Impaired awareness of hypoglycemia in type 1 diabetes

The role of lactate

Hanne M.M. Rooijackers
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Hanne Margaretha Maria Rooijackers
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Chapter 1

General introduction and outline of the thesis
Introduction

Diabetes mellitus is a highly prevalent condition that is characterized by chronically elevated blood glucose levels (*hyperglycemia*). Fueled by unfavorable lifestyle changes, the number of adults with diabetes mellitus has globally quadrupled from 108 million in 1980 to 422 million in 2014, and is projected to increase by at least 50% in the next 20 years (1). Diabetes is generally classified into two main types: type 1 and type 2 diabetes. In the Netherlands, there were over one million people living with diabetes in 2015 (2), with type 1 diabetes accounting for approximately 10% of cases (3). Although type 1 diabetes is not related to lifestyle, its incidence and prevalence are also increasing (4; 5). Since the present thesis focuses on patients with type 1 diabetes, type 2 diabetes will not be discussed in further detail here.

Type 1 diabetes typically develops in childhood or early adulthood, but may present at any age. It is an autoimmune disease characterized by near-complete destruction of the insulin producing pancreatic beta-cells and results in an absolute insulin deficiency (6; 7). In healthy individuals, insulin is rapidly released into the circulation in response to a meal, and promotes the uptake of glucose from the circulation into the liver, skeletal muscle and adipose tissue, while simultaneously suppressing glucose production by the liver (8). These actions result in stable blood glucose levels in healthy people. In people with type 1 diabetes, insulin deficiency leads to hyperglycemia and necessitates the use of insulin replacement therapy. However, therapeutic insulin is a relatively poor replacement of endogenous insulin, in part due to a slower onset of action and a more prolonged glucose-lowering effect (9). In addition, injection of identical insulin doses may result in different glucose responses within the same individual at different times, because of variability in insulin absorption and metabolic effects (10). As a consequence, glucose fluctuations and – on average – chronic hyperglycemia remain part of everyday life in these patients.

Chronic hyperglycemia, even when mild, increases the risk for microvascular complications, including retinopathy, nephropathy and neuropathy (11). Chronic hyperglycemia is also a risk factor for atherosclerotic disease of the large vessels, so-called macrovascular complications, causing cardiac, cerebral and peripheral vascular disease. Despite major medical advances over the past 20 years, the estimated life expectancy for people with type 1 diabetes at the age of 20 is still approximately 12 years less compared with the general population without diabetes. The largest percentage of the estimated loss in life expectancy is due to cardiovascular complications (12).
There is ample evidence that intensive insulin treatment aimed at maintaining near-normal blood glucose levels substantially reduces the risk of microvascular and macrovascular complications in patients with type 1 diabetes (13-15). Guidelines therefore recommend tight, near-normal glycemic control to prevent or delay these complications (16), but this comes at a price, i.e. an increased risk for low blood glucose levels (hypoglycemia) (17). As a consequence, the glycemic goal in a patient with diabetes is almost by definition a compromise between preventing complications associated with hyperglycemia and minimizing the problems associated with iatrogenic hypoglycemia (18). In other words, iatrogenic hypoglycemia is the main barrier in the glycemic management of type 1 diabetes (19).

**Hypoglycemia and glucose counterregulation**

Insulin-induced hypoglycemia in people with diabetes is called non-severe if the individual is able to self-treat such an event, usually by ingesting carbohydrates. Severe hypoglycemia is defined as an event that requires the help from another person for recovery (20). Patients with type 1 diabetes experience on average two non-severe episodes per week and one severe, potentially hazardous event, every year (21; 22). Although the rate and severity of hypoglycemia varies greatly between persons (21), this means that people with type 1 diabetes are exposed to many thousands of hypoglycemic episodes over a lifetime of diabetes.

