimerizes and translocates into the nucleus where it regulates gene transcription. There is strong evidence that by this mechanism, corticosteroids inhibit the effects of pro-inflammatory transcription factors, such as activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which regulate the expression of genes that code for many inflammatory proteins, such as cytokines, inflammatory enzymes, adhesion molecules and inflammatory receptors.(McKay *et al.*, 1999; Karin *et al.*, 2001).

2.5.2 Neuronal pathways

Lymphoid organs including bone marrow, thymus, spleen and lymph nodes are all innervated by fibers of the autonomic nervous system. Classic neurotransmitters such as catecholamines and ACh are released from neurons into the lymphoid microenvironment and contribute to neuroimmune modulation. Many immune cells express specific receptors for neurotransmitters. These receptors have been shown to respond in vivo and/or in vitro to the neuronal substances and their manipulation can alter immune response. These findings demonstrate the anatomical and functional basis of interactions between nerves and immune cells.

Catecholamines released from the sympathetic nervous system (SNS) directly affect immune cells in bone marrow (Madden *et al.*, 1994), thymus (Singh *et al.*, 1976), spleen (Felten *et al.*, 1987), and lymph nodes (Madden *et al.*, 1994). The released catecholamines norepinephrine and epinephrine mainly stimulate cell surface β-adrenergic receptors, to some extent also α-adrenergic receptors, present on a wide variety of immune cells (Sanders *et al.*, 1997) (Spengler *et al.*, 1990). Through binding to adrenergic receptors of immune cells, norepinephrine and epinephrine inhibit the production of pro-inflammatory cytokines, such as IL-12, TNF-α and IFN-γ and stimulate the production of anti-inflammatory cytokines like IL-10 and transforming growth factor (TGF)- β (Elenkov *et al.*, 1999). In vitro experiments with macrophages harvested from spleen and lymph nodes have shown that via β-adrenergic receptors, norepinephrine can dramatically inhibit the production and secretion of TNF-α in response to lipopolysaccharide (LPS) (Ignatowski *et al.*, 1996). Moreover, interruption of sympathetic innervation of immune organs has been shown to modulate the outcome of inflammatory diseases and increase susceptibility to infectious diseases (Lorton *et al.*, 1996). The development of a β2-adrenergic-receptor deficient mouse was considered a breakthrough: it was hoped that this model would convincingly demonstrate that the expression of this receptor on immune cells was immunologically relevant. However, the

mouse phenotype was normal before and after immunization, which led to the conclusion that dual activation of the SNS and HPA axis is required for full modulation of host defenses to infection. (Sanders *et al.*, 2003)

Activation of the parasympathetic nervous system (PNS) results in activation of the cholinergic nerve fibers of the efferent vagus nerve and subsequently leads to release of ACh at the synapses. Studies by Tracey and colleagues have suggested that the efferent vagus nerve, representing the PNS plays a unique role in regulating systemic and localized inflammatory processes, primarily by inhibiting macrophage production of TNF-α, but also affecting the production of other pro-inflammatory cytokines such as IL-1ß, IL-6 and IL-18 (Borovikova *et al.*, 2000). The molecular mechanism by which cytokine production is inhibited is attributable to ACh, the major vagus nerve neurotransmitter. Macrophages, which are believed to be the main source of pro-inflammatory cytokines during inflammation, but also other immune cells express ACh receptors (AChRs), which transduce an intracellular signal that inhibits cytokine release. The major receptor identified as a suppressor of cytokine release from immune cells is the α7 subunit of the nicotinic AChR (α7-nAChR). Vagus nerve stimulation, or administration of α7-nAChR agonists, inhibits production of pro-inflammatory cytokines, however, if mice are deficient in α7-nAChR (α7-nAChR knockout mice) or vagus nerve activity (vagotomy), these animals fail to suppress cytokine release after exposure to endotoxin (Wang *et al.*, 2003) (Wang *et al.*, 2004). In addition, the vagus nerve also has an immune-sensory function transmitting information from the immune system to the CNS. Immune stimuli activate vagal sensory neurons. For example, administration of bacterial endotoxins and IL-1 has been shown to induce expression of c-Fos, a marker of neuronal activation in vagal sensory ganglia, and vagotomy abolishes these early activation gene responses (Gaykema *et al.*, 1998; Goehler *et al.*, 1998).

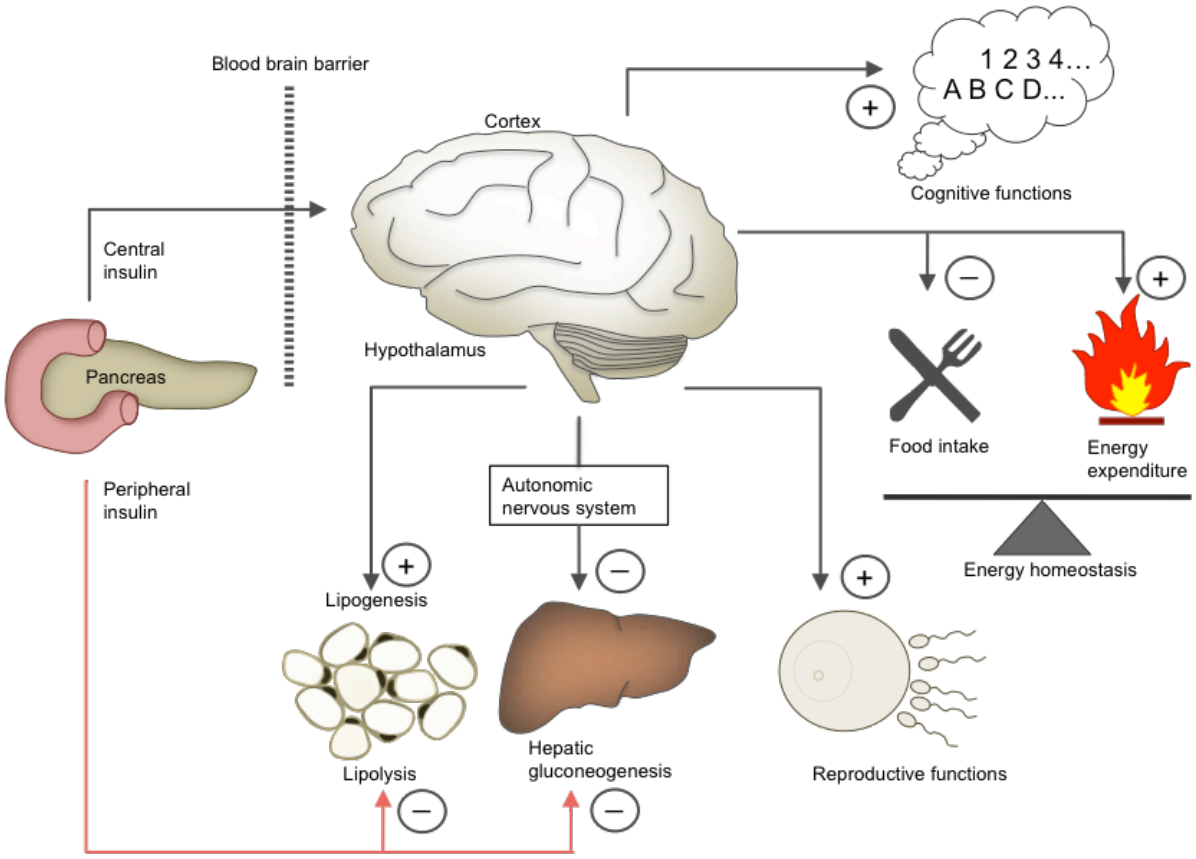
Together, these findings lead to the concept of the cholinergic anti-inflammatory pathway which is part of the inflammatory reflex. This is described as a rapid mechanism by which inflammatory signals reach the brain, to which the brain subsequently responds with a rapid anti-inflammatory action through cholinergic nerve fibers. (Tracey, 2002)

2.6 Central Insulin

While insulin is well known for its peripheral effects on glucose, fat, and protein metabolism, in recent years it has become widely acknowledged that insulin also has profound effects on the CNS. Insulin is secreted by pancreatic β-cells and readily transported from the periphery across the blood brain barrier (BBB) by a saturable, receptor-mediated process (Banks *et al.*, 1997). Insulin receptors are widely present throughout the brain, but the highest concentrations of receptors are in the cerebral cortex, olfactory bulb, hippocampus,

amygdala, cerebellum and the hypothalamus (Havrankova *et al.*, 1978; Unger *et al.*, 1991). Central actions of insulin include effects on food intake, energy balance, reproductive functions and the autonomic nervous system (Figure 3).

Figure 3 Schematic model of central nervous insulin effects. Insulin secreted from the pancreas crosses the BBB. By acting on relevant brain structures, insulin improves cognition while reducing food intake and promoting energy expenditure, thereby modulating energy homeostasis. In parallel, central nervous insulin contributes to the inhibition of gluconeogenesis and, together with peripheral insulin, regulates adipocyte metabolism. Modified from (Hallschmid *et al.*, 2009)



2.6.1 Energy balance

A first model for the physiological mechanism underlying the long-term regulation of bodyweight by the brain was proposed two decades ago by Porte and Woods. In their study the concentration of insulin found in cerebrospinal fluid was increased by systemic infusions of glucose or insulin and was shown to be proportional to its concentration in plasma. Moreover, infusion of insulin (10 and 10 μU/kg/day) into the lateral cerebral ventricles of free-feeding baboons caused a dose dependent suppression of food intake and bodyweight suggesting that the amount of insulin secreted per day modulates food intake to maintain a constant bodyweight. (Porte *et al.*, 1981)

However, it has been difficult to discern whether these metabolic consequences are directly due to the manipulation of central insulin action. Further studies, however, have generated experimental support for a direct role of hypothalamic insulin signaling in energy homeostasis. The arcuate nucleus of the hypothalamus contains proopiomelanocortin / cocaine- and amphetamine-regulated transcript (POMC/CART)-expressing neurons, which suppress feeding when activated and this has been identified as an important site for regulating energy balance. In contrast, activation of neuropeptide Y/agouti-related protein (NPY/AgRP)-expressing neurons, also present in the arcuate nucleus, stimulate feeding. Indeed insulin affects the expression of NPY: in fasted animals ICV administration of insulin decreases neuropeptide Y (NPY) and its messenger ribonucleic acid (mRNA) in the arcuate nucleus, suggesting a potential molecular mechanism by which central insulin affects feeding behavior as observed in earlier studies (Schwartz *et al.*, 1992). It has been shown that insulin action in the CNS not only controls bodyweight but also controls peripheral glucose and fat metabolism. The infusion of insulin in the third cerebral ventricle suppressed glucose production and this occurs independently of any change in circulating levels of insulin or other glucoregulatory hormones. The efferent mechanism coupling insulin action in the brain to hepatic glucose metabolism is unknown, but is presumed to involve autonomic innervation of the liver or other tissues (Obici *et al.*, 2002). Recently, it was demonstrated by our group that insulin administered centrally to Sprague-Dawley rats increases white adipose tissue lipogenic protein expression, inactivates hormone-sensitive lipase, and suppresses lipolysis. Conversely, mice that lack the neuronal insulin receptor exhibit unrestrained lipolysis and decreased de novo lipogenesis in white adipose tissue. Thus, brain and, in particular, hypothalamic insulin action play a pivotal role in white adipose tissue functionality (Scherer *et al.*, 2011).

2.6.2 Reproductive functions

There is increasing evidence to support a central role of insulin in the control of reproductive functions, beyond maintaining the homeostatic and metabolic balance. To study the physiological role of insulin signaling in the brain, Brüning introduced a mouse model with a neuron-specific disruption of the insulin receptor gene (NIRKO). They discovered that inactivation of the neuronal insulin receptor had no impact on brain development or neuronal survival. NIRKO mice not only showed increased food intake and developed diet-sensitive obesity, but their results also reveal an important link between brain insulin signaling and reproduction. It was shown that loss of CNS insulin receptor leads to decreased fertility and a significant reduction in reproductive hormone levels. NIRKO mice exhibited impaired spermatogenesis and ovarian follicle maturation due to hypothalamic dysregulation of luteinizing hormone. (Bruning *et al.*, 2000). Further studies have shown that insulin

administered centrally, into the third ventricle, is completely sufficient to maintain hormone-facilitated reproductive behavior in female rats. Diabetic female rats have decreased ovulation, reproductive behavior and luteinizing hormone surges. Insulin administered intracerebroventricular (ICV) at doses that did not correct the peripheral metabolic changes associated with T2DM caused a modest increase in the lordosis quotient, which has been employed as a measure of female rat sexual motivation in a large number of studies and serves as an accurate parameter for measuring the intensity of sexual motivation or drive. Moreover, ICV insulin also increased the quality of lordosis among diabetic rats. These experiments show that ICV insulin infusion is able to completely restore reproductive behavior in diabetic female rats (Kovacs *et al.*, 2003).

2.6.3 Autonomic nervous system

Extensive research results indicate that brain insulin also influences the autonomic nervous system. Central insulin primarily increases the activity of multiple sympathetic nerves. The first study designed to determine whether acute central infusion of insulin increases peripheral sympathetic nervous outflow was performed in 1994. In this study insulin was infused at three different concentrations (0.1 μU/min, 10 μU/min and 100 μU/min) into the third cerebral ventricle of rats while recording the activity of the lumbar, renal and adrenal nerves. The results demonstrated that centrally administered insulin produces a considerable increase in lumbar sympathetic nerve activity without inducing changes in blood glucose or plasma insulin. (Muntzel *et al.*, 1994). In a later study, insulin was infused into the lateral ventricle of anesthetized rats instrumented for recordings of mean arterial pressure, heart rate and lumbar sympathetic nerve activity. The results of this study confirmed the previous findings and, moreover, demonstrated that ICV insulin influences baroreflex control of heart rate by increasing gain and maximum baroreflex levels (Pricher *et al.*, 2008). There is evidence that centrally administered insulin not only affects the sympathetic part of the autonomic nervous system but also influences the parasympathetic branch. As described earlier, it has been shown that ICV insulin suppresses hepatic glucose production. These central effects of insulin require innervation of the liver by the PNS, as surgical resection of the hepatic branch of the vagus nerve negates the effects of central insulin (Pocai *et al.*, 2005).

2.6.4 Cognition

Recent findings suggest that central insulin may also play a role in brain cognition phenomena such as learning and memory, and the association of insulin receptor deterioration with brain degenerative dementia (e.g., Alzheimer's disease) has attracted increasing interest. Insulin has been shown to exert a memory enhancing effect in both

humans and experimental animals. One experiment determined whether ICV administration of insulin improves memory formation in rats. To this end, rats were trained on a step-through passive-avoidance task, in which they were either shocked or not after entering a darkened compartment. After training, the animals received an ICV injection of 4 mU insulin, heat-deactivated insulin or saline vehicle. After 24 hours, the animals were tested for retention of the task. The results show that the shocked animals receiving insulin had a greater increase in their test latency to enter the compartment than animals receiving control solutions. These observations suggest that brain insulin enhanced the performance of rats on memorizing the negative consequences of entering the compartment. Because insulin was given after acquisition of the experience, it most likely contributed to processes underlying memory consolidation (Park *et al.*, 2000). Recently, intranasal administration of insulin to humans has been reported to be an advantageous delivery route. Delivery of insulin to human subjects by this method induced sharp and rapid increase in cerebrospinal fluid insulin concentrations, without affecting blood insulin and glucose levels (Kern *et al.*, 1999). This mode of administration was used in a study to determine whether treatment with insulin induces improvement particularly in hippocampus dependent types of declarative memory. To study changes in memory associated with a prolonged increase in brain insulin activity in humans, the effects of 8 weeks of intranasal administration of insulin (human regular insulin 4 x 40 U/d) on declarative memory (immediate and delayed recall of word lists), attention and mood was evaluated in 38 healthy subjects. Delayed recall of words significantly improved after 8 weeks of intranasal insulin administration and, moreover, subjects receiving insulin reported signs of enhanced mood. These finding indicate a direct action of prolonged intranasal administration of insulin on brain functions, thus improving memory and mood in the absence of systemic side effects (Benedict *et al.*, 2004).

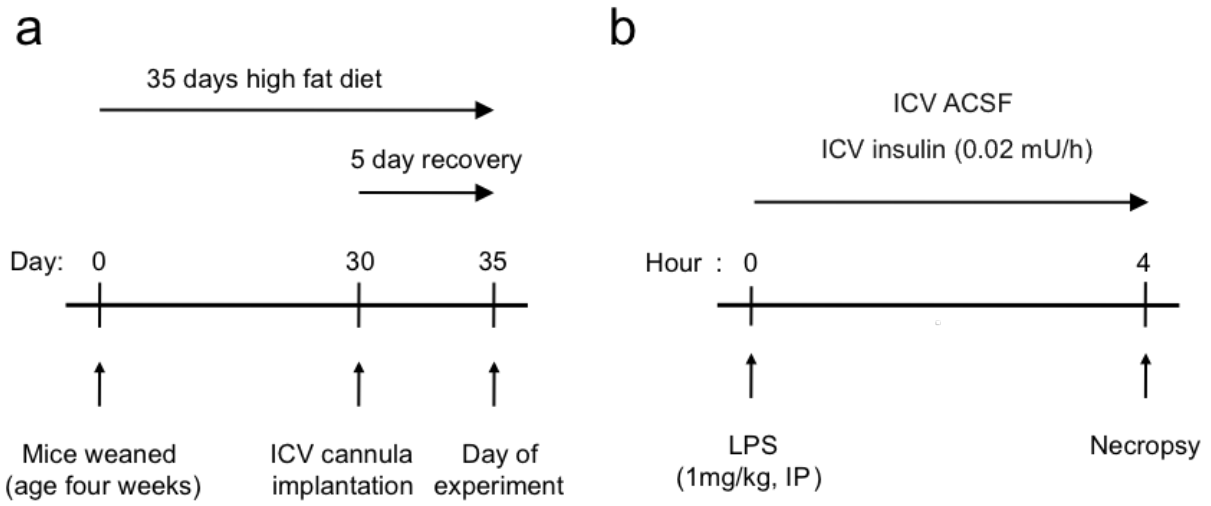
3 Objectives

Insulin acts as a direct anti-inflammatory agent. Administered to critically ill patients insulin can reduce morbidity and mortality in surgical and medical ICUs. The anti-inflammatory mechanisms involved are, however, poorly understood. Brain insulin has well defined effects on several peripheral parameters including food intake, cognitive functions, energy balance, reproductive functions and the autonomic nervous system. It is also known that by regulating autonomic nervous system activity the brain modulates the inflammatory state. Since brain insulin modulates the autonomic nervous system, we speculate that centrally administered insulin has systemic anti-inflammatory effects and we have tested this hypothesis in a mouse model of LPS induced critical illness.

3.1 Experimental design

The aim of the first study was to investigate whether ICV infusion of insulin modulates the inflammatory response in endotoxemic mice in a model of overfeeding-induced insulin resistance. We expected that brain insulin would suppress pro-inflammatory markers and enhance anti-inflammatory cytokines. In a second series of studies, animals were injected with a lethal dose of LPS, followed by an infusion of central insulin, in order to determine whether survival of animals in the insulin treatment group would be significantly higher than in the vehicle-treated group.

Figure 4 (a) Experimental protocol for HFD feeding and ICV cannula implantation. (b) Protocol for infusion studies. 4-week old male C57BL/6 mice were fed a high-fat diet for 4 weeks and ICV cannulas were implanted. After 5 days recovery from surgery infusion studies were performed. Mice were injected with LPS (1 mg/kg, IP) and randomly assigned to receive ICV insulin or vehicle infusions for 4 hours until necropsy.



3.1.1 Aim No 1

To determine whether ICV infusion of insulin affects the inflammatory response after injecting insulin-resistant animals with a sublethal dose of LPS.

Male C57BL/6 mice are weaned at 4 weeks of age and fed a high-fat diet (HFD) for 35 days to induce insulin resistance. After 30 days of HFD feeding, the mice undergo stereotactic surgery to implant ICV guide cannulas followed by a 5-day recovery period until infusion experiments are carried out. After complete recovery from surgery animals are placed in individual cages and an endotoxemic reaction is induced by intraperitoneal (IP) injection of a sublethal dose of E. coli LPS (1 mg/kg bodyweight). Without delay, an ICV infusion of either insulin (0.02mU/h) or artificial cerebrospinal fluid (ACSF) is continuously administered for a period of four hours. Serial sampling of plasma allows us to assess circulating cytokine levels by enzyme-linked immunosorbent assay (ELISA). Additionally, metabolic parameters such as insulin and glucose concentrations are monitored during the experiment. At the end of the studies, the animals are sacrificed and liver, kidney, spleen, muscle, lung and adipose tissue are freeze clamped in liquid nitrogen and stored until further analysis. Frozen tissue samples are used to measure concentrations of pro-inflammatory and anti-inflammatory molecules by western blot analysis.

Injecting mice with LPS leads to an endotoxemic reaction with increased signs of inflammation. The ICV infusion of insulin should attenuate the inflammatory response, indicated by lower concentrations of inflammatory markers in peripheral tissues and serum.

Two groups of animals (n=8 in each group) are studied:

1) High-fat diet for 35d + sublethal dose LPS IP injected + ICV vehicle (ACSF)

2) High-fat diet for 35d + sublethal dose LPS IP injected + ICV insulin (total dose 0.08 mU)

Table 4: Treatment protocol of animals studied to determine whether ICV infusion of insulin affects the inflammatory response after injecting insulin-resistant animals with a sublethal dose of LPS.

Group	Animals	Diet	Infusion	LPS
Insulin	Male C57BL/6, 8-10 weeks old	HFD, 35 days	Insulin (0.02 mU/h), 4 h	1 mg/kg, IP
Control	Male C57BL/6, 8-10 weeks old	HFD, 35 days	ACSF, 4 h	1 mg/kg, IP

3.1.2 Aim No 2

To determine whether central insulin administration improves survival in insulin-resistant mice injected with a lethal dose of LPS.

We first determine on the basis of a dose-response curve the lethal LPS dose. After 35 days of HFD feeding male C57BL/6 mice are randomly assigned to four groups of animals (n=6) receiving IP injections of varying doses of LPS (1, 5, 10 or 15 mg/kg bodyweight) and overall survival in each group is assessed twice daily for the following five days. The LPS dose causing death in over 80% of the animals serves as the lethal dose (LD 80). Subsequently, the mice are injected with the lethal LPS dose and receive central administration of either insulin or vehicle via ICV cannula. The overall survival in each group is assessed over a period of five days.

Two groups of animals (n=8 in each group) are studied:

1) High-fat diet for 35d + LPS LD 80 IP injected + ICV vehicle (ACSF)

2) High-fat diet for 35d + LPS LD 80 IP injected + ICV insulin (total dose of 2.4 mU)

Table 5: Treatment protocol of animals studied determine whether central insulin administration improves survival in insulin-resistant mice injected with a lethal dose of LPS.

Group	Animals	Diet	Infusion	LPS
Insulin	Male C57BL/6, 8-10 weeks old	HFD, 35 days	Insulin (0.02 mU/h), 5 days	5 mg/kg, IP
Control	Male C57BL/6, 8-10 weeks old	HFD, 35 days	ACSF, 5 days	5 mg/kg, IP

The aim of this experiment is to demonstrate that the anti-inflammatory effect of central insulin not only influences the molecular signs of inflammation but also has “clinical

relevance” by increasing overall survival. Based on our results from the first experiment we expect a significant reduction in mortality after five days in the group of animals treated with central insulin.

4 Materials & Methods

4.1 Protein extraction

4.1.1 Tissue homogenization

Tissue for western blot analysis is removed from freshly sacrificed animals and immediately clamped and snap frozen in liquid nitrogen to prevent specific or nonspecific degradation of proteins by autolytic and/or proteolytic processes. After freezing the tissue is transferred to labeled tubes and cryopreserved at -80°C until further analysis. For protein extraction from frozen tissue, the samples are weighed on dry ice to avoid thawing then transferred to 5 mL tubes appropriate for homogenization with the tissue sharer. Lysis buffer is added to the tissue sample according to the type and weight of tissue used for protein extraction (Table 6). The lysis buffer contains the detergent Tergitol-type NP-40 which breaks up the cytoplasmic but not the nuclear membrane and is therefore used to obtain the cytoplasmic contents of the cells. Additional constituents are chelating agents to deactivate metal-dependent enzymes, buffers to stabilize the pH and different inhibitors of protein phosphatases to preserve the phosphorylation of proteins of interest by inhibiting endogenous phosphatases present in the cell lysate mixture. The sample is completely immersed in lyses buffer and homogenized with a high-torque motor driven tissue grinder at highest speed using up-down strokes for 30 seconds. To break up any remaining cell or organelle membranes an additional method for cell disruption is applied to the sample using ultrasound (20-50 kHz) (sonication). To this end the homogenized tissue samples are carefully exposed to ultrasound energy produced by a sonicator for 15 seconds in order to agitate particles while avoiding excess heat production and are then stored on ice at 0°C.

Table 6: Showing correct lysis buffer and tissue sample weight ratio for protein extraction.

Tissue	Liver	Spleen	Lung	Fat
Sample weight	100 mg	30 mg	50 mg	100 mg
Lysis buffer	1 mL	500 µL	1 mL	500 µL

4.1.2 Centrifugation

The homogenates are transferred to micro centrifuge tubes and centrifuged for 20 minutes at 13,000 rpm, 4°C. The resulting supernatant (total cell extract) is removed and stored on ice

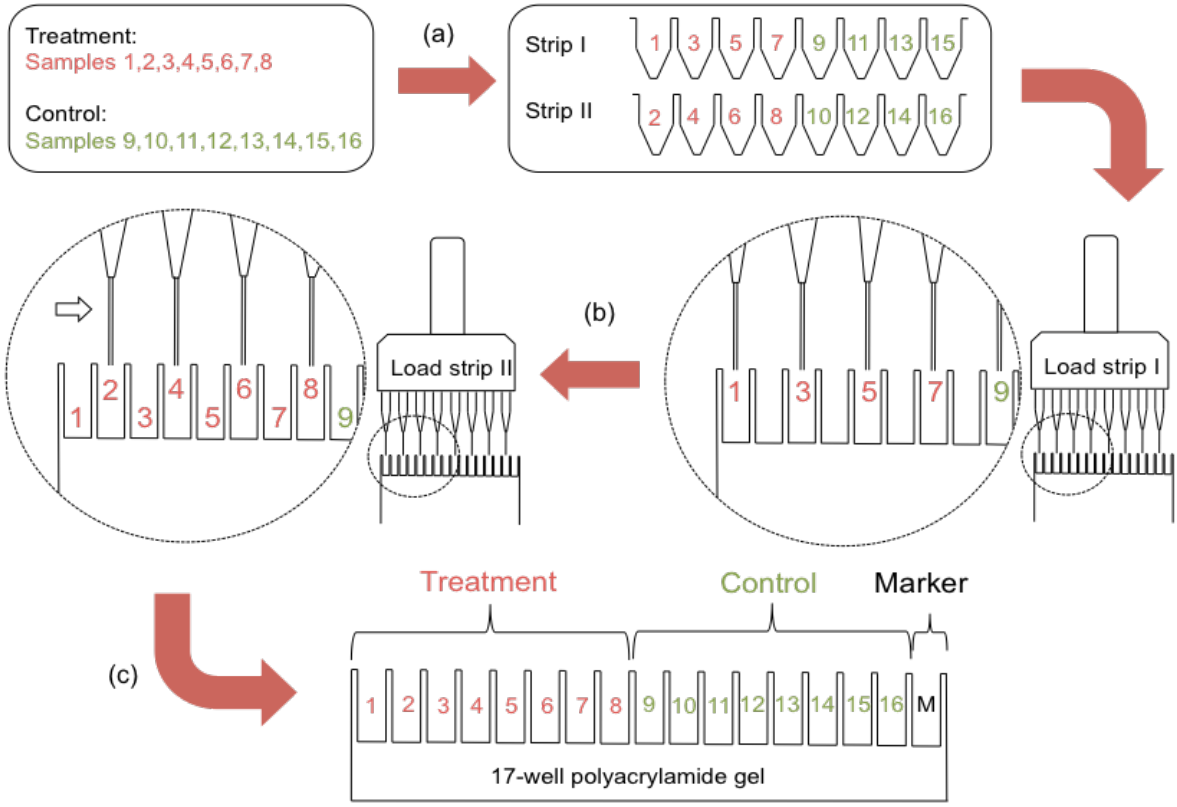
at 0°C for further procedures, e.g. analysis by western blot. The resulting pellet is snap frozen and stored at -80°C. The differential centrifugation by velocity has to be strong enough to remove large organelles, cell debris and connective tissue even if it means accepting a partial loss of the desired smaller proteins.

4.1.3 Sample preparation

A bicinchoninic acid (BCA) assay is used to determine the total protein concentration of each sample. The wells of the 96-well plate are filled with duplicates of samples, distilled water and increasing concentrations of bovine serum albumin (BSA). In order to determine the protein concentration of the samples, the assay response of each sample is compared to a BSA protein standard of known concentration. To this end the BCA protein assay reagent is added to all wells, the plate is covered with a plate sealer and incubated at 37°C for 30 minutes. The total protein concentration is expressed by purple coloration of the sample solution in proportion to protein concentration, which can then be measured by spectrophotometry. The coloration is based on two separate reactions. The first step is a reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium. The amount of Cu^{1+} produced is proportional to the amount of protein present in the solution. In a second step of color development reaction, BCA reacts with the cuprous ion (Cu^{1+}) that was formed in step one. The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{1+}). The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm. The protein concentration of each sample is determined using a standard curve produced with BSA. Varying amounts of lyses buffer are added to each sample depending on the detected protein concentration to normalize each sample to 2 μg protein per μL solution. Preparing the samples at approximately the same protein concentration will ensure that the samples are comparable and that differences of detected antigens between samples are due to changes in protein expression in the cells.

Sample buffer containing a tracking dye, lithium dodecyl sulfate (LDS) and dithiothreitol (DTT) is added to each sample. This is important to make sure that small proteins do not run off the gel during electrophoresis and to obtain an electrophoretic separation by mass rather than net charge. To be able to load the 17-well gel with an 8-channel pipette the samples need to be arranged in 8-tube strips so that they can be loaded in the correct order (Figure 5). Before loading the gel the 8-tube strips are vortexed and heated at 85°C for 5 minutes. By heating the protein samples at 85˚C in the presence of excess LDS and thiol reagent, disulfide bonds are cleaved between cysteine residues and the protein is fully dissociated into its subunits.

Figure 5 Illustrating preparation of samples to perform electrophoresis using a 17-well polyacrylamide gel and an 8-channel pipette. (a) Control and treatment samples are placed in a specific order and loaded into two 8-tube strips, using an 8-channel pipette, strip I and II are subsequently loaded onto the gel beginning with strip I on the left, (b) followed by strip II to the right so that (c) treatment samples are loaded on the left and control samples on the right of the 17-well polyacrylamide gel. Well 17 is used for the molecular weight marker.



Materials

High-torque motor driven tissue grinder
Sonicator, Branson Sonifier 250
BCA quantification kit, Pierce
1.5mL microcentrifuge tubes
Heating block, PTC-100 Programmable Thermal Controller MJ Research
Spectrophotometer, Titertek Multiskan MCC130 Type 347
Microcentrifuge, Eppendorf Minispin (2004) 5452
Scale, Mettler Toledo PB153
Container with dry ice
Markers, ink and pens
Clean Forceps
Clean Scalpels
Liquid Nitrogen

Lysis buffer

*20 mM MOPS (*3-(N-morpholino) propanesulfonic acid)
2 mM EGTA (ethylene glycol tetraacetic acid)
5 mM EDTA (ethylenediaminetetraacetic acid)
30 mM Sodium fluoride
40 mM β-glycerophosphate
10 mM Sodium pyrophosphate
2 mM Orthovanadate
0.5 % NP-40
complete protease inhibitor cocktail tablet

Sample buffer

NuPAGE® LDS Sample Buffer (4x)
106 mM Tris HCl
141 mM Tris base
2 % LDS

10 % Glycerol
0.51 mM EDTA
0.22 mM SERVA® Blue G250
0.175 mM Phenol Red
pH 8.5

NuPAGE® Sample Reducing Agent (10x)
500 mM DTT

4.2 Electrophoresis

Electrophoresis is defined as the transport of charged molecules through a solvent by an electrical field. It is used to separate complex mixtures of proteins, investigate subunit compositions or purify proteins for use in further applications. Most biological molecules e.g. proteins, deoxyribonucleic acid (DNA), RNA carry a net charge at any pH other than their isoelectric point and will migrate at a rate proportional to their charge density. In polyacrylamide gel electrophoresis (PAGE), proteins migrate in response to an electric field through pores in the gel matrix which acts as a porous media and functions like a molecular sieve. The combination of pore size of the gel and protein charge, size, and shape determines the migration rate of the protein. The higher the acrylamide concentration, the smaller the pore size, resulting in resolution of lower molecular weight molecules and vice-versa. Proteins separated on gels can be subsequently analyzed by immunoblotting.

We use an electrophoresis apparatus for vertical electrophoresis of pre-cast polyacrylamide gradient gels to separate proteins. The gel has 17 wells each 1.0 mm wide and consists of a gradient of increasing polyacrylamide concentrations. Unlike single concentration gels, gradient gels resolve a much wider size range of proteins than standard single-concentration gels. They separate proteins evenly over a wide molecular weight range of 10 to 200 kDa. The protein bands are also much sharper, particularly in the low molecular weight range. The comb is carefully removed without tearing the sides of the wells and the gel is placed into the electrophoresis apparatus. In a next step the gel is immersed in running buffer that provides ions to carry a current and to maintain the pH at a relatively constant value. Here we use a discontinuous buffer system which utilizes different gel buffer and running buffer. Chloride (-) supplied by the gel buffer serves as the leading ion due to its high affinity to the anode. 3-(N-morpholino) propanesulfonic acid (MOPS) (-) serves as the trailing ion. Bis-Tris (+) is the common ion present in the gel and running buffer. This buffer system provides a neutral pH 7.0 environment resulting in maximum stability of proteins giving sharper bands and accurate results. Electrophoresis is performed under denaturating conditions using the anionic detergent sodium dodecyl sulfate (SDS) and under reducing conditions using DTT. SDS denatures and unfolds proteins by wrapping around the hydrophobic portions of the protein. The intrinsic charges of the polypeptide are insignificant compared to the negative charges

provided by the bound detergent so that the SDS-polypeptide complexes have essentially the same negative charge and shape and migrate through the gel strictly according to polypeptide size. The reducing agent DTT completely unfolds the denatured proteins into their subunits by cleaving the disulfide bonds between cysteine residues. An antioxidant is added to the running buffer in the upper buffer chamber. The antioxidant migrates with the proteins during electrophoresis preventing the proteins from re-oxidizing and maintaining the proteins in a reduced state.

The wells are carefully rinsed with running buffer to remove unpolymerized monomer. Using a 20 μL eight-channel pipette an appropriate volume (15 μL) of sample is loaded by carefully applying the samples as a thin layer at the bottom of each well. Next the control well is loaded with a one-color weight marker fluoresce in the 700 nm channel containing ten protein bands of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa (LI-COR Biosciences). The electrophoresis apparatus is attached to the power supply and run at 110 V constant voltage until the tracking dye of the samples has reached the bottom of the gel (approx. 2 hours). The negative charges flow from the negative cathode (black) terminal into the upper buffer chamber, through the gel, and into the lower buffer chamber. The lower buffer chamber is connected to the positive anode (red) terminal to complete the circuit. Thus, negatively charged molecules, such as SDS-polypeptide complexes, move from the negatively charged upper buffer chamber to the positively charged lower buffer chamber. Understanding how a gel apparatus is connected to the power supply requires a basic knowledge of Ohm's law: voltage = current x resistance (*V=IR).* A gel can be viewed as a resistor and the power supply as the voltage and current source. We use a MOPS SDS running buffer system where the fast moving highly conductive chloride ions in the gel are gradually replaced by the slower moving, less conductive MOPS ions from the running buffer as the gel runs. As a result the resistance of the gel increases and the current decreases to satisfy Ohm's law. Operating electrophoresis with constant voltage has two advantages over constant current. As resistance increases during electrophoresis current and watts (wattage = current x resistance, or W=IV) decreases throughout the run providing a safety margin for overheating and dangerous high voltage. Another advantage is that the same voltage settings can be used regardless of the number or thickness of gels undergoing electrophoresis because the current will automatically adjust via the power supply.

Materials:

Protein samples on ice
NuPAGE® Novex 4-12% Bis-Tris Gels 1.0 mm, 17 well, Invitrogen
Electrophoresis apparatus XCell SureLock Mini-Cell, Invitrogen
Constant voltage power supply, Invitrogen Zoom Dual Power ZP10001

Buffers and solutions:

Running buffer:

NuPAGE® MOPS SDS Running Buffer (20x)
50 mM MOPS
50 mM Tris base
0.1% SDS
1 mM EDTA
pH 7.7

NuPAGE® Antioxidant
Molecular weight marker

4.3 Immunoblotting and Immunodetection

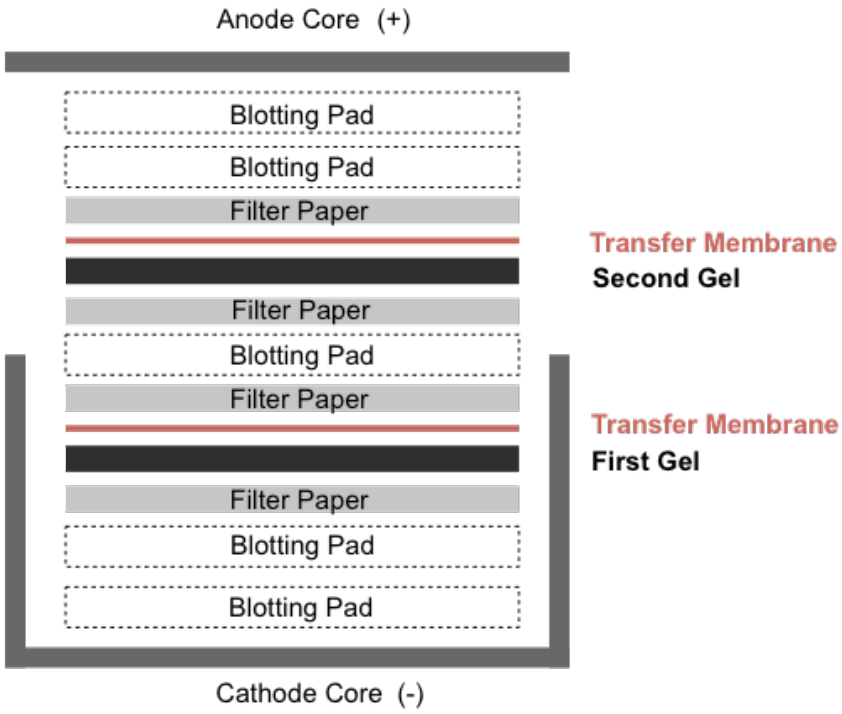
Immunoblotting (often referred to as western blotting) is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. Protein samples are separated by SDS-PAGE. The antigens are then electrophoretically transferred in a tank to a polyvinylidene fluoride (PVDF) membrane. The transferred antigens are bound to the surface of the membrane, providing access for reaction with immunodetection reagents. All remaining binding sites are blocked by immersing the membrane in a solution containing a protein blocking agent. After probing with the primary antibody, the membrane is washed and probed with the appropriate secondary anti-IgG antibody. Secondary antibodies are directly labeled with near-infrared (IR) dyes for two-colored detection with IR fluorescence. A laser imaging instrument with imaging membranes in two different IR channels is used to generate digital images for each fluorescent channel. Finally membranes are stripped and reprobed several times.

4.3.1 Protein Blotting

In this procedure proteins are electrophoretically transferred using a tank blotting unit. Two transfer buffer-soaked blotting pads are placed on the cathode (-) core of the blot module. The polyacrylamide gel is carefully removed from the plastic casing and picked up with a sheet of half-soaked filter paper. The uncovered side of the gel is overlaid with a sheet of membrane precut to the size of the gel. This membrane is then overlaid with another sheet of soaked filter paper. Potential air bubbles that might interfere with the transfer are removed by rolling a glass pipette over the membrane surface. The gel/membrane sandwich is placed on top of the two blotting pads and covered with an additional pre-soaked blotting pad. A second gel/membrane sandwich is placed on top, covered with two additional blotting pads and the anode (+) core. The two gel/membrane sandwiches are held securely between the two halves of the blot module and the entire assembly is placed into a tank containing transfer buffer. The blot module is filled with ice to prevent overheating and connected to the power supply. Transfer is achieved by applying a 200 mA current for 2 hours. Negatively charged

proteins are transferred from the gel onto the membrane because the membrane is positioned on the anode side of the gel (Figure 6).

Figure 6 Depicting assembly of two gel/membrane sandwich with blotting pads and filter paper inside the blot module for transfer of proteins from the gels onto the transfer membranes.