The maintenance of adequate plasma glucose levels is particularly important for the brain, because the brain relies on a continuous supply of glucose as its primary energy source. To this extent, a hierarchically coordinated system of physiological mechanisms, commonly referred to as **counterregulatory responses**, exists in healthy, non-diabetic individuals, that effectively prevents hypoglycemia in virtually all circumstances (23) (figure 1). The initial response to a decrease in plasma glucose levels within the physiological range is suppression of endogenous insulin secretion. When plasma glucose levels fall just below the physiological range (<3.9 mmol/L), the second response is an increase in glucagon secretion by pancreatic alpha-cells, which stimulates hepatic glycogenolysis and gluconeogenesis. The third response of the system occurs at similar plasma levels, but only becomes critical when glucagon secretion is insufficient, and concerns the release of adrenaline by the adrenal medulla (23). Adrenaline stimulates glycogenolysis and gluconeogenesis, mobilizes gluconeogenic substrates and limits peripheral glucose uptake. Growth hormone and cortisol are released at slightly lower plasma glucose levels and are aimed at preventing prolonged hypoglycemia, but are not essential for the acute response (24).
Introduction

When counterregulatory hormones fail to prevent hypoglycemia and plasma glucose levels drop below ~3.2 mmol/L, this further increases sympathoadrenal responses and induces autonomic warning symptoms, such as hunger, palpitations and sweating, aimed at initiating an appropriate behavioral response: the ingestion of carbohydrates (23). The most worrisome symptoms of hypoglycemia for patients (25) occur at even lower plasma glucose levels and result from glucose deprivation in the brain. These so-called neuroglycopenic symptoms range from difficulty in concentrating to alterations in consciousness and eventually seizures or coma, which requires third-party assistance for recovery. Hypoglycemia is detected by both peripheral and cerebral glucose sensing neurons, with the most important sensors located in the central nervous system (CNS) (26). A fall in cerebral glucose levels is generally sensed as a result of a change in energy status (ATP/ADP and AMP/ATP ratios) in specific hypothalamic neurons (27). Counterregulatory responses (including neuroendocrine, autonomic and behavioral responses) are also predominantly initiated and coordinated by the CNS.

Defective counterregulation and impaired awareness of hypoglycemia

In patients with type 1 diabetes, the normal counterregulatory responses to hypoglycemia are impaired (23) (figure 1). First, the loss of insulin secreting capacity disrupts the insulin response to declining plasma glucose levels, and insulin levels remain elevated due to the use of exogenous insulin therapy. Second, within a few years after diagnosis, patients fail to generate an adequate glucagon response to hypoglycemia, presumably because of the loss of the paracrine interaction between alpha- and beta-cells (28), but islet-selective loss of sympathetic nerves may also contribute (29). In the absence of the glucagon response, patients depend predominantly on adrenaline for glucose counterregulation. Unfortunately, the adrenaline response also becomes attenuated and is activated at lower glucose levels in most patients with longer diabetes duration (30). Failure of this third line defense against hypoglycemia causes defective glucose counterregulation and markedly increases the risk of severe hypoglycemia (23).

A defective adrenaline response is a marker for an attenuated sympathoadrenal (or autonomic) response (23), and is associated with, but not necessarily the cause of, defects in the emergence of autonomic symptoms of hypoglycemia (31; 32). A diminished ability to perceive the onset of acute hypoglycemia is known as the clinical syndrome of impaired awareness of hypoglycemia (IAH).
This syndrome includes reduced symptom intensity and an altered symptom profile, as well as failure to interpret hypoglycemic symptoms correctly. IAH occurs in up to one third of patients with type 1 diabetes and increases the risk of severe hypoglycemia by a factor 6 or more (33; 34). In the past, the term ‘hypoglycemia unawareness’ has often been used, but IAH is preferred, because very few patients are completely unaware of hypoglycemia, meaning that they have a total loss of symptoms of hypoglycemia under all circumstances.

**Figure 1. Physiology of glucose counterregulation.** A simplified illustration of the mechanisms that normally prevent or rapidly correct hypoglycemia, and defects in these mechanisms in type 1 diabetes. NA, noradrenaline, Ach, acetylcholine. Figure adapted from (23).
Pathophysiology of IAH

Antecedent hypoglycemia

For many years, IAH was thought to result from peripheral autonomic neuropathy. Although these conditions may coexist in patients with longstanding type 1 diabetes, most people with IAH have no signs of (severe) autonomic neuropathy (35-37). Instead, IAH is probably a functional disorder, rather than the result of a structural (e.g. neuropathic) defect.

Currently, it has been firmly established that hypoglycemia itself initiates a process of habituation that attenuates counterregulatory hormone and symptomatic responses to hypoglycemia occurring in the subsequent 24-48 hours (38; 39). Reduced counterregulatory responses in the context of therapeutic hyperinsulinemia increase