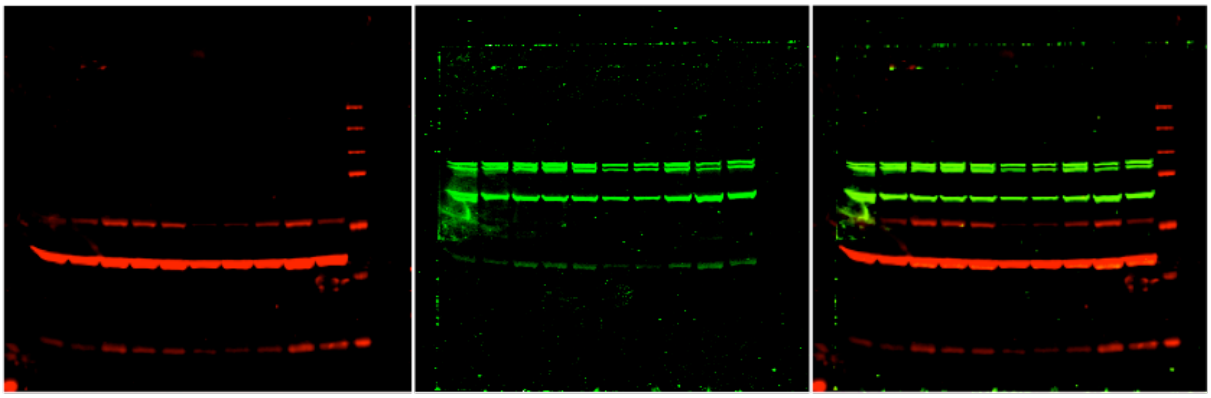
4.3.2 Immunoprobing

After blotting the transferred proteins bound to the surface of the membrane are probed with specific antibodies to identify and quantify any antigens present. The gel is discarded and the membrane is immersed in blocking buffer for 1 hour on the rocking shaker to fill all protein-binding sites with nonreactive protein, thus preventing nonspecific binding of antibodies. The membrane is then placed in a solution containing the antibody directed against the antigen (primary antibody solution) and incubated for 12 hours at 4°C. The blot is washed three times in tris base buffered saline containing tween 20 (TBS/T) for 5 minutes to remove any nonspecific bound primary antibody. The membranes are then exposed to an IR dye conjugated antibody directed against the primary antibody (secondary antibody; e.g. goat anti-rabbit immunoglobulin G (IgG). The boxes containing the membranes are covered with aluminum foil due to light sensitivity of the secondary antibody and left on the rocking shaker for 1 hour. After a further three washing steps in TBS/T and one in tris based buffered saline (TBS) antigens are identified and quantified by IR fluorescence of the antigen/primary antibody/secondary antibody/fluorophore complex bound to the membrane.

4.3.3 Quantitative, two-color visualization with infrared fluorescence

By selecting appropriate primary and secondary antibody reactivity and using spectrally distinct IR fluorophores, we analyzed antigens by two-color IR fluorescence. A laser imaging instrument - the Odyssey IR Imaging System (LI-COR Biosciences) - was used for IR detection. Antigens are visualized with antibodies directly conjugated with IR fluorophores such as IRDye 800CW (excitation 778 nm, emission 795 nm), DyLight 680 (excitation 682 nm, emission 715 nm) and DyLight 800 (excitation 770 nm, emission 794 nm). Because these dyes fall into two classes (approx. 700 nm or 800 nm emission, with 100 nm separation) they can be used simultaneously for multiplex analysis of two targets. Mouse, rabbit and goat primary antibodies were incubated with the blot together, and the same was done for dye-labeled goat anti-mouse (approx. 700 nm emission), goat anti-rabbit and donkey anti-goat secondary antibodies (approx. 800 nm emission). The blots were imaged with the IR imager in both 700 and 800 nm channels in a single scan. Mouse antibodies appeared red and rabbit antibodies appeared green on the scan making it easy to distinguish and detect different antigens in one scan (Figure 7). Quantification of the bands was performed with the analysis software provided.

Figure 7 Images of membranes scanned with IR imager.Using 680 nm (left) and 800 nm (middle) channels and the combined image (right). Using dyes attached to the secondary anti-body that fall into two classes allows simultaneous use of several targets on one membrane for multiplex analysis.



4.3.4 Stripping and reusing membranes

Reprobing PVDF membranes that have already been exposed to antibodies is simple and straight forward. All residual antibodies are removed from the membrane by rewetting it in TBS/T and briefly treating it with stripping solution containing sodium hydroxide (NaOH) for 15 minutes. Although repeated reprobing can cause inconsistent protein loss that compromises quantification, up to five reprobings are generally feasible. The ability to quantitatively detect several targets on the same blot increases experimental throughput.

Materials:	*Odyssey Infrared Imaging System, LI-COR Biosciences* *Immobilon-FL Membrane, Millipore* *Tank blotting unit XCell II Blot Module, Invitrogen* *Constant voltage power supply, Invitrogen Zoom Dual Power ZP10001*
Buffers and solutions:	*Transfer buffer (10x):* *25 mM Tris base (trishydroxymethylaminomethane)* *200 mM Glycine* *5 M Methanol* *TBS (20x):* *0.4 M Tris base* *2.7 M Sodium chloride* *pH 7.6* *for TBS/T add 0.1 % Tween 20 to TBS (1x)* *Primary antibody solution:* *Blocking buffer mixed with TBS/T at a 1:1 ratio* *primary antibody* *Secondary antibody solution:* *TBS/T* *0.001 ‰ SDS* *DyLight 800 Goat Anti-Rabbit (1:6000), Thermo Fisher Scientific Inc.* *DyLight 680 Goat Anti-Mouse (1:8000), Thermo Fisher Scientific Inc.* *IRDye 800CW Donkey Anti-Goat (1:10000)* *Blocking buffer* *Odyssey Blocking Buffer mixed with TBS at a 1:1 ratio* *Stripping solution* *0.2 M Sodium hydroxide*
Antibodies:	*IL-1β (SC 7884), Santa Cruz Biotechnology, Inc.* *Phospho-Stat1 (CS 9171), Cell Signaling Technology* *Phospho-Stat3 (CS 9131), Cell Signaling Technology* *COX II (SC 1745), Santa Cruz Biotechnology, Inc.* *IKK-γ (SC 8330), Santa Cruz Biotechnology, Inc.* *GAPDH (G8795), Sigma-Aldrich Co.* *β-actin (A5441), Sigma-Aldrich Co.* *α-tubulin (ab7291), Abcam Inc.*

4.4 Enzyme-Linked Immunosorbent Assay

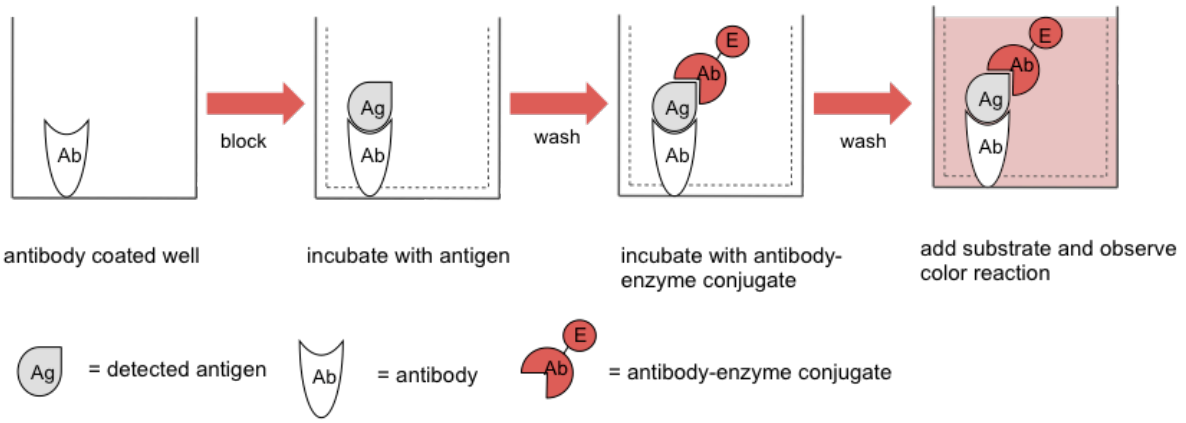
The enzyme-linked immunosorbent assay is a solid-phase immunoassay used to detect the presence and concentration of a specific soluble target molecule. The target molecule (e.g. antigen) is first immobilized onto an insoluble substrate, often composed of polystyrene, in a 96-well microtiter plate configuration. Ordinarily, this immobilization is a non-specific binding event and must be followed with a blocking reagent to saturate any other non-specific binding sites on the substrate. Once the surface has been blocked, the complementary molecular partner (cognate antibody covalently coupled to an enzyme) can be added to the surface and allowed to selectively interact with the target molecule. Unbound antibody-enzyme conjugates are washed out and an appropriate substrate for the enzyme used is added. As the substrate is hydrolyzed by the bound enzyme conjugate, a colored or

fluorescent product that can be measured in a microtiter plate reader is generated. The amount of product generated is proportional to the amount of antibody-enzyme conjugate that has bound to the surface and is hence proportional to the amount of the target molecule in the test mixture.

4.4.1 Antibody-sandwich ELISA

If the antigen is present in a heterogeneous sample mixture that also contains other molecules (e.g. blood serum samples) the actual detection of antigen will be influenced by the competitive binding of those different molecules from the sample. For this reason an ELISA that employs a capture antibody (antibody-sandwich ELISA) is more commonly used (Figure 8). To detect the target molecule, the wells of a microtiter plate are coated with specific (capture) antibody followed by incubation with the test mixture containing the antigen. Irrelevant molecules will be left unbound. After washing away unbound molecules, antigen that remains bound to the surface can then be detected with a different antigen-specific antibody conjugated to an enzyme. Substrate is added and after another incubation cycle, the degree of substrate hydrolysis is measured. The amount of substrate hydrolyzed is proportional to the amount of antigen in the test solution. The advantage of antibody-sandwich ELISA is that the sample does not have to be purified and the assay can be very sensitive due to the fact that the target molecule is identified by two specific antibodies.

Figure 8 Antibody-sandwich ELISA to detect antigen. Ag=antigen; Ab=antibody; E=enzyme.



4.4.2 ELISA procedure

The number of antibody-coated micro-well strips required to test the desired number of serum samples plus appropriate number of wells needed for running blanks and standards are set up. 100 μL of standard is pipetted into the wells. It is important to include wells for non-specific binding controls and for negative-antigen control reactions where no antigen is

added to the wells. Several dilutions of the serum samples are prepared and added to the wells to ensure that at least one of the dilutions can be accurately measured with the standard curve. For accurate quantization standards, controls and samples should be assayed in duplicate. When the correct dilution for the serum samples is determined by performing a preliminary ELISA the remaining samples are similarly diluted and 100 μL of each sample is added to the wells together with 50 μL of the detection antibody against the target protein, the plate is then covered with a plate sealer and incubated for 3 hours at room temperature. In this assay the antibody is coupled to biotin and the labeling reagent horse radish peroxidase (HRP) is coupled to streptavidin (avidin). When the avidin-enzyme conjugate is added to the biotinylated antibody the biotin binds specifically to the streptavidin hence coupling the enzyme to the antibody. A biotin/streptavidin (avidin) system provides significant sensitivity and low background. Due to its high affinity bond, it minimizes nonspecific interactions with other proteins and permits more complete washing, without the risk of lowered signal due to antibody detachment. After incubation the plate is emptied and each well is rinsed three times with 300 μL washing solution and residual liquid is removed by gently flicking it face down onto several paper towels. 100 μL of HRP-streptavidin is added to each sample. The plate is again covered and incubated for 30 minutes to allow binding of HRP-streptavidin to the detection antibody. After incubation the wells are again emptied and washed three times. 100 μL of substrate solution 3,3´,5,5´-tetramethylbenzidine (TMB) is added to each well and the plate is incubated in the dark for 25 minutes at room temperature. After incubation the enzyme substrate reaction is stopped by quickly pipetting 100 μL of sulfuric acid (H_2SO_4) stop reagent into each well and this completely inactivates the enzyme. Immediately after the addition of H_2SO_4 the results must be read with a spectrophotometer at a primary wavelength of 450 nm. TMB is a chromogen that yields a blue color when oxidized with hydrogen peroxide (catalyzed by HRP). The color changes to yellow with the addition of sulfuric acid with maximum absorbance at 450 nm. A standard curve is constructed from the data produced by serial dilutions of the standard antigen. Antigen concentrations are plotted on the x-axis and fluorescence on the y-axis. The concentration of antigen in the serum samples is interpolated from the standard curve.

Materials:	*Multichannel pipette and disposable pipette tips, Eppendorf* *Microwell strips pre-coated with capture antibody* *Microtiter plate frame for microwell strips* *Microtiter plate reader, Spectrophotometer with 450 nm filter* *Paper towels* *Timer* *Serum samples*
Reagents and solutions:	*Standard buffer diluents* *Standards* *Dilution of biotinylated antibody* *Dilution of streptavidin-HRP* *Washing buffer*

ELISA:	*IL-6 murine ELISA kit (ab46100), Abcam Inc., Cambridge; MA* *IL-6 murine ELISA kit (ab100578), Abcam Inc., Cambridge; MA* *TNF-alpha Quantikine ELISA Kit (DTA00C), R&D Systems Inc., Minneapolis; MN*

4.5 Animal techniques

4.5.1 Animals

Male and female C57BL/6 mice, three to four weeks old, are purchased from The Jackson Laboratory, Bar Harbor, ME. For breeding two female and one male animal are housed in one cage in a temperature- and light (12-h light-dark cycle)-controlled facility. The mice have access to regular chow diet (RCD) and water ad libitum. Nestlets are added to the cage as nesting material. The males are left with the females at all times to prevent stressing the female mice and subsequent cannibalization of the litter. After six weeks the breeding cages are checked frequently for newly delivered litter. At four weeks of age the litters are separated from the parenting cage. The litters are then separated by sex and kept in groups of three to five animals per cage. Animals are fed either with RCD or HFD and water at libitum for four weeks until the experiments are performed. In this study only C57BL/6 male mice, eight to ten weeks of age, are used for experimental procedures.

4.5.2 Blood sampling

During the 4-hour infusion protocol blood is collected from the tail vein at several time points while continuously infusing the animal. Each mouse is placed in a restraining device so the blood draw can be performed by one person. A small incision is made on the side of the tail near the end. Mineral oil is used to gently massage the length if the mouse's tail to promote blood flow. Accumulating blood droplets are touched with a glass capillary tube to allow uptake of blood into the tube by capillary action. After collection the cut is compressed with gauze and the animal is returned to its cage. The collection tube is filled with the anticoagulant Ethylenediaminetetraacetic acid (EDTA) to prevent coagulation which removes fibrinogen or varying amounts of other proteins from the blood. Plasma is collected by centrifugation of EDTA blood at 13,000 rpm for 2 minutes at room temperature. The blood separates into three phases with the bottom phase consisting of red blood cells, the top phase which is collected consisting of plasma and an interphase resembling a thin layer containing mainly immune-type cells. The plasma (approximately 100 µl per draw) is ready for analysis and, if necessary, can be stored at -20°C. At the end of the ICV infusions, the animal is sacrificed with a lethal dose of the volatile halogenated ether isoflurane and blood

is collected from the retro-orbital sinus where blood draw quantity is higher (approximately 900 μL). The blood is mixed with EDTA, and centrifuged for 2 min at 13,000 rpm at room temperature. Plasma is stored at -20°C until further analysis.

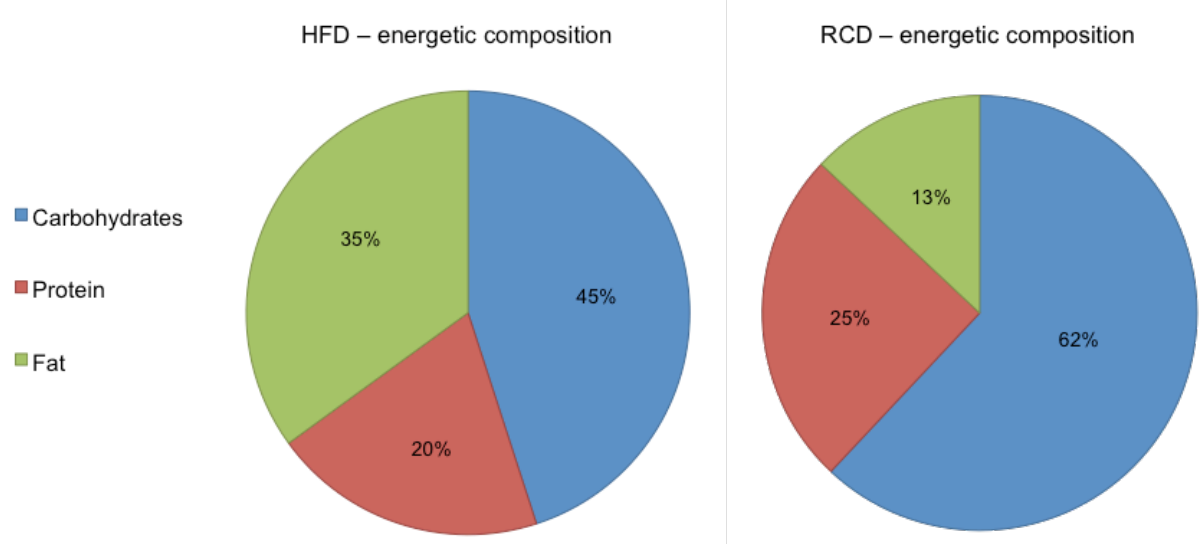
Materials:

Mice, C57BL/6, Stock-No 000664,The Jackson Laboratory, Bar Harbor, Main, USA
Mouse cages
Restraining device
Surgical blade
Scissors
Microhematocrit capillary tubes (with EDTA, Microhematocrit Kimble
Microcentrifuge, Eppendorf Minispin (2004) 5452
Forceps
Gauze
Mineral oil light white, MP Biomedical

4.5.3 Model of diet-induced insulin resistance

Maintaining metabolic homeostasis relies on the balanced intake of nutrients from food. Consequently, diet composition strongly impacts whole-body physiology. Dietary formulations with strong nutrient imbalances can lead to metabolic disorders and chronic diseases including obesity, metabolic syndrome, T2DM and cardiovascular disease. RCD is formulated to closely match the daily caloric requirements of mice (10 to 15 kcal/day) when provided ad libitum. The caloric intake from the RCD used in our experiment is 3000 kcal/kg. The approximate energetic composition (specified as percent of the total energy content) of RCD is made of fat 13%, proteins 25% and carbohydrates 62%. The energetic composition of the HFD we used to induce insulin resistance differed significantly from the RCD, delivering 35% of energy as fat (Figure 9). When fed with a HFD the excess energy is stored as triglycerides primarily in adipose tissue. Subsequently triglycerides also accumulate in peripheral tissues (e.g. the liver or the muscle) and exert deleterious effects on insulin signaling leading to broader pathology where glucose homeostasis is also altered. The C57BL/6 mouse strain is prone to develop metabolic disorders when challenged by a diet enriched in fat (Surwit *et al.*, 1988). To induce mild insulin resistance C57BL/6 male mice, four weeks of age, were challenged with a HFD for four weeks before experiments were carried out.

Figure 9 Diagram depicting energetic composition of high fat and regular chow diet as percentage of total energy content.

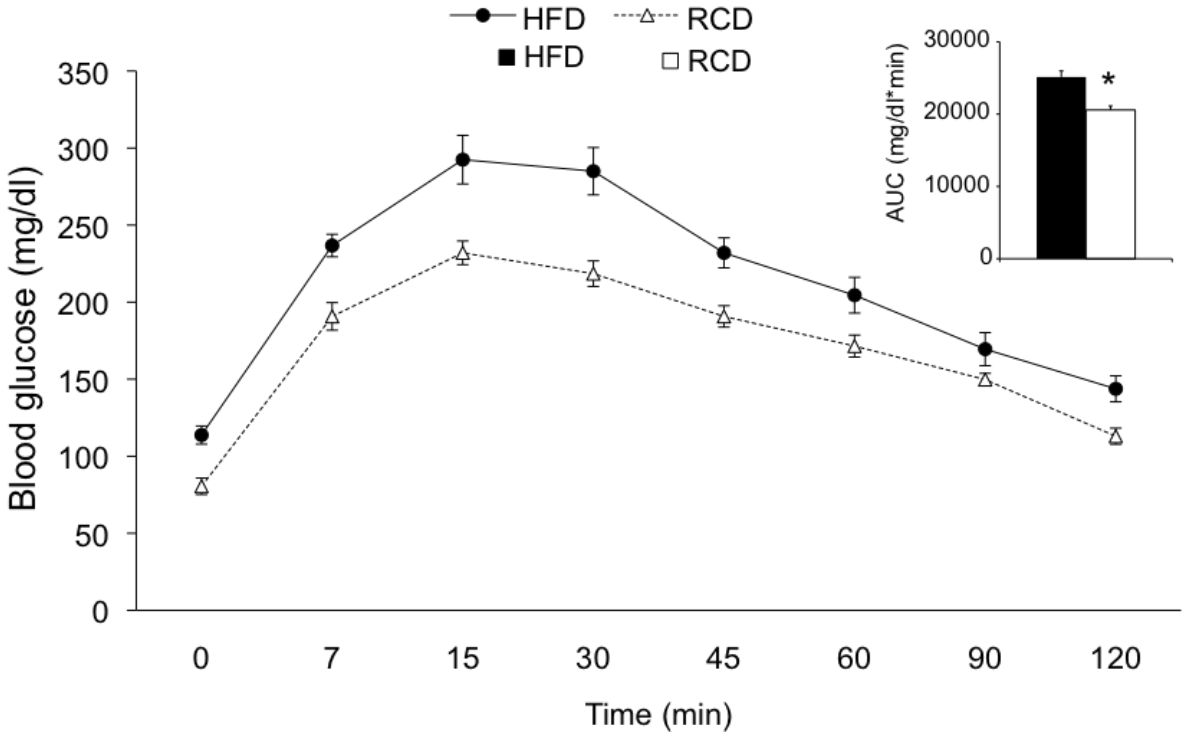


4.5.4 Glucose tolerance test

Consistent with the literature, compared to mice fed RCD, mice fed HFD did not show altered bodyweight after four weeks of feeding. The reason is that impact of dietary manipulations is typically time-dependent and progressive. A HFD will induce mild glucose intolerance after only four weeks, but this intolerance will gradually increase with time and the final symptoms (e.g. obesity and T2DM) will be observed only later, after at least 10 weeks on the diet. To prove that mice fed a HFD for four weeks already show signs of metabolic disorders e.g. glucose intolerance we performed an intraperitoneal glucose tolerance test (IPGTT). The IPGTT measures the clearance of a standardized intraperitoneally injected glucose load from the body. To this end, animals fasting for 16 hours are administered a glucose solution and blood glucose is measured at different time points. IPGTT is performed on 8 week-old, C57BL/6 male mice, fed either a HFD or RCD for 4 weeks (5 in each group). The mice are fasted for 16 hours (access to drinking water at all times), weighed and placed in individual cages with only water (no food) available. The volume of a 20% glucose solution required for IP injection of 1 g glucose/kg bodyweight is calculated and the syringes, containing the calculated glucose prepared for each animal with the needles (26-G, 3/8 intradermal needle) already attached. Blood glucose concentrations are measured with a blood glucose monitor and associated glucose test strips. The basal glucose concentration (t = 0) of each mouse is measured by removing one mouse at the time from its cage, making a small incision over the tail vein using dissecting scissors and placing a blood sample directly onto the test strip in the blood glucose monitor. After all mice have been measured the glucose solution is administered intraperitoneally to each animal at 35-second intervals between animals. At t =15 min the blood glucose is measured again, starting with the first mouse injected and

using the same time interval as that used for IP administration until all animals have been measured. Measurements are repeated at t = 7, 15, 30, 45, 60, 90, and 120 min after injection. For visual presentation glucose values are plotted against time (Figure 10). For statistical analysis area under the curve is calculated by adding the areas under the glucose-time curve for the intervals 0-7, 7-15, 15-30, 30-60, 60-90 and 90-120 min. A Student's t test is used to compare group means.

Figure 10 Intraperitoneal glucose tolerance test (IPGTT) showing a significant difference between high fat and regular chow fed mice. After 4 weeks on the diet, 8-week-old male C57BL/6 mice are fasted overnight, basal blood glucose measured (0 min) before intraperitoneal administration of 1 g/kg glucose. Blood glucose is determined with a glucometer.



Materials:

C57BL/6 mice, 8 weeks old, fed either HFD (n=4) or RCD (n=4) for four weeks
Mouse cages
Animal scale
Blood glucose monitor, Freedom-lite Freestyle
Glucose test strips, Freedom-lite Freestyle
1mL Syringe, BD
26-G 3/8 intradermal bevel
Dissecting scissors
Timer
RCD (20 5053), PicoLab Rodent
HFD (D12336), Research Diets, Inc.

Reagents and solutions:

20 % (w/v) aqueous glucose solution:
Dissolve 20 g D-glucose in 100 mL distilled water.

4.6 Animal Model of sepsis

The aim of using an animal model of endotoxic shock is to faithfully reproduce clinically relevant pathogenesis similar to sepsis or septic shock observed in critically ill humans. The model described here is among the most widely used and is represented by exogenous administration of the toxin LPS. LPS is a compound of the cell wall of Gram-negative bacteria and is the major mediator that triggers the massive cellular and humoral responses observed in endotoxic shock induced by Gram-negative bacteria. LPS compromises two components: the O-specific chain and the core. The O-specific chain accounts for the antigenic variability among species and strains of Gram-negative bacteria. The core is composed of an oligosaccharide bound to a molecule lipid called lipid A. Most of the toxicity of LPS resides in the lipid A moiety. Immune pathology of endotoxicosis models using bolus injections is characterized by an overwhelming innate immune response, inflammatory cytokines such as TNF-α representing crucial mediators. The model used here employs a large IP bolus dose of LPS administered to mice in the absence of any supportive therapy, such as intravascular volume resuscitation, intubation and mechanical ventilation or infusion of inotropic agent. The endpoint of this model is either survival in case of high LPS dose or immune regulation of cytokines for low LPS dose.

A stock solution of LPS from E. coli serotype 0111:B4 at a concentration of 1 μg/μL is prepared. To this end LPS is dissolved in saline, sonicated for 5 min at room temperature and separated into 1 mL aliquots. This stock solution is kept frozen at -20° C for several months and is sonicated each time after thawing and before use. LPS from various sources can be used, and the toxicity of the specific LPS must be checked in pilot experiments.

To find the optimal dose of the LPS pilot experiments with three groups of five mice each are performed. The weight of 8-week-old male C57BL/6 mice fed HFD and water ad libitum for four weeks is determined with a scale. The stock solution of LPS (1 μg/μl) is diluted with saline and injected intraperitoneally using a total volume of 200 μl per animal. Mortality is checked twice a day for the following three days. Based on the results the LPS doses are chosen. For survival experiments the LPS dose that produces 80-100% lethality (5 mg/kg bodyweight) is chosen and for immune regulation studies a sublethal dose of 1 mg/kg bodyweight is selected.

Materials	*Male C57BL/6 mice, 8 weeks old, fed HFD for 35d* *Mouse cages* *Animal scale* *LPS E. coli-serotype 0111:B4, Sigma Aldrich* *1mL syringes, BD* *26-G x 3/8 intradermal needles*

4.7 Intracerebroventricular infusion methods

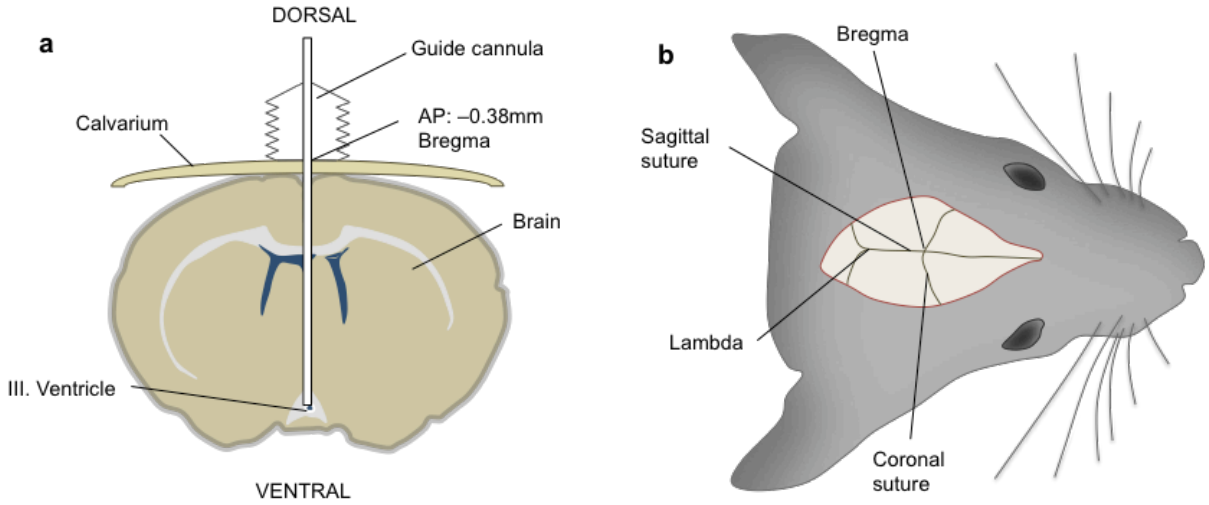
ICV infusion was first used as a method to provide enhanced estimation of central actions of drugs. For this purpose the ICV route of administration is particularly useful in at least two respects: first, in the study of drugs with relatively low blood-brain barrier permeability following systemic administration, and second, as a tool in assessing the central versus peripheral components of drug action. The first method of intracerebroventricular injection described in conscious animals, consisted of creating a trephine hole in the skull, sewing the skin over the bone and, after the wound had healed, injecting the substances through the skin and brain tissue. There are major drawbacks with this method: (1) the injection is painful and the animal has to be restrained forcefully thus inducing stress; (2) puncture of the ventricle has to be ascertained each time while the animal is restrained; and (3) each injection produces fresh brain injury. In summary, this method induces great stress in the animal making it difficult to assess the exact effect of the injected substance. In 1953 Feldberg and Sherwood (Feldberg *et al.*, 1953) were one of the first groups to use implanted cannulas for injection of drugs into the lateral ventricles of cats, rather than direct puncture of the injection site. With implanted cannulas they successfully avoided the drawbacks of earlier methods described above. The cannulas were implanted in anaesthetized animals and after an adequate recovery period of eight to ten days injections were made with a hypodermic needle through the cannula without the cat being aware of it.

The first commonly used ICV injection technique in conscious mice was that of Haley and McCormick (Haley *et al.*, 1957). The researchers used a line drawn through the anterior base of the ears as an injection site landmark. However, because of looseness of the skin, the base of the ears is not a fixed landmark with respect to the skull, so the potential for inaccuracy is considerable and the method therefore requires a great amount of practice to obtain acceptable degrees of accuracy. For this reason, some methodological refinements to this method were introduced later. Using the bregma, a fixed point on the skull located at the intersection of the coronal suture and the sagittal suture on the superior middle portion of the calvarium as the injection site obviated the difficulties mentioned above (Figure 11b). (Laursen *et al.*, 1986).

Stereotaxic surgery is a well-established technique for localizing specific brain structures within the brain of living animals. We use this technique for the chronic implantation of a cannula within these structures. These surgical interventions allow the assessment of the structure-specific effects of insulin within the brain. For surgery we use a high precision stereotaxic frame, capable of 10-μm precision. Boney features on the surface of the skull are used as reference points for determining the location of internal brain structures. The features used are bregma and lambda, the intersections of the sagittal suture with the

coronal and lambdoidal sutures, respectively (Figure 11). A mouse stereotaxic atlas of a C57BL/6 mouse brain (Franklin *et al.*, 1997) and additionally an online brain atlas based on a 51-day old C57BL/6 male mouse with a bodyweight of approx. 20 g is used to determine the anterior-posterior (AP), medial-lateral (ML), and dorsal-ventral (DV) coordinates of brain structures from measurements of the skull features (R. W. Williams, 2003, The Mouse Brain Library (www.mbl.org, August 9, 2010). Targeting the third ventricle the coordinates are AP: -0.38mm, ML: 0mm and DV -5.5mm relative to bregma. Since the exact coordinates of the target brain structure can vary with the strain and age of the mouse, pilot studies were carried out to refine these coordinates by trial and error. To this end, dye was injected followed by a histological examination of the target brain region. The refined coordinates are used to implant cannula, which allow the inception of substances in awake, freely moving mice.

Figure 11 Depicting location of guide cannula and landmark structures. (a) depicting a transversal section through the skull and brain of a mouse at the insertion site of the guide cannula; (b) Graphic showing a top view of a mouse head with a 1 cm long midline incision revealing the underlying skull and its landmark structures: bregma (intersection of the coronal suture with the sagittal suture) and lambda (intersection of the lambdoidal suture with the sagittal suture).



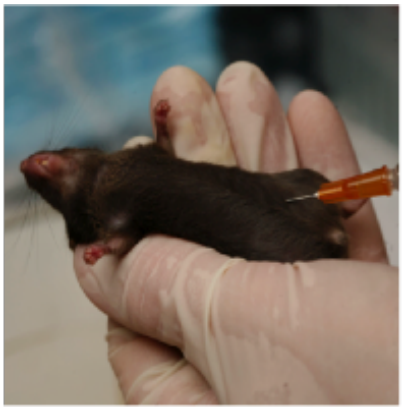
The mouse is gently restrained and injected intraperitoneally with an appropriate dose of ketamine/xylazine-cocktail (ketamine 100 mg/kg and xylazine 10 mg/kg). The correct level of anesthesia has been reached when the mouse shows abdominal breathing but no response to testing pain reflexes by pinching footpads with forceps, generally within approximately 5 minutes of injection. If the mouse still responds to pain stimuli a supplemental dose of anesthetic is administered (usually half of the original dose). The total volume of the injection should be limited to 750 μL to prevent discomfort to the animal due to abdominal distention. Prior to mounting the animal onto the stereotaxic instrument the animal's head is shaved.

To mount the animal the incisor bar and nose clamp is moved out of the way and one of the ear bars is locked at the 5 mm position. The head, neck and upper body of the mouse is grasped loosely with one hand and placed on to the stereotaxic apparatus. For correct positioning and subsequent surgery, the mouse must be elevated and should be level with the ear bars. The operator typically uses a folded blue pad for this purpose. While the animal's head is supported from beneath, the tip of the locked ear bar is guided into the external auditory meatus and held firmly. Keeping the animal in the same position and maintaining pressure the second ear bar is gently inserted into the auditory canal while moving the head slightly up and down. When no further movement of the head is possible the position of the second ear bar is locked. If the animal is correctly positioned movement should be possible only in the DV plane. Now the incisors are placed on the bar and the nose clamp is swung back into position. The nose bar is gently closed by screwing it lightly in place. It is essential to tighten the nose bar only lightly because too much pressure will prevent the mouse from breathing properly. Ophthalmic ointment is applied to the eyes of the mouse to prevent drying out and protect against accidental spills.

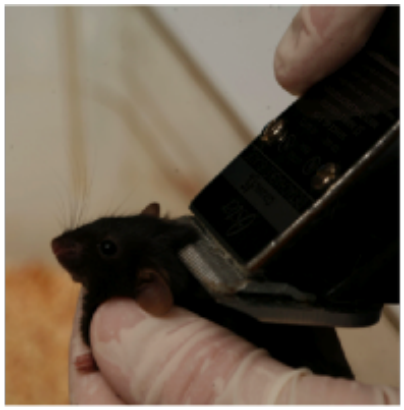
The head of the mouse is disinfected with alcohol pads. A 1-cm long midline incision is made using the point of a scalpel blade to pierce the skin. The incision is made with one stroke over the skull. If necessary the incision is extended forward to the back of the eyes and backwards between the ears. Retractors are placed in the incision to expose the skull and the transparent pericranial tissue (fascia, connective tissue) is scraped off with a cotton swab. Bregma (intersection of the coronal suture with the sagittal suture) and lambda (intersection of the lambdoidal suture with the sagittal suture) are identified. Bregma and lambda should be in the same plane, which is called the flat skull position, and most brain atlases are constructed using this plane. To check the lambda and bregma plane, the guide cannula is fitted to the stereotaxic frame and DV measurements at bregma and lambda are made by lowering the cannula until it is just touching these structures. Bring the bregma and lambda coordinates within 0.1 mm of each other by either raising or lowering the incisor bar.

The coordinates used for the implantation of the guide cannula targeting the third ventricle are AP: -0.38 mm, ML: 0 mm and DV: -5.5 mm relative to bregma. Mark the correct AP and ML coordinates with a 20-gauge hypodermic needle. Carefully drill a hole through the skull of the animal with the tip of the needle. Exercise caution when drilling so that the meninges and underlying brain tissue are not damaged. Bleeding can be reduced by application of pressure with gauze or cotton swab. Attach the guide cannula (precut to the correct length of 5.5 mm so that it will not extend over the DV coordinates) into the stereotaxic holder and straighten. Center the cannula over the drill hole and slowly lower into the target region until the cannula base is just about to rest on the skull. Apply one or two drops of instant adhesive to the base of the guide cannula. Lower the guide cannula further until the base of the cannula rests on

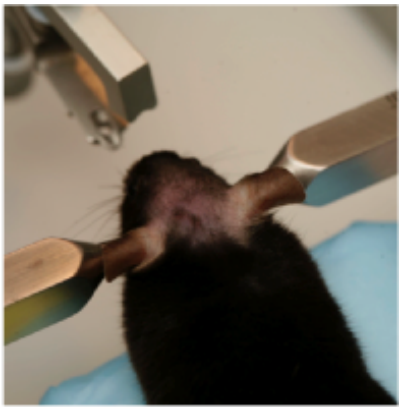
the skull in its final position. Hold this position for one or two minutes, allowing the instant adhesive to dry. Carefully remove the holder once the instant adhesive is dry avoiding any change of position of the cannula. Remove the skin retractors and dry the skull again if necessary. Prepare and apply a small amount of dental acrylic cement around the cannula, taking care to avoid cementing the opening of the cannula or creating sharp edges that may interfere with post-operative healing. Allow the dental acrylic cement to dry fully (approx. 3 to 5 minutes) and apply a protective dummy cannula into the guide cannula before the animal is removed from the stereotaxic frame. Keep the animals warm while they are recovering from the anesthetic by placing cages on a heating pad set at low (approx. 35°C). House the mice individually after surgery to prevent them from chewing each other's wounds or removing each other's cannulas.



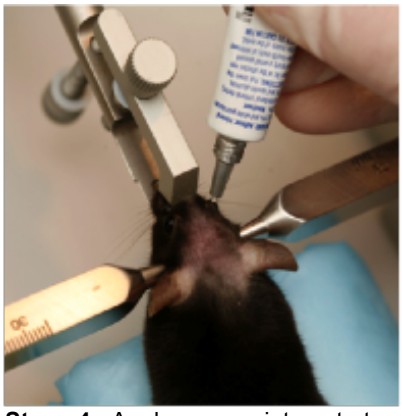
Step 1 Anesthetize the mouse with an intraperitoneal injection of a solution of xylazine and ketamine.



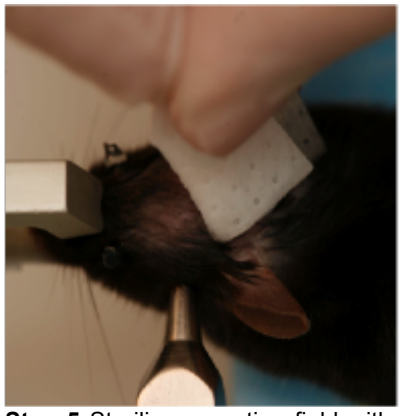
Step 2 After adequacy of the anesthesia is ensured, shave the operating field.



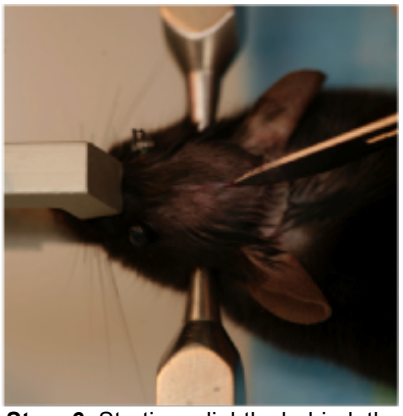
Step 3 Fit the animal into a stereotaxic frame.



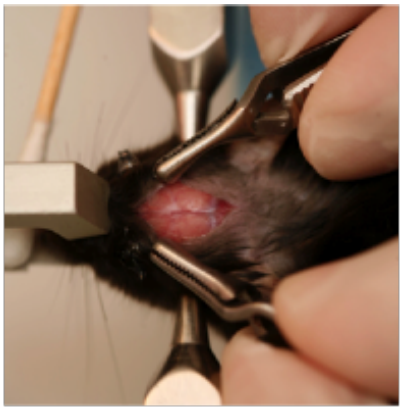
Step 4 Apply eye ointment to prevent eye damage during surgery.



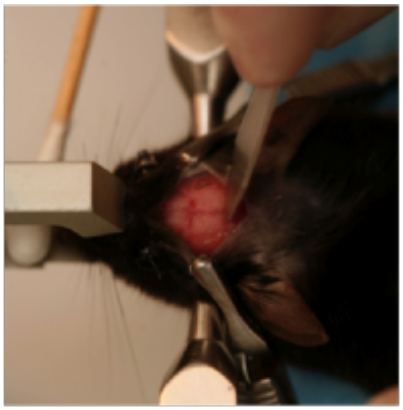
Step 5 Sterilize operating field with antiseptic wipes.



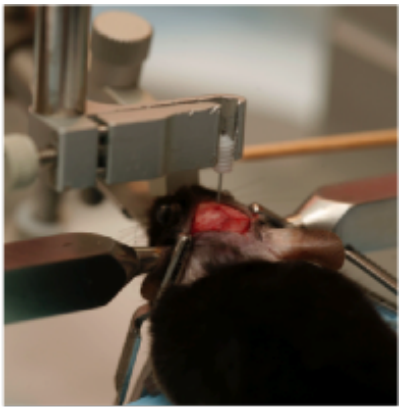
Step 6 Starting slightly behind the eyes make a 1cm midline sagittal incision.



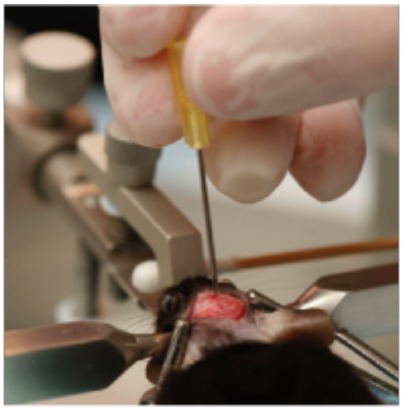
Step 7 Attach micro dissecting clips to ensure access to the skull bone.



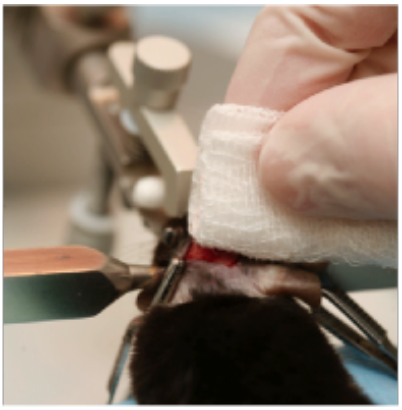
Step 8 With a scalpel lightly scrape the exposed skull area. Scraping should remove the periosteal connective tissue which adheres to the skull, permitting good adhesion of the dental cement used later (Step 14) to secure the cannula.



Step 9 Identify the bone suture junctions bregma and lambda. With these as reference points ensure that the skull is aligned flat laterally and anteroposteriorly by using the stereotaxic apparatus.

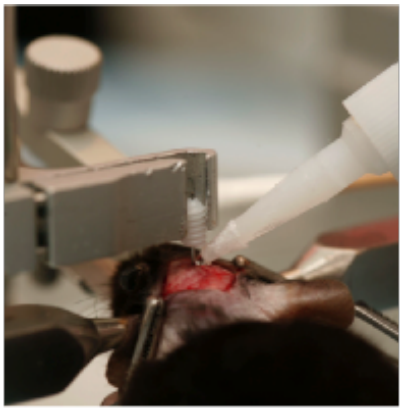


Step 10 With bregma as a reference point determine and mark the location for cannula placement using the stereotaxic coordinates determined with a brain anatomy atlas (here 0.38 mm anteroposterior from bregma). Using a 20-gauge needle drill a hole 1 mm in diameter through the skull at the marked stereotaxically correct location. This hole will receive the cannula.

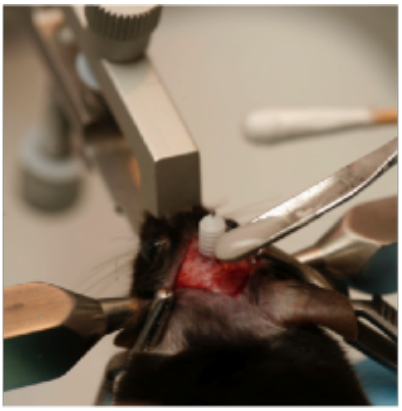


Step 11 Completely dry the skull surface from blood with sterile gauze. This can take several minutes until bleeding stops.

Step 12 Using the midline hole, insert the cannula through the skull to one third of the stereotaxically correct depth.

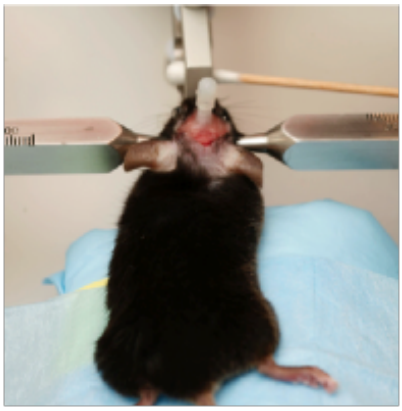


Step 13 Apply a small drop of instant adhesive onto the skull around the hole and Insert the guide cannula to the stereotactically correct depth (here 5.5mm). This ensures temporarily fixation of the cannula. Remove the placement tab from the top of the cannula.



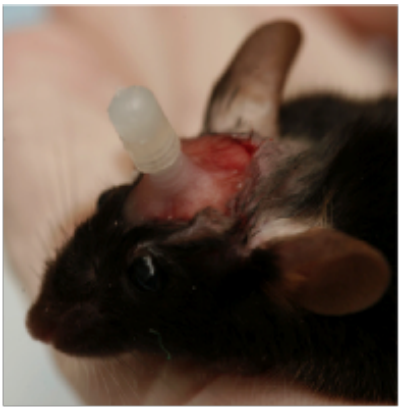
Step 14 Cover the cannula, the entire cannulation site and the exposed skull bone with acrylic dental cement. The powdered dental cement can be mixed with its acrylic solvent in a dish. Care should be taken not to drip any dental cement into the animal's eye.

Step 15 Allow the dental cement to harden for three minutes and insert a cannula dummy to seal the top of the guide cannula and prevent tissue, blood or liquor entry potentially causing occlusion of the guide cannula.

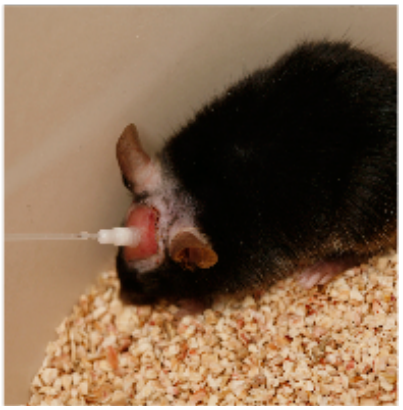


Step 16 Remove the animal from the stereotaxic apparatus.

Photos © by Helen Stevens, 2008



Step 17 Check for bleeding and correct position of the guide cannula and place the animal back into its cage onto a heating pad for one hour to prevent hypothermia during the recovery phase.



Step 18 Allow the animal to recover for at least 5 d and check food intake and bodyweight. Animals not reaching the presurgical weight cannot be used for infusion experiments.

Materials

Male C57BL/6 mice, 8-10 weeks of age at time of surgery
Small animal stereotactic frame, ASI Instruments
Tweezers
Shaver
Spatula
Heating pad
Skin retractors
26-G guide cannula cut 5.5 mm below pedestal, Plastics one
Protective dummy cannula for guide cannula, Plastics one
Surgical blade
20-G needle, Braun
Cotton swabs
Gauze
Light
454 Prism Instant Adhesive, Loctite
Dental acrylic cement
Alcohol pads
Blue pads
Ketamine/xylazine cocktail
1.5 mL xylazine (100mg/mL)

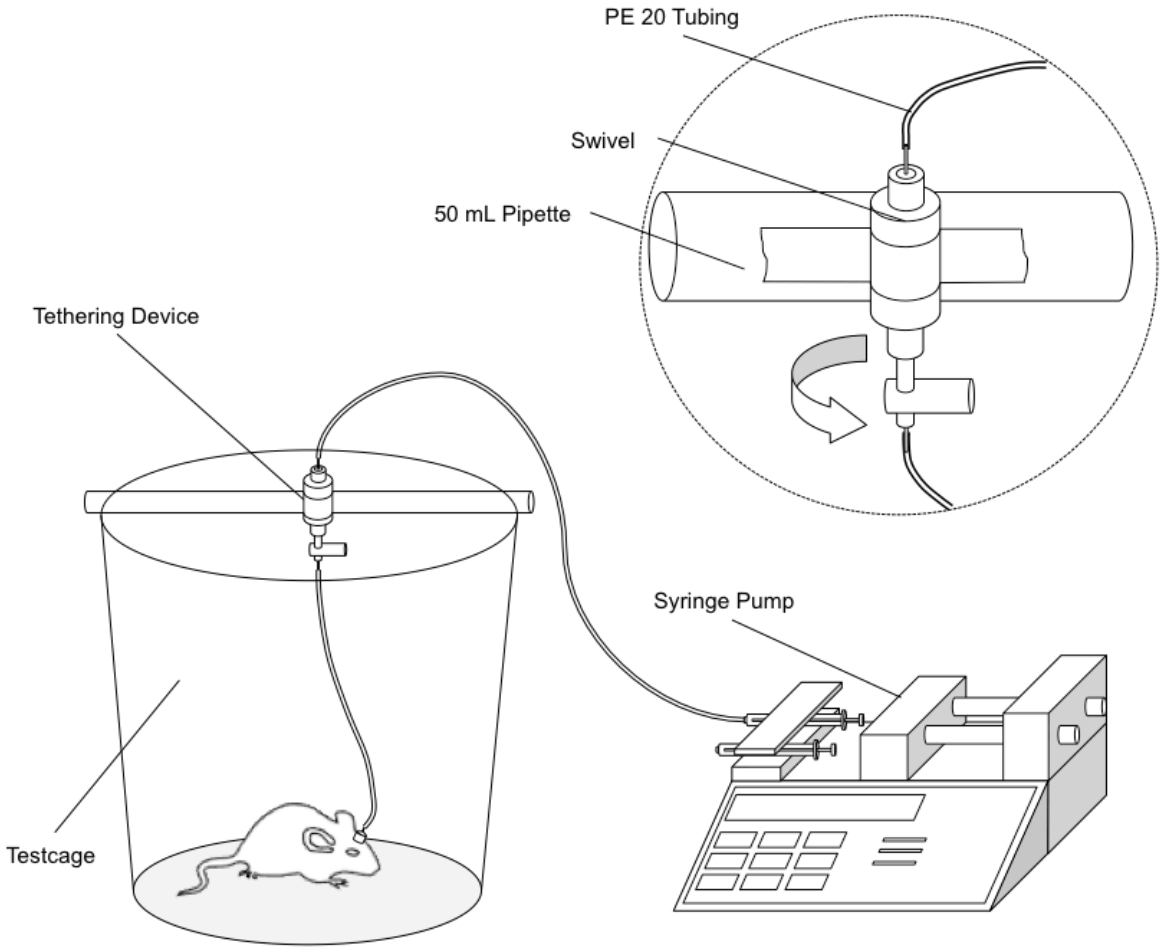
10 mL ketamine (100mg/mL)
dilute 1:4 with sterile saline (0.9% NaCl)

4.8 Intracerebroventricular infusion

Infusion studies can be conducted in the anesthetized or conscious animal. Although the anesthetized preparation has advantages for handling the animal, there is evidence that anesthesia can affect the release of several cytokines in a model of LPS induced sepsis (Adams *et al.*, 2008). Therefore, infusion studies here are preformed in conscious animals. In order to perform infusions in conscious animals, a tethering system designed to enable free movement of the animal in the test cage while protecting the inflow tubes from tension and tangling is necessary during the ICV infusion.

A tethering system essential for protection of the probe and movement of the animal in a test cage consists of a liquid swivel and a swivel mounting device. A serological 50 mL pipette is used to build a swivel-mounting device that centers the swivel on top of the test cage. A swivel is a water-tight device that rotates to keep the infusion lines from tangling. In our infusion system a lighter-weight swivel is used suitable for the weight and strength of a mouse. The infusion system consists of a Hamilton syringe, an air-tight connector, PE 20 tubing, a swivel, again PE 20 tubing and an injector cannula (Figure 12). It is assembled by attaching PE 20 size tubing to each sides of the swivel. On the animal end of the inflow line the PE 20 tubing connecting the swivel and the animal is cut to a length depending on the height of the test cage walls. If the tubing is oversized the animal is able to grab the line and bite through, if it is undersized the tubing is under tension and there is the potential risk of the guide cannula being displaced during infusion. An injector cannula is attached to the animal end of the tubing. After ensuring that the line is long enough to reach the pump, a connector is attached to the pump end of the inflow line so that there is an airtight connection between the PE 20 tubing and the syringe on the syringe pump driver.

Figure 12 Graphic depicting infusion system setup with syringe pump and test cage. Note swivel and leaver arm assembly permitting free movement of unrestrained and conscious mouse during infusion.



To prepare the infusion system for chronic infusions the PE 20 tubing is connected to a 10 mL syringe filled with 100% ethanol and flushed. The syringe is then replaced by another 10 mL syringe filled with sterile distilled water and flushed again. The syringe is refilled with sterile distilled water and used to slowly fill the infusion system with water leaving the 10 mL syringe connected to the PE 20 catheter. It must be ensured that the system is not leaking and that no air bubbles are trapped in the system. The 50 μL Hamilton syringes are filled with sterile distilled water without trapping any air inside. The 10 mL syringe is now carefully removed from the PE 20 catheter and replaced by the water filled Hamilton syringe. The Hamilton syringe is completely emptied then the plunger is gently pulled to trap a small air bubble in the injector cannula. The air bubble serves to separate the distilled water from the insulin or control solution. The injector cannula is immersed in the solution and 50 μL infusion solution is drawn into the chronic infusion system by slowly pulling the plunger of the Hamilton syringe as far as possible. The Hamilton syringe is mounted to the syringe pump and the system checked for free flow using a low infusion rate (e.g. 1 μL per hour). The infusion device is labeled according to the solution it contains.

After 4-5 days recovery from surgery the mouse is weighed to check for potential loss of bodyweight and to inject the animal with the correct amount of LPS. A loss of more than 10% bodyweight indicates prolonged recovery from the stereotaxic surgery and when this is detected the mouse is sacrificed or used for other experiments. If the animal can be used, hold the mouse restrained in one hand and inject LPS, IP. The dummy cannula is carefully removed from the guide cannula and the injector cannula is inserted using tweezers. After the injector cannula is inserted one drop of instant adhesive is applied to make sure that it stays attached to the guide cannula during the infusion period. Once connected, the animal is placed in the circular testing cage, the swivel-mounting device is attached to the cage and the syringe pump is started. A 1 μL bolus is applied initially to overcome resistance of the system and the pump is then set to the desired infusion rate (e.g. 2 μL per hour). Up to twelve animals are infused simultaneously using two syringe pumps connected to twelve chronic infusion systems.

Materials:	*Tweezers* *CMA 100 Microinfusion pump, Carnegie Medicine* *Glass gas-tight 50 μL micro syringes, Hamilton* *10 mL syringes* *1 mL syringes with 20-G needles* *microcentrifuge tubes* *454 Prism Instant Adhesive, Loctite* *Chronic infusion system:* *Circular testing cages (diameter 28 cm; height 30 cm)* *25-G Single-channel swivel,375/25PS, Instech Laboratories, Inc.* *Standard PE 20 catheter tubing, Harvard Apparatus* *33-G injector cannula, 0.5 mm projection, Plastics one* *Connector* *50 mL pipette*
Reagents and solutions	*Ethanol 100 %* *Sterile distilled water* *Insulin 100 mU/μL (Humulin R), Eli Lilly and Co* *dilute 1:10.000 with ACSF and infuse at 2 μL/h (0.02 mU Insulin per hour)* *ACSF, microfiltered and sterile, Harvard Apparatus* *150 mM Sodium* *3 mM Potassium* *1.4 mM Calcium* *0.8 mM Magnesium* *1.0 mM Phosphorus* *155 mM Chloride*

4.9 Statistics

All data are presented as mean ± SEM. Comparisons among groups are made using one-way ANOVA followed by unpaired two-tailed Student's t tests if not otherwise indicated. Differences are considered statistically significant at $p < 0.05$

5 Results

5.1 Central insulin improves survival of mice challenged with a lethal LPS dose

The aim of the pilot experiment was to determine the lethal dose of LPS in a model of HFD induced insulin resistance. Two groups of animals (n = 9) fed either a HFD to induce insulin resistance or RCD were injected with varying doses of LPS (3, 5, 10 mg/kg bodyweight) and overall survival in each group was assessed after 5 days. As previously described (Huang *et al.*, 2007) we found that HFD increases sensitivity of mice to LPS. The dose of 5 mg/kg bodyweight caused death within five days in more than 80% of the HFD fed animals but not in the RCD fed animals (Figure 13). The LPS dose of 5 mg/kg was therefore selected as the lethal dose (LD) in further experiments.

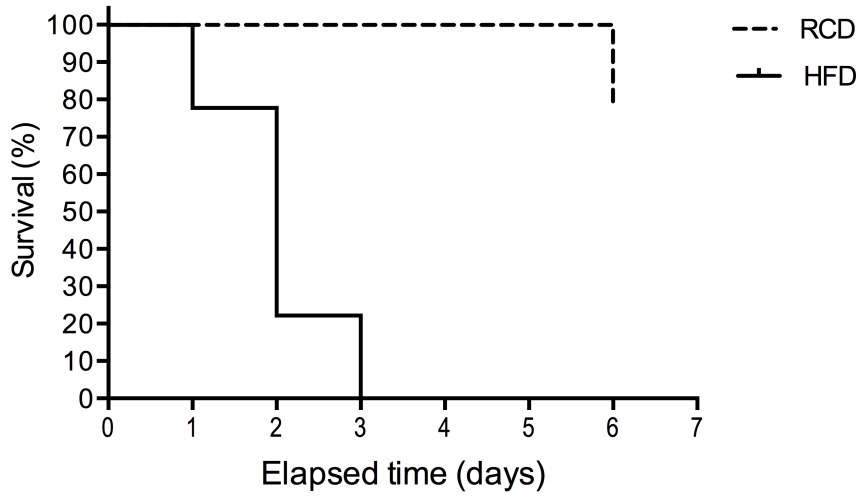


Figure 13: HFD increases sensitivity of mice to LPS. C57BL/6 mice, 4 weeks of age were fed either HFD to induce insulin resistance or RCD for 4 weeks. At 8 weeks of age the mice were injected with LPS (5 mg/kg, IP) and survival in each group was assessed daily over the following 6 days. *n = 9* per group. *p<0.0001*

To asses whether central insulin improves survival in a model of critical illness, 8-week-old male C57BL/6 mice, fed with a HFD for 35 days, were challenged with a lethal LPS dose and randomly assigned to receive a continuous ICV infusion with insulin or ACSF over a period of five days. Blood was taken from the tail vein at several time points (2 h, 6 h and 12 h) and each animal also received an IP injection of 300 μl sterile saline solution to prevent dehydration. In the present study, significant differences in mortality were determined between the control and ICV insulin-treated group. As depicted in Figure 14, ICV infusion of insulin significantly improved five-day survival rate after LPS induced sepsis. A survival rate of 5% after five days of continuous infusion was found in the control animals. In comparison,

the survival rate of animals treated with central insulin was 52%, suggesting that central insulin significantly improved survival in this endotoxemic mouse model of critical illness.

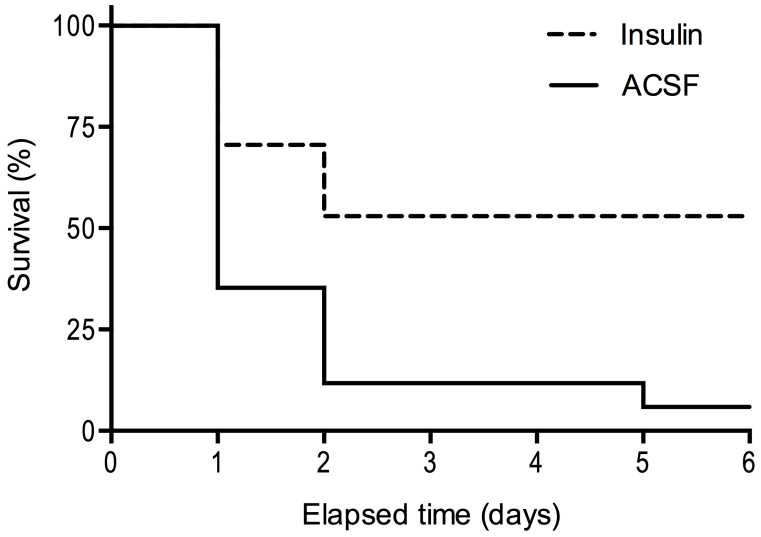
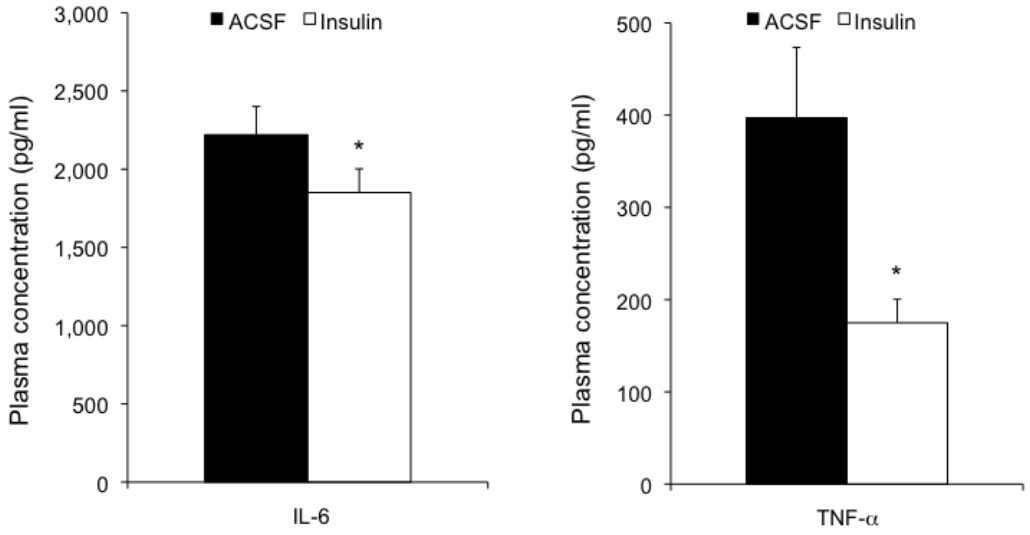


Figure 14: ICV insulin improves survival in a mouse model of sepsis. 4-week-old C57BL/6 mice were fed high-fat diet for 4 weeks and at the age of 8 weeks ICV cannulas were implanted. The mice were injected with LPS (5 mg/kg, IP) and randomly assigned to receive ICV insulin or vehicle infusions for the following 5 days. Survival was assessed daily. ICV insulin significantly improved survival compared to control group. *n = 17 per group. p = 0.0027*

5.2 Central insulin attenuates systemic inflammatory response

The effect of central insulin on systemic inflammatory response was assessed by measuring concentrations of key pro-inflammatory cytokines that mediate effects of LPS. Plasma TNF-α of mice challenged with LPS (1 mg/kg bodyweight) was significantly lower after 4 hours of ICV insulin treatment compared to controls receiving ICV infusion of ACSF. Moreover, IL-6, another important mediator of the inflammatory response to LPS, was also lowered by ICV insulin (Figure 15). The data obtained demonstrate that central insulin attenuates systemic inflammatory response of mice to endotoxemia by decreasing levels of key pro-inflammatory cytokines in the circulation.

Figure 15 ICV insulin treatment reduces circulating pro-inflammatory cytokine levels in mice injected with LPS. Eight-week-old C57BL/6 male mice injected with LPS (1mg/kg bodyweight, IP) received intracerebroventricular infusion of either central insulin (0.02mU/h) or ACSF for 4 hours and were subsequently sacrificed for blood collection as described. Serum concentrations of TNF-α and IL-6 were determined by ELISA. *n = 12-14 per group,*p < 0.05*



5.2.1 Central insulin reduces cytokine signal transduction in response to LPS

To investigate whether central insulin reduces cytokine signaling, we measured expression and phosphorylation of signal transducer and activator of transcription (Stat) in liver, spleen and lung tissue by western blot. The Stat signaling pathway is a primary signal transduction pathway used by type 1 cytokines and IFNs. After ICV insulin infusion, we observe a marked inhibition of LPS induced Stat1 phosphorylation in liver, spleen and lung tissue. Furthermore, Stat3 activation in liver and spleen tissue is significantly decreased. Lower levels of the pro-inflammatory cytokine IL-1β are detectable in liver, spleen and lung tissue of the ICV insulin infused animals.

Figure 16 ICV insulin treatment reduces LPS induced pro-inflammatory protein expression in spleen tissue. Quantification of western blot analyses from spleen tissue lysates expressed as fold change to vehicle (ACSF) and normalized over the housekeeping gene β-actin. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as a loading control. Activation of the transcription factors Stat1 and Stat3 is decreased. The pro-inflammatory markers IL-1β, Cyclooxygenase 2 (COX2) and I-kappa-B kinase subunit gamma (IKK-γ) are reduced in the animals infused centrally with insulin. *n = 6 per group ,*p < 0.05*

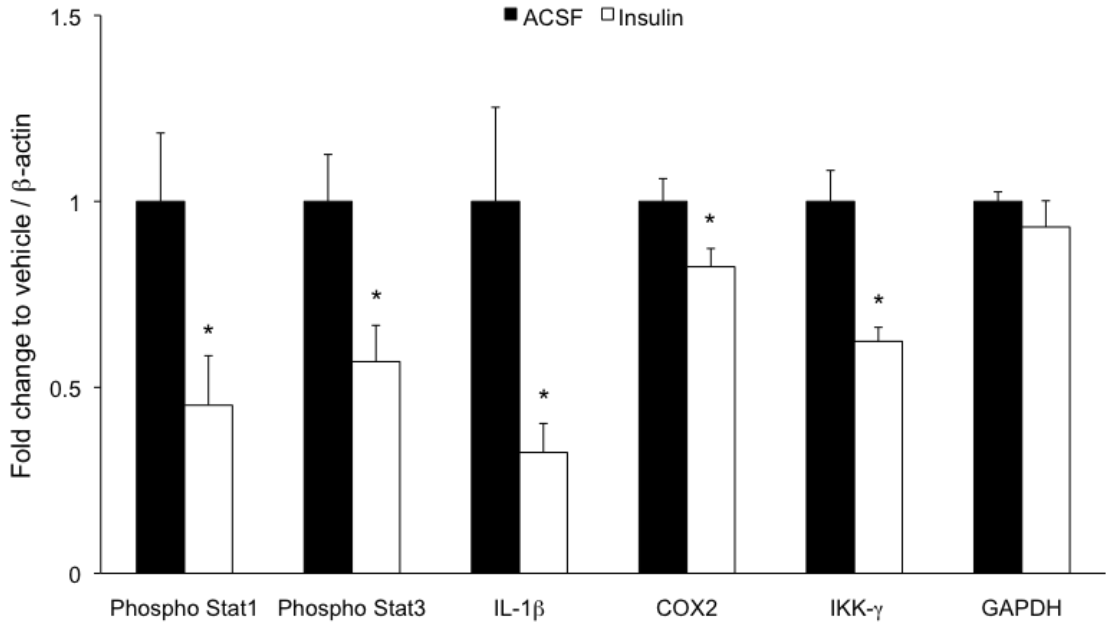


Figure 17 ICV insulin treatment reduces LPS induced pro-inflammatory proteins in liver tissue. Expression of the pro-inflammatory cytokine IL-1β and activation of the transcription factors Stat1 and Stat3 assessed by western blot are reduced by central insulin. Quantification of western blot analyses from liver tissue lysates expressed as fold change to vehicle (ACSF) and normalized over the housekeeping gene β-actin. GAPDH was used as loading control. *n = 6 per group, *p < 0.05*

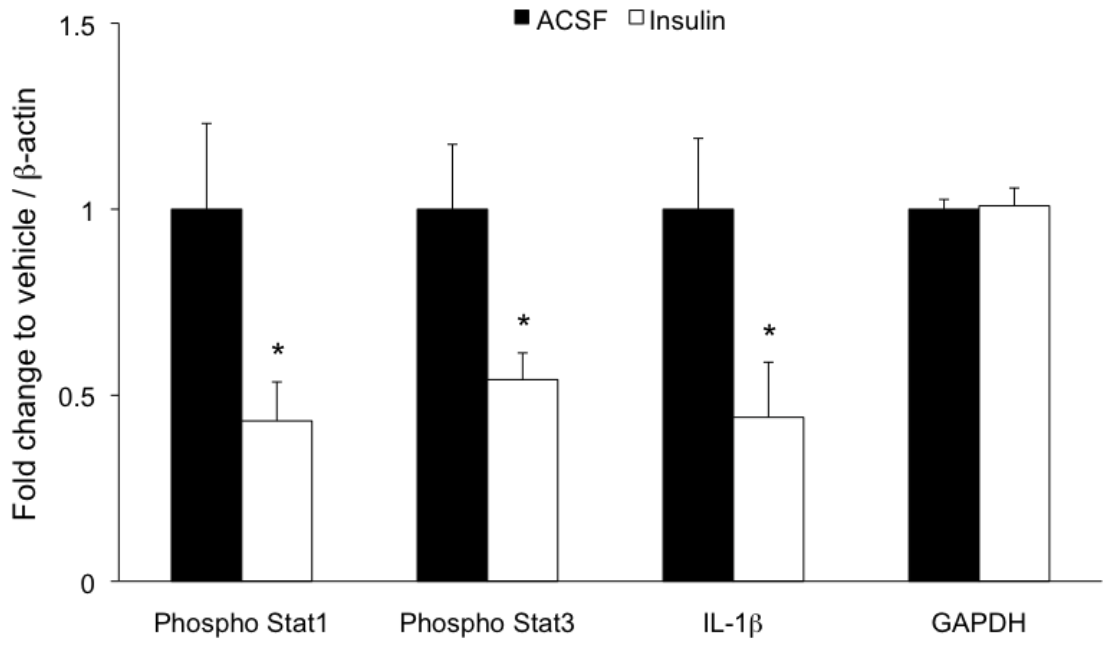
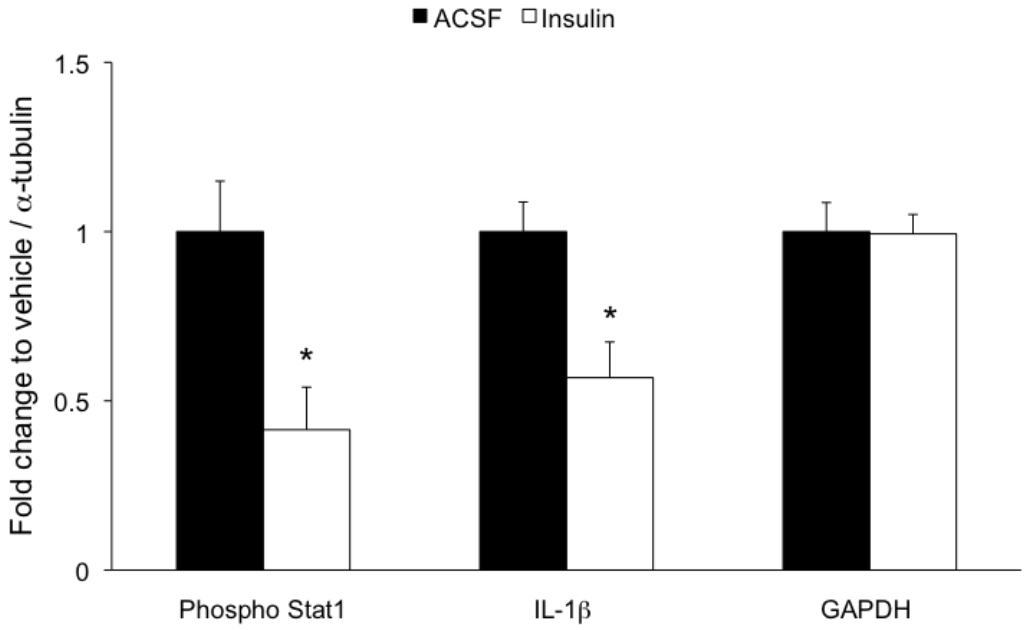


Figure 18 ICV insulin treatment reduces expression of LPS induced pro-inflammatory proteins in lung tissue. Western blot analyses of lung tissue lysates shows decreased Stat1 activation by central insulin. Lower levels of the pro-inflammatory cytokine IL-1β are detectable in the animals infused with ICV insulin. Quantification of western blot analyses from lung tissue lysates expressed as fold change to vehicle (ACSF) and normalized over the housekeeping gene α-tubulin. GAPDH was used as loading control. *n = 6 per group, *p < 0.05*

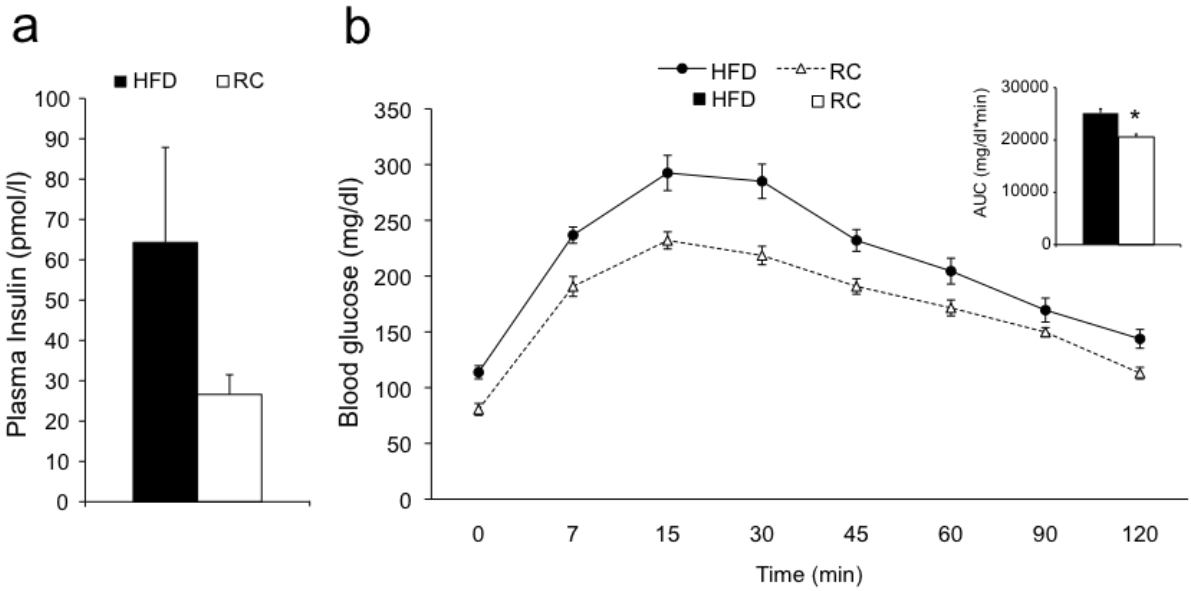


5.3 Effects of central insulin on metabolic parameters

5.3.1 Blood glucose levels

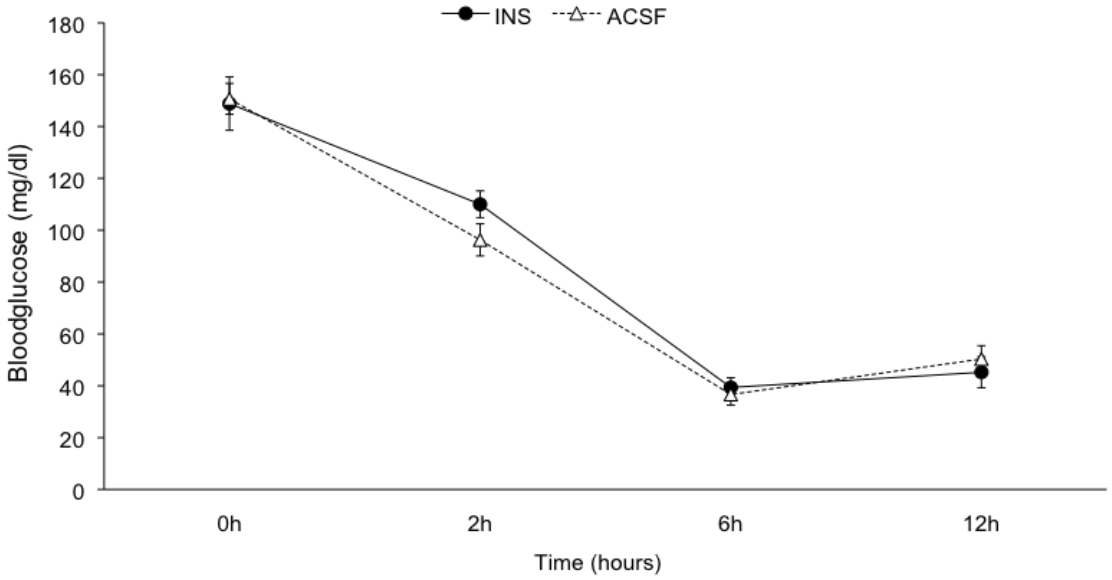
Male C57BL/6 mice were fed a HFD for 4 weeks. To evaluate the effect of HFD on glucose homeostasis in the fasting state, IPGTTs were performed as described earlier. Overnight-fasted mice exhibited impaired glucose tolerance, manifested by higher plasma glucose levels at 7, 15, 30, 45, 60, 90 and 120 min after glucose administration. After 16 hours fasting insulin levels were higher in the animals on a HFD.

Figure 19 (a) Basal insulin levels differed between high fat and regular chow fed mice. (b) IPGTT. After 4 weeks on the diet, 8-week-old male C57BL/6 mice were fasted overnight and basal insulin levels measured by ELISA. IGPTT showed a significant difference between high fat and regular chow fed mice. After 4 weeks on either RCD or HFD, 8-week-old male C57BL/6 mice were fasted overnight, basal blood glucose was measured (t = 0 min) before IP administration of 1 g/kg glucose. Blood glucose was measured with a glucometer. Data points represent the mean ± SE. Estimation and comparison of variance for AUCs created by HFD fed and control mice indicated a significant difference between the two curves.**p<0.05, n = 6-8 per group*.



Among the various responses elicited by administration of LPS in vivo is modulation of glucose metabolism, which results in dose-dependent hypoglycemia in mice. Additionally, pro-inflammatory cytokines like IL-1 and TNF-α have been demonstrated to induce hypoglycemia when administered to mice in vivo. After LPS (5 mg/kg, IP) injection and ICV infusion of insulin or ACSF, blood glucose levels fell significantly in both ICV treatment groups over the following 12 hours. There was no difference in blood glucose between the insulin and ACSF infused animals after LPS administration (Figure 20).

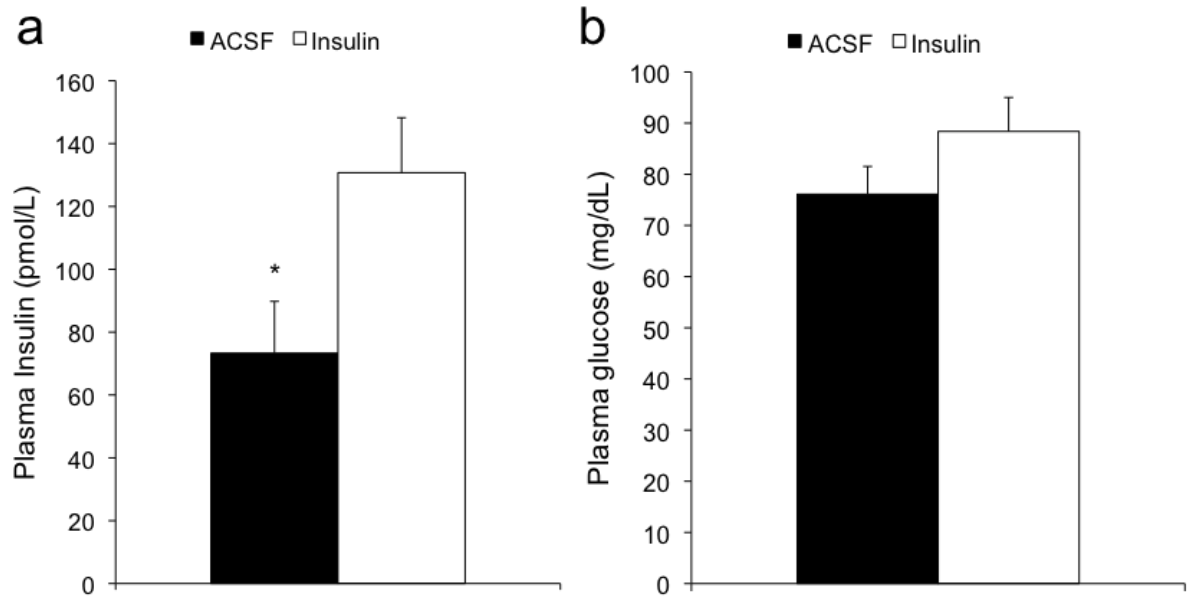
Figure 20 ICV insulin has no effect on blood glucose levels in a mouse model of sepsis. 4-week-old C57BL/6 mice were fed high-fat diet for 4 weeks and ICV cannulas implanted. The mice were injected with LPS (5 mg/kg, IP) and randomly assigned to receive ICV insulin or vehicle infusions over the following 12 hours. Glucose was measured with a glucometer at the different time points depicted (*n = 11 per group).*



5.3.2 Peripheral insulin levels

After LPS administration followed by four hours of either ICV insulin or ACSF infusion, we checked peripheral insulin levels. In our ICV infusion experiment we use a premade insulin solution with an insulin concentration of 100 mU/μL and dilute the solution 1:10.000 with ACSF. The final concentration of the infused insulin-solution is 0.01 mU/μL. A syringe pump is set at an infusion rate of 2 μL/h and runs for four hours, so that each animal receives a total dose 0.08 mU insulin (equivalent to 4 mU/kg bodyweight). After four hours of ICV insulin infusion, to our surprise, we found a significant difference in peripheral insulin levels between the insulin and ACSF infused animals (Figure 21a), but at the same time no difference in peripheral glycemia, thus confirming our previously obtained results (Figure 20 and 21b).

Figure 21 (a**)** ICV insulin treatment increases plasma insulin concentration. Male C57BL/6 mice are injected with LPS (1 mg/kg, IP), infused with central insulin (0.02 mU/h) or vehicle for 4 hours and sacrificed. Plasma concentrations of insulin are determined by ELISA (b) ICV insulin does not change plasma glucose levels significantly as measured with glucometer (*n = 12-14 per group,*p < 0.05).*



6 Discussion

In this work we investigated whether centrally administered insulin regulates systemic inflammation and improves survival in a septic mouse model of critical illness and impaired metabolic control. We show here for the first time that brain insulin regulates peripheral immune functions, resulting in decreased mortality in an animal model of critical illness and impaired metabolic control. This could be of great translational interest, as it has been shown recently that systemic insulin treatment decreases morbidity and mortality in critically ill patients, even though the mechanism of the beneficial effects seen yet remains unexplained. In a model of high-fat diet induced insulin-resistance combined with critical illness, we have also gained further insight into the connection between impaired metabolic control and inflammation. We have confirmed that peripheral insulin-resistance results in higher susceptibility of mice to lipopolysaccharide induced mortality. Our results demonstrate that central infusion of insulin partly restores immune functions making the treated mice significantly more resistant to the devastating effects of lipopolysaccharide. Although these effects are significant and clearly measurable in survival studies, we are unable to fully explain the mechanism involved. It is clear that centrally administered insulin acts like an anti-inflammatory agent, suppressing major pro-inflammatory cytokines in the circulation and regulating molecules that participate in cytokine signaling in several immunologic organs.

Our results are of particular interest in light of the fact that although extensive research efforts have focused on strategies to prevent adverse outcome in critically ill patients, many results have been less encouraging and some of the mechanisms remain unexplained (Marshall, 2003). Recent data suggest, however, that strict glycemic control with insulin may restore the balance between pro- and anti-inflammatory mediators and improve outcome in critically ill patients (Marik *et al.*, 2004). A prospective, randomized, controlled landmark study by Van den Berghe demonstrated that IIT reduced morbidity and mortality in surgical ICU patients (van den Berghe *et al.*, 2001). The authors suggest that metabolic control as reflected by normoglycemia, rather than the infused insulin dose, correlates with the beneficial effects of intensive treatment with insulin (Van den Berghe *et al.*, 2003). Further studies revealed that maintaining normoglycemia with IIT during critical illness protects the endothelium and exerts anti-inflammatory effects (Langouche *et al.*, 2005).

There is also evidence to suggest that insulin treatment itself, rather than maintaining normoglycemia, decreases morbidity and mortality in the context of critical illness. Analysis of 451 patients who received prolonged IIT revealed changes in serum levels of acute phase proteins, such as CRP and mannose-binding lectin (MBL) (Hansen *et al.*, 2003). Multivariate

logistic regression analysis, corrected for other determinants of outcome, demonstrates a direct anti-inflammatory action of insulin as indicated by suppressed serum levels of CRP, which would largely explain the beneficial effects of IIT on organ failure and mortality. Insulin treatment in diabetic patients, but not improved glycemic control per se, has been shown to reduce circulating CRP (Yudkin *et al.*, 2000). Endothelial function is, however, highly variable and as the authors state in their references acute hyperglycemia attenuates vasodilatation (Williams *et al.*, 1998). Application of insulin once or twice daily may have caused fluctuating concentrations of glucose and therefore provoked changes in endothelial function or endothelial markers that could have masked the author's findings. Another limitation to the study is that the authors have not stated how the possible existence of mild inflammation or infection has been excluded. The large variation in CRP values of patients included in the study suggests a possible impact on the concentration of other markers of inflammation. Overall, it remains unclear whether lowered blood glucose is the primary effect and responsible for improved patient outcome or if a glycaemia-independent role of insulin contributes to the observed improvement in morbidity and mortality of critically ill patients.

Regardless of the mechanism behind improved outcome of critically ill patients treated with IIT, recent data have shown that IIT leads to an increased risk of serious adverse events related to hypoglycemia, unfortunately counteracting the beneficial effects of this promising new therapeutic approach. Possible connections between hypoglycemic episodes and increased risk of death or complications in critical illness are well documented (Lacherade *et al.*, 2009). A possible explanation could be that mechanisms that usually counteract the physiological response to hypoglycemia are impaired in critically ill patients. For instance, the expected increased release of counterregulatory hormones such as glucagon, epinephrine and cortisol when blood glucose drops beneath the physiological range may be impaired (Taborsky *et al.*, 2012). In a state of critical illness, levels of these hormones are typically already elevated thus preventing an adequate physiological response. A large, multicenter, randomized, controlled trial was carried out to confirm the results of the landmark study. This trial had to be terminated prematurely, however, due to high incidence of severe hypoglycemia (Brunkhorst *et al.*, 2008). In addition, a meta analysis of fourteen trials has shown that IIT leads to a six-fold increase in the risk of severe hypoglycemic events (Griesdale *et al.*, 2009). It has also been demonstrated that severe hypoglycemia is related to ICU mortality, suggesting the possibility of a causal relationship (Krinsley *et al.*, 2007). A recently published observational cohort study was carried out to determine whether mild or moderate hypoglycemia in critically ill patients is independently associated with an increased risk of death. To this end, the relationship between milder hypoglycemia and mortality was investigated in a population of 4946 patients with glycemic targets of 108 to 180 mg/dL admitted to two ICUs. It was shown that even mild hypoglycemia (defined as blood glucose <

72 mg/dL) was associated with an increased risk of death in critically ill patients. (Egi *et al.*, 2010). These findings have since been confirmed in an even larger international cohort of critically ill patients, suggesting that efforts to reduce hypoglycemia in critically ill patients may also reduce mortality. (Krinsley *et al.*, 2011)

Many studies have investigated a possible increased risk of morbidity and mortality in T2DM patients in the ICU setting and there is evidence that treating hyperglycemia in critically ill patients with T2DM improves outcome. (Malmberg, 1997) (Slynkova *et al.*, 2006). Abnormal inflammatory responses in T2DM might contribute both to an increased susceptibility to infection and also the severity of infections in diabetic patients. Many authors have shown that infection is more serious, and possibly more difficult to control, in diabetic subjects (Geerlings *et al.*, 1999) (Alba-Loureiro *et al.*, 2007) (Stoeckle *et al.*, 2008). The literature is, however, currently inconsistent on this topic with some data showing neutral or even decreased risk for patients with pre existing T2DM admitted to the ICU. Recent studies of two large mixed ICU populations (Graham *et al., 2010*) and - more specifically - sepsis patients (Stegenga *et al., 2010*) found no differences in mortality when diabetic patients were compared with non-diabetic patients, despite a higher morbidity in the former group. Thus the role of IIT in critically ill patients with T2DM is unclear and requires further research.

To address the question as to whether central insulin not only improves outcome in critical illness but is also beneficial in an insulin-resistant state, i.e. to mimic ICU patients with T2DM, we developed an endotoxemic mouse model combined with overfeeding-induced insulin resistance. Data presented here show that ICV insulin infusion significantly improves survival in insulin-resistant mice suffering from a lethal LPS dose. Moreover, in animals administered sublethal doses of LPS to mimic the systemic inflammatory response of Gram-negative sepsis, central insulin considerably suppresses systemic levels of two major pro-inflammatory cytokines, IL-6 and TNF-α, and also down-regulates expression of the proteins Stat1, Stat3, which are important for cytokine signaling.

A fundamental feature of the innate immune system is the production and release of cytokines. Cytokines are low molecular weight glycoproteins mainly produced by the cells of the immune system and involved in the regulation of immune system response and intercellular signaling. Cytokines act via paracrine and autocrine routes and, after binding to specific receptors, affect immune cell differentiation, proliferation and activity. Absolute cytokine production is usually very low thus limiting the effects in local tissue. With persistent activation of immune cells as seen in severe bacterial infections, however, excessive production of cytokines and overspill into the systemic circulation occurs resulting in widespread activation of inflammatory cascades, provoking a systemic inflammatory response syndrome (SIRS). This evokes further activation via inflammatory pathways

resulting in a downward spiral of organ dysfunction and ultimately leads to the multiple organ dysfunction syndrome (MODS) responsible for the high mortality rates in critically ill patients. The role of cytokines in SIRS and MODS was originally demonstrated in animal models, where injection of TNF-α (then known as cachectin) induced a syndrome clinically identical to shock (Tracey *et al.*, 1986). The study of individual cytokines, however, is largely academic.

The clinical significance of elevated cytokine levels in sepsis has been studied intensively and although many cytokines act together as part of a complex network that is not yet fully understood, there is substantial evidence that TNF-α, IL-1β and IL-6 in particular are strongly implicated as mediators of sepsis. Raised levels of pro-inflammatory cytokines appear generally to correlate with severity of illness and outcome. Elevated IL-6 is associated with non-survival in intra-abdominal and Gram-negative bacterial sepsis (Wakefield *et al.*, 1998) (Calandra *et al.*, 1991). Levels of IL-6 correlate with levels of TNF-α and patient outcome. TNF-α levels are higher in septic shock than in severe sepsis (Damas *et al.*, 1992) and soluble tumor necrosis factor receptor (TNF-sR), a natural antagonist of TNF-α, is also associated with non-survival if present at higher concentrations. Data presented here show that infusion of insulin into the third ventricle of insulin-resistant mice suffering from a sublethal dose of LPS (to mimic the SIRS of Gram-negative sepsis) significantly suppresses circulating levels of the pro-inflammatory cytokines IL-6 and TNF-α. Since raised levels of pro-inflammatory cytokines, in particular circulating levels of IL-6, generally appear to correlate with severity of illness and outcome, decreasing plasma IL-6 and TNF-α levels might be a potential mechanism by which central insulin signaling can significantly improve survival of endotoxemic mice as described in our second experiment. Further support for this concept is obtained from other promising research studies in which attenuating inflammatory response with anti-inflammatory agents or antibodies against pro-inflammatory cytokines have increased survival rates in animal septicemia models (Tracey *et al.*, 1987) (Jeschke *et al.*, 2004).

Stat proteins are transcription factors mediating cytokine signaling. Cytokines bind to homo- or hetero-dimeric receptors, which bind janus kinase (JAK) proteins. Activated JAKs phosphorylate cytokine receptors, allowing Stats to bind via SH2-phosphotyrosine interactions. Stats in turn are phosphorylated and following dimerization activated Stats are then transported into the nucleus. Inside the nucleus the activated Stat dimers bind to cytokine-inducible promoter regions of the DNA and regulate gene expression.

To determine whether central insulin exerts its effects on cytokines directly by modulating signal transcription factors, we measured expression and phosphorylation of hepatic, splenic and lung signal transcription factors by western blot. Our results show that ICV insulin affects

the intracellular signal cascade in the liver, spleen and the lung in a model of systemic inflammation caused by endotoxin.

Stat1 is a central mediator of inflammatory response. Stat1 is mainly activated by IFNs and acts as a pro-inflammatory factor by inducing expression of genes that contribute to the activation and recruitment of immune cells to inflammatory sites. IFN-γ through Stat1 leads to expression of pro-inflammatory proteins such as intercellular adhesion molecule (ICAM), important for leukocyte adhesion on endothelial cells (Dustin *et al.*, 1988) and chemokine (C-X-C motif) ligand 10 (CXCL10) which acts as a chemoatractant for T-cells (Strieter *et al.*, 1995). Stat1 also induces pro-inflammatory mediators, such as inducible nitric oxide synthases (iNOS) and COX2 in macrophages (Jacobs *et al.*, 2001). Activation of STAT1 through type I and II IFNs promotes antigen presentation by increasing expression of major histocompatibility complex (MHC) class II (Lee *et al.*, 1996) and enhancing antigen processing (Marques *et al.*, 2004). Stat1 mediates IFN-gamma signaling. In a model of endotoxin-induced sepsis, IFNγR-/- mice and IFN-/- mice were resistant to lethality compared with wild-type (WT) mice, a result that was associated with reduced production of TNF-α (Car *et al.*, 1994). Since IFN is mediated by Stat1, it is reasonable to speculate that Stat1 plays a role in endotoxic shock. At the same time, blockade of the Stat1 pathway at the right time and at the appropriate point prevented the lethal effects of endotoxin in a mouse sepsis model. (Kamezaki *et al.*, 2004).

There is evidence that Stat3 is a transcription factor that mediates anti-inflammatory cytokine signaling. It has been shown that Stat3 deficiency in macrophages and neutrophils fails to respond to the anti-inflammatory cytokine IL-10 and high levels of TNF-α are secreted after stimulation with LPS and IL-10, suggesting that Stat3 expressed in macrophages and neutrophils may negatively regulate the inflammatory response (Riley *et al.*, 1999). Moreover, conditional Stat3 knockout mice were highly susceptible to endotoxemia due to increased levels of pro-inflammatory cytokines and succumbed to septic peritonitis induced by cecal ligation and puncture (CLP) (Matsukawa *et al.*, 2003). There is also evidence that Stat3 appears to be a key transcription factor in inflammation activated by IL-6 (Niehof *et al.*, 2001) and it is possible that insulin exerts its anti-inflammatory effects on systemic inflammation through down-regulation of Stat3 and Stat5 (Jeschke *et al.*, 2004). IL-6 and related pro-inflammatory cytokines signal through phosphorylation of the transcription factor Stat3 (Zhong *et al.*, 1994). A recent study investigated the potential role of JAK2 and Stat3 in regulation of the systemic inflammatory response during sepsis. This in vivo study used a CLP model to induce sepsis in rats, with subsequent inhibition of JAK2 and Stat3 by AG490 or rapamycin. The results showed that inhibition of JAK2/Stat3, seen by marked down-regulation of phosphorylation of Stat3 and JAK2, ameliorates multiple organ damage and improves outcome in CLP-induced sepsis, suggesting that JAK2/Stat3 is involved in

regulation of the inflammatory response in the septic setting. The underlying mechanisms of JAK2/Stat3-regulation of organ damage and lethality in sepsis still require explanation (Hui *et al.*, 2009).

In the context of sepsis, excessive systemic inflammation is fatal to the host but this can be weakened by deactivating pro-inflammatory signal transcription factors Stats. We show here that central insulin down-regulates Stat1 and Stat3 activation in several reticuloendothelial organs. Augmenting the innate immune response by controlling JAK/Stat pathways throughout the body might therefore be one of the mechanisms that could explain how central insulin considerably improves prognosis in patients with severe sepsis.

Strategies in treatment of sepsis and septic shock focus on a multimodal approach and involve early resuscitation, appropriate antimicrobial medication, aggressive debridement of necrotic tissue and supportive care in case of organ dysfunction. The multiple mediators and pathways involved in the inflammatory response to sepsis have lead to studies of numerous therapeutic approaches in this pathology. Some approaches include antagonizing individual pro-inflammatory cytokines such as IL-1 or TNF-α by targeting them with monoclonal antibodies or, in case of IL-1, by using a recombinant IL-1 receptor antagonist. These interventions, despite showing promising results in animal models, were unfortunately not effective in human studies. (Fisher *et al.*, 1994; Reinhart *et al.*, 2001). One problem with targeting individual cytokines might be that cytokines and inflammatory mediators are players in a complex and delicately balanced, network. Most cytokines exert both beneficial and deleterious effects. TNF-α, for example, promotes endothelial migration of neutrophils to sites of inflammation and infection and at the same time stimulates superoxide production in neutrophils, which is important for killing bacteria (Lukacs *et al.*, 1995) (Moore *et al.*, 1991). Patients go through many different stages in the development of sepsis during which either pro- or anti-inflammatory effects of one cytokine may be dominant and needed to ensure effective immune response. Therapies directed at an individual cytokine may cause imbalance of this delicate network and could therefore prove beneficial in some septic patients but deleterious in others.

The only established agents used in routine clinical practice for the treatment of patients with sepsis are anti-coagulants such as activated protein C and immune modulating agents such as low-dose steroids. Low-dose hydrocortisone has been shown to be beneficial in patients with sepsis (Bernard *et al.*, 2001) (Keh *et al.*, 2003) (Oppert *et al.*, 2005) Treatment with low-dose hydrocortisone accelerates the reversal of shock and exhibits immune modulatory effects. In contrast to blocking a single cytokine, as with immune modulators which have proved unsuccessful in tests, low-dose steroids reduce levels of a broad spectrum of pro-inflammatory cytokines. This could well be the reason for the promising results obtained with

this therapeutic approach, now established in clinical practice for the treatment of septic patients.

Similar to the effects of low-dose steroids, it has been demonstrated in our work that central insulin also exerts a broad spectrum of anti-inflammatory effects throughout the body's periphery. Central insulin considerably suppresses systemic levels of important pro-inflammatory cytokines and at the same time down-regulates expression of the signaling molecules that are decisive for the inflammatory response. Due to the similarities observed between low-dose steroids and central insulin with regard to their broad range of anti-inflammatory effects, one might speculate that treatment with central insulin could represent a new therapeutic approach to this devastating disease.

Brain insulin concentrations in different species, including humans, are normally finely tuned to circulating concentrations of insulin (Wallum *et al.*, 1987). Obese humans show elevated plasma insulin levels as a consequence of peripheral insulin resistance. However, a corresponding increase of insulin levels in the CSF as described in healthy subjects is not detectable, resulting in a decreased CSF to plasma insulin ratio (Kern *et al.*, 2006). Similar observations have been made in animals with diet-induced (Kaiyala *et al.*, 2000) and genetic obesity (Stein *et al.*, 1987). These findings support the notion that there is reduced transport of insulin across the BBB in states of peripheral insulin resistance. Since peripheral insulin resistance not only occurs in obesity but also during critical illness ('diabetes of injury') impaired BBB transport of insulin resulting in decreased central insulin signaling in the brain might also affect critically ill patients. With IV administration of insulin to critically ill patients, as in IIT, central anti-inflammatory effects of insulin as described here, might be the principal mediator of the inflammation reduction and explain improved outcome in this patient group. It can also be speculated that insulin resistance at the BBB and subsequent lower CSF insulin concentrations might attenuate the anti-inflammatory effects of central insulin, which could explain why patients with T2DM do not seem to benefit from IIT during ICU treatment (Van den Berghe *et al.*, 2006). One explanation could be that pre-existing diabetes potentiates 'diabetes of injury' in the context of critical illness thus further impairs the BBB transport of IV administered insulin. This would result in reduced central insulin signaling in the brain, ultimately attenuating the anti-inflammatory properties of central insulin.

A number of methods to overcome central nervous insulin resistance have been tested in basic research and results are promising. Intranasal administration of insulin to target brain functions might be of particular therapeutic relevance because it bypasses the BBB and increases central nervous availability of the hormone. For example, insulin was detectable in the human CSF just 10 minutes after intranasal administration (Born *et al.*, 2002). Intranasal administration of insulin acutely improved memory functions in adults with Alzheimer's

disease (Born *et al.*, 2002) and reduced HPA axis secretory activity (Bohringer *et al.*, 2008) potentially offering a therapeutic benefit in declarative memory function or preventing hyperactivity of the HPA system. In obese men intranasal insulin had no influence on bodyweight (Hallschmid *et al.*, 2008), but similar to the effects seen in men of normal weight, declarative memory and mood were improved and HPA activity was reduced. Accordingly, the catabolic impact of ICV insulin in animals with diet-induced obesity is reduced (Posey *et al.*, 2009). This pattern suggests that in an insulin-resistant state, brain structures involved in energy homeostasis show decreased sensitivity to the effects of insulin, whereas other functions of central insulin signaling remain unaffected. Given that intranasal insulin influences memory functions and HPA axis activity in subjects with peripheral insulin resistance, it might also modulate the inflammatory response during critical illness (also a state of insulin resistance) by suppressing pro-inflammatory pathways as described in this thesis. Systemic administration of insulin increases the incidence of hypoglycemic episodes, counteracting the beneficial effects of IIT in critically ill patients. Administering insulin intranasally has two major advantages over the current IIT protocols. First, reduced uptake of insulin across the BBB in insulin-resistant states such as critical illness (‘diabetes of injury’) would be resolved, allowing increased insulin signaling in the brain in the absence of systemic insulin effects. Second, as described in this thesis, stimulation of brain insulin signaling modulates the inflammatory response and improves survival in an animal model of critical illness. In summary, isolated stimulation of brain insulin signaling without raising systemic insulin levels has broad anti-inflammatory properties and may represent a treatment for critically ill patients without the risk of hypoglycemia. Changing the route of insulin administration might not only reduce the risk of hypoglycemia for all patients but also increase the beneficial effect for diabetic or elderly patients who do not benefit - or are even harmed by - conventional IIT.

7 Limitations

7.1 Animal model of sepsis

One problem inherent in sepsis research in animal models is to faithfully reproduce the complex pathophysiological and biochemical changes that are hallmarks of different stages of sepsis in humans. Endotoxicosis models are well established and widely used because of their many advantages. LPS is a stable, relatively pure compound that is also convenient to use thus simplifying some aspects of experimental design. There is accumulating evidence that LPS is pathophysiologically important in humans. It has been shown, for example, that injections of low-dose endotoxin in human volunteers mimic the pathophysiologic changes seen in sepsis patients. In healthy subjects, very low doses of LPS (4 ng/kg) induce hemodynamic, hematologic and also some metabolic changes similar to those observed in septic patients; these include fever, tachycardia, elevated cardiac output, hypotension, decreased systemic vascular resistance and leukocytosis (Revhaug *et al.*, 1988), (Suffredini *et al.*, 1989). Despite the similarities observed, there are overt differences between chemically-induced septic shock in animals and clinical disease in humans. Two major drawbacks are the time-scale of disease development and the lack of supportive therapeutic interventions. The onset and progression of sepsis from SIRS to MODS occurs in hours to days in animal models, whereas in human patients this progression occurs over days to weeks. Moreover, human patients are promptly treated with various standard therapies such as intubation and mechanical ventilation, fluid replacement, antibiotic and vasopressor therapy and nutritional support. The questionable clinical relevance of the LPS model can also be derived from other observed differences in the effects of endotoxin seen in animals and humans. Bolus injections of LPS in animals cause low cardiac output and elevated or normal systemic vascular resistance (Brackett *et al.*, 1985), whereas the hemodynamic changes observed in human sepsis, termed ‘hyperdynamic’, are just the opposite, with cardiac output being typically elevated and systemic vascular resistance generally abnormally low (Abraham *et al.*, 1983) (Ahmed *et al.*, 1991). A major problem inherent in our study is thus the opposing effects of critical illness on energy metabolism in animals and humans. As described above, following LPS administration gluconeogenesis is suppressed and hypoglycemia occurs in the experimental sepsis induced in animals (Copeland *et al.*, 2005). In line with these findings, we observed a marked decrease of blood glucose levels after IP administration of LPS to mice, although there was no difference in blood glucose concentrations detectable in animals receiving central insulin infusion and controls (Figure

19). Unlike the animal model, critical illness in humans causes hyperglycemia which is thought to result from a combination of increased hepatic glucose production and decreased peripheral glucose utilization (Marik *et al.*, 2004). A solution to this problem could be a different animal sepsis model which more accurately mimics the disease in humans, e.g. the CLP model. First described by Wichterman et al. (Wichterman *et al.*, 1980) sepsis is induced by a surgical procedure involving laparotomy, exteriorization of the cecum, ligation of the cecum distal to the ileocecal valve and puncture of the ligated cecum. This results in disruption of the protective barriers that normally shield sterile compartments from pathogens, allowing leakage of fecal contents into the peritoneum. Although this model recreates the hemodynamic and metabolic phases of human sepsis more accurately than our LPS model, there are also major drawbacks. Variability within the CLP model itself and the magnitude of the host response to CLP make it difficult to compare experimental results between medium to small laboratory groups. For example, two studies evaluating the role of IL-10 in sepsis used the same mice and double punctured CLP with a 20- and 22-gauge needle. (van der Poll *et al.*, 1995) (Oberholzer *et al.*, 2002). Contrary to the well known positive correlation of needle size and mortality in the CLP model, the results showed that survival was significantly greater in the group double-punctured with the larger 20-gauge needle. Moreover, the amount of fecal material that leaks from the puncture is difficult to control and this can lead to variability in the severity of the disease. Another argument against use of the CPL model for our studies is that it involves major surgery and is therefore much more time consuming than an IP injection of LPS. This would have made our experimental protocol much longer and technically more difficult, at the price of lower experimental turnover and decreased sample size. Moreover, the two major obstacles of other animal models of sepsis, time-scale of disease development and lack of supportive therapeutic interventions, are also inherent in the CPL model.

In summary, the well-established LPS model has contributed significantly to our understanding of the host response to infection and the role of inflammatory mediators in the pathophysiology of sepsis. It is a good compromise between commitment of resources and accuracy in mimicking the clinical features of sepsis. However, given the lack of supportive interventions and the fact that sepsis models in animals often do not closely mimic the pathophysiological changes of human disease, caution must be exercised before efficacy results from animal studies are extrapolated to human disease. Particularly the fact that in our animal model of sepsis mice became hypoglycemic, whereas humans present with hyperglycemia during critical illness, limits justifiable extrapolation of our results to human disease.

7.2 Peripheral insulin levels

Insulin released by pancreatic β-cells into the circulation is a major regulator of carbohydrate and lipid metabolism. Insulin target tissues, such as skeletal and cardiac muscle, white adipose tissue and liver tissue, show significantly enhanced glucose uptake when stimulated by insulin. Furthermore, liver and muscle tissue store glucose in the form of glycogen. Apart from these well-known effects, it has become evident during the last decades that circulating insulin also has unexpected and novel non-metabolic effects. One of the non-metabolic actions of peripheral insulin is the direct regulation of immune functions via different mechanisms. Insulin administered peripherally affects the immune system in various ways: it induces hemodynamic changes via nitric oxide-dependent vasodilatation (Steinberg *et al.*, 1994), has anti-inflammatory effects on the endothelial cells of arteries and veins (Aljada *et al.*, 2001) and also on mononuclear leukocytes (Dandona *et al.*, 2001). Circulating insulin even has an antithrombotic effect (Aljada *et al.*, 2002). In an endotoxemic animal study similar to our model, peripheral infusion of insulin modulated the inflammatory response by decreasing pro-inflammatory cytokines and increasing serum levels of anti-inflammatory cytokines, independently of glucose or electrolyte levels (Jeschke *et al.*, 2004). Altogether this underlines the fact that circulating insulin clearly interacts with the immune system. Although the anti-inflammatory effects of insulin described in many *in vivo* studies could, at least in part, be due to central insulin effects. It has also been shown in numerous studies that insulin directly interacts with and regulates leukocytes and endothelial cells thus changing the body's immunologic response to inflammatory stimuli.

In our study we wanted to investigate the immune regulating effects of brain insulin in critically ill mice, as opposed to any peripheral effects of insulin. To this end, instead of administering insulin peripherally we infused it via an ICV cannula directly into the third ventricle. To reduce the likelihood of a direct peripheral pharmacological effect of the centrally infused insulin, we chose a dose similar to that used by other research groups investigating various central effects of insulin in mice (Table 7).

Table 7: Comparison of insulin doses used in different studies investigating central effects of insulin in mice.

Study	Animals	Central insulin dose
(Brown *et al.*, 2006)	Male C57BL/6, 6-7 weeks old	0.05, 0.1 or 0.4 μU injection
(Morgan *et al.*, 2010)	Male agouti obese mice and male C57BL/6, 12-16 weeks old	20 μU or 100 μU injection
(Muller *et al.*, 2011)	CF1 mice, 8 weeks old	0.5 mU (500 μU) or 5 mU (5000 μU) injection
(Koch *et al.*, 2008)	Male C57BL/6, 11 weeks old	200 μU/d (8 μU/h) over a period of 7 days
Present study	Male C57BL/6, 8-10 weeks old	0.02 mU/h (20 μU/h), over a period of 4 hours

Although we used a dose of insulin for ICV infusion similar to that selected by other researchers, to our surprise 4 hours of chronic ICV insulin infusion caused a mild but significant increase in circulating insulin levels (Figure 20). It is well known that centrally administered insulin stimulates pancreatic insulin secretion. This effect was first described in 1975 in a study performed to investigate the effect of cerebral ICV insulin on pancreatic insulin secretion. An extracorporeal pancreatic blood circuit was established after laparotomy of six dogs to monitor blood flow and insulin concentration directly from the superior pancreaticoduodenal vein. ICV infusion of insulin (0.2 U/kg) into the left lateral cerebral ventricle caused a significant increase of pancreatic output as early as five minutes post injection. From the results obtained, this group concluded that insulin-sensitive cells of the CNS somehow communicate with the insulin secreting β-cells of the pancreas (Chen *et al.*, 1975). Further studies have revealed that the secretion response as well as the control of the beta-cell mass are influenced by external hormonal and neuronal signals. There is evidence for autonomic nervous system control of pancreatic insulin secretion. For example, electrical stimulation of the vagal nerve, the main parasympathetic branch, causes an increase in pancreatic insulin secretion, whereas activation of the sympathetic nerves such as the splanchnic nerve leads to a reduction in pancreatic insulin output (Holst *et al.*, 1986). Since ICV insulin modulates autonomic nervous response, this could potentially also be a mechanism by which central insulin infusion stimulates pancreatic insulin secretion.

Elevated circulating insulin levels in our ICV insulin infused mice might me caused by elevated parasympathetic nerve activity. However, the fact that we measured raised peripheral insulin levels in our centrally infused animals, together with the knowledge that

circulating insulin itself is a potent immune-regulating agent, poses a problem. Under these circumstances it is difficult to determine, whether brain insulin has a direct anti-inflammatory effect or, alternatively, the observed improved survival and reduced inflammatory response to LPS is due to indirectly by raised peripheral insulin levels.

A possible way to overcome this dilemma would be to establish pancreatic clamps in the animals during ICV infusion experiments. To establish a basal insulin clamp, plasma insulin concentrations are maintained at a base level (e.g. 80 pmol/L) in both study groups by continuous infusion of insulin. At the same time blood glucose levels are kept constant by a variable glucose infusion until steady-state is achieved. This involves a significantly more complex and resource-intensive experimental protocol than the one we used. First, major additional surgery is needed because intravenous and intra-arterial lines for glucose and insulin infusion and blood sampling are required. Second, to achieve a steady-state during basal insulin clamps, a complex setup involving several costly infusion pumps that also require constant supervision for accurate performance would be needed. More man power would be essential as glucose and insulin levels need to be monitored constantly and infusion flow rates of glucose or insulin increased or decreased almost instantly. These resources were not available at the time we performed our experiments, but for future studies to confirm our observations a setup of this type must be considered and may well be necessary in order to fully differentiate the central from the peripheral anti-inflammatory effects of insulin.

8 Conclusion

Insulin acts as a direct anti-inflammatory agent. Administered to critically ill patients insulin can reduce morbidity and mortality in surgical and medical ICUs. The anti-inflammatory mechanisms involved are, however, poorly understood. Brain insulin has well defined effects on several peripheral parameters including food intake, cognitive functions, energy balance, reproductive functions and the autonomic nervous system. It is also known that by regulating autonomic nervous system activity the brain modulates the inflammatory state. Since brain insulin modulates the autonomic nervous system, we speculate that centrally administered insulin has systemic anti-inflammatory effects and we have tested this hypothesis in a mouse model of LPS induced critical illness.

We show here for the first time that brain insulin regulates peripheral immune functions, resulting in decreased mortality in an animal model of critical illness and impaired metabolic control. This could be of great translational interest, as it has been shown recently that systemic insulin treatment decreases morbidity and mortality in critically ill patients. Employing a model of high-fat diet induced insulin-resistance combined with critical illness we have also gained further insight into the connection between impaired metabolic control and inflammation. We have confirmed that peripheral insulin-resistance results in higher susceptibility of mice to lipopolysaccharide induced mortality. Central infusion of insulin partly restores immune functions, making these mice significantly more resistant to the devastating effects of lipopolysaccharide. Although these effects are significant and clearly measurable in survival studies, we are unable to fully explain the mechanism involved. It is clear that centrally administered insulin acts like an anti-inflammatory agent, suppressing major pro-inflammatory cytokines in the circulation and regulating molecules that participate in cytokine signaling in several immunologic organs

How these small amounts of centrally administered insulin regulate immune functions on such a broad basis has yet to be elucidated. The brain affects peripheral immune functions through neuronal and hormonal pathways and central insulin signaling is known to influence many pathways connecting the brain to the periphery. If pathways that are influenced by brain insulin are also involved in central control of immune functions, this could be an explanation of how centrally administered insulin might act as an immune regulating substance. Central insulin signaling certainly holds a promise for a possible new therapeutic approach in critical illness.

We should not forget the limitations of our experimental model. The physiological responses and metabolic parameters of septic humans may differ from those observed in insulin-resistant C57BL/6 mice injected with lipopolysaccharide. Although lipopolysaccharide administration is a well known and established model for sepsis, it is fair to question the suitability of the method as an appropriate model for mimicking critical illness when addressing the question of potential beneficial effects of insulin in septic patients. Another experimental concern is that central infusion of insulin also slightly raises peripheral insulin levels. Because systemic insulin has known anti-inflammatory effects, is it possible under these circumstances to adequately separate central from peripheral effects of insulin on immune functions? Modification of the experimental protocol, including use of a different *in vivo* model, might provide answers to these questions. A more accurate animal model for sepsis involving cecal puncture and legation and use of a basal insulin clamp allowing better separation of the central from the peripheral effects of insulin, might well answer these questions.

In conclusion, we believe that the results obtained in this work provide a novel insight into the physiology of the central effects of insulin and the link between impaired metabolic control and inflammation. These initial results require thorough investigation and validation in a more accurate animal model of human sepsis. Further investigations will also need to clarify whether the anti-inflammatory effects observed are solely a consequence of central insulin activity, in contrast to the well known immune regulating systemic properties of insulin. A future study with an improved design could provide a more realistic basis for further investigation in clinical trials involving critically ill humans. The crucial step to deliver proof of therapeutic benefit of brain insulin in critically ill humans might then appear feasible. Central insulin administration already exists as a therapeutic strategy and is currently the subject of investigations in humans. Ongoing research investigating effects of intranasally administered insulin on cognitive functions in patients with Alzheimer's disease has delivered promising results.

9 Summary

9.1 Background

Critically ill patients are not only at high risk of death, but often develop what is commonly referred to as “stress diabetes”. Stress diabetes, describing an impaired metabolic state characterized by hyperglycemia and insulin resistance, is often present in critically ill patients hospitalized in intensive care units. Moreover, it is known that critically ill patients with type 2 diabetes mellitus have a poorer prognosis than non-diabetic patients. Critical illness thus induces a state of impaired metabolic control, and type 2 diabetes exacerbates critical illness. A recent landmark study has shown that intensive insulin therapy significantly increases survival and reduces morbidity in critically ill patients, although the mechanisms accounting for the beneficial effect are not yet understood.

Insulin receptors are present extensively in the central nervous system and circulating insulin is readily transported into the central nervous system, crossing the blood brain barrier. Insulin signaling in the brain has many effects on the body, such as regulating cognition, fertility, energy metabolism and the activity of the autonomic nervous system. Growing evidence suggests that the central nervous system also plays an important role in the regulation of innate immunity. A recent series of elegant studies has shown that the central nervous system inhibits pro-inflammatory cytokine production and systemic inflammation via the efferent vagus nerve. Additionally, it has been established that the brain effects peripheral immunity through hormonal signaling, e.g. via the hypothalamus-pituitary-adrenal axis.

9.2 Objective

Central insulin has well known peripheral effects, one of which is modulation of the autonomic nervous system. It has been shown that the brain affects peripheral immune functions via at least two major routes including neuronal and hormonal signaling. These two findings taken together suggest that brain insulin, in addition to other effects, might also regulate the immune system in the periphery by signaling through hormonal or neuronal pathways.

Based on the fact that critically ill patients clearly benefit from intensive insulin therapy, we speculate that some of the benefits of intensive insulin therapy on the survival of critically ill patients are, at least in part, due to immune regulating mechanisms of brain insulin.

To this end, we investigated in this work whether centrally administered insulin regulates systemic inflammation and improves survival in a septic mouse model of critical illness and impaired metabolic control.

9.3 Design

In a first experiment we observed significant differences in inflammatory markers such as systemic cytokine levels and molecules participating in cytokine signaling between the control and insulin-treated animals. After four weeks on a high-fat diet, male C57BL/6 mice received lipopolysaccharide as an intraperitoneal injection followed by a 4-hour intracerebroventricular infusion of insulin or vehicle, and were then sacrificed. Markers of systemic inflammation such as plasma cytokines levels were measured with ELISA. We measured protein expression and activation by phosphorylation of molecules involved in cytokine signaling pathways in several organs by immunoblotting.

A second experiment was carried out to determine whether central insulin administration improves survival in insulin-resistant mice challenged with lipopolysaccharide. In this experiment mice were injected with a lethal dose of lipopolysaccharide and randomly assigned to receive continuous intracerebroventricular insulin or vehicle infusions for the following 5 days. Survival was assessed daily during the 5-day period of continuous intracerebroventricular infusion.

9.4 Results

Low-dose centrally administered insulin regulates the systemic inflammatory response of insulin-resistant mice challenged with lipopolysaccharide. After 4 weeks on a high-fat diet, male C57BL/6 mice received lipopolysaccharide (1mg/kg) as an intraperitoneal injection followed by a 4-hour intracerebroventricular infusion of insulin or vehicle, and then sacrificed. Plasma levels of tumor necrosis factor alpha and interleukin 6 were significantly decreased as a result of the central insulin treatment. In spleen tissue central insulin infusion decreased interleukin 1 beta and cyclooxygenase 2 protein expression, phosphorylation of signal transducer and activator of transcription 1. ICV insulin reduced the protein levels of interleukin 1 beta in lung tissue. In liver tissue, phosphorylation of Stat1, Stat3 and concentration of interleukin 1 beta was lower in the intracerebroventricular insulin-treated group. In a second experiment insulin-resistant mice were injected with a lethal lipopolysaccharide dose (5 mg/kg) and randomly assigned to receive continuous intracerebroventricular insulin or vehicle infusions for the following 5 days. Survival was assessed daily on each of the consecutive 5 days of continuous intracerebroventricular

infusion. Our results show that after administration of a lethal dose of lipopolysaccharide, brain insulin signaling significantly improves survival of insulin-resistant critically ill mice.

9.5 Conclusion

Central administration of insulin to mice suffering from impaired metabolic control and severe systemic inflammation - also present in critically ill humans - is an effective way of regulating the devastating effects of an overshooting immune response. These anti-inflammatory effects are measurable on the basis of lower levels of systemic pro-inflammatory markers such as cytokines and other important molecules involved in immune signaling. Even more significant is the beneficial effect of brain insulin in a worse state of critical illness. Continuous intracerebroventricular infusion of insulin to mice challenged with a lethal dose of lipopolysaccharide significantly improved survival compared to controls. What is more, the fact that insulin-resistant-mice are more susceptible to lipopolysaccharide than control mice suggests that there is a link between impaired metabolic state and reduced immune functions. Because central insulin signaling is decreased in impaired metabolic state and overcoming central insulin resistance by infusing insulin directly into the third ventricle improves immunologic functions, this suggests that one of the factors contributing to the poorer prognosis of critically ill type 2 diabetics might be decreased central insulin signaling leading to a reduced immune regulating effect.

The data obtained from this research provide a basis for future investigation of central insulin, especially its role in regulating peripheral immune functions. The results also suggest that brain insulin might play an important therapeutic role - currently an area of fervent investigation - in the alleviation of cognitive impairment in patients suffering from Alzheimer's disease. Intranasal administration of insulin to critically ill patients in intensive care units might well improve the patient's outcome. Many other novel therapeutic strategies introduced over the past decades have failed in this respect.

10 Table of figures

11 References

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12 Acknowledgement

Mein besonderer Dank gilt Herrn Prof. Dr. Christoph Buettner für die Aufnahme in seine Arbeitsgruppe, die Bereitstellung des interessanten Themas sowie die hervorragende wissenschaftliche Betreuung. Er verstand es, mich durch seine eigene Begeisterung für dieses Thema stets zu motivieren und unterstütze meine tägliche Arbeit durch gute fachliche Betreuung und tatkräftige Hilfestellung bei allen Experimenten und wissenschaftlichen Fragestellungen.

Mein Dank gilt ebenfalls Herrn Prof. Dr. Hans Hauner, der sich freundlichst dazu bereit erklärt hat als offiziellen Betreuer der Dissertation an der Technischen Universität München zu fungieren. Für sein Vertrauen und für die kritische Durchsicht meiner Arbeit danke ich Ihm sehr. Des weiteren war Herr Prof. Hauner eine große Hilfe bei der Beschaffung finanzieller Mittel, die es mir erst ermöglich haben diese Projekt im Ausland fertigzustellen. Ein großes Dankschön an Boehringer Ingelheim Fonds (BIF) und den Deutsche Akademischen Austauschdienst (DAAD) für die großzügige finanzielle Unterstützung des Projekts.

Herzlichen Dank auch an Linghong Chen für ihre geduldige Anleitung beim Erlernen vieler Arbeitstechniken und ihre Unterstützung im Laboralltag. Entsprechender Dank auch an Thomas Scherer, Bob Cheng und Claudia Lindtner für die angenehme Arbeitsatmosphäre, die ständige Hilfsbereitschaft und die konstruktiven Diskussionen, die zum Gelingen dieser Arbeit maßgeblich beigetragen haben. Letztgenannte standen mir nicht nur im Labor stets mit fachlicher Kompetenz, Rat und Hilfe zur Seite, sondern machten diesen Forschungsaufenthalt in New York auch außerhalb des Labors zu einem sehr abwechslungsreichen und einmaligen Erlebnis. Über das bis Heute andauernde freundschaftliche Verhältnis freue ich mich sehr. Bedanken möchte ich mich auch bei Ruslan Novosyadlyy und Hayden Courtland aus der Arbeitsgruppe von Herrn Prof. Dr. Derek LeRoith für die fachliche und mentale Unterstützung.

Mein größter Dank gilt allen voran meinen Eltern, ohne dessen bedingungslose Unterstützung ich diese herausfordernde Aufgabe nur schwer gemeistert hätte. Ein besonderer Dank gilt hierbei meinem Vater für die kritische Durchsicht meiner Arbeit. Nicht zuletzt danke ich all meinen Freunden und all denjenigen, die mich auf ihre persönliche Art und Weise auf diesem Weg begleitet und unterstützt haben.

Thomas Milsom, Berlin 2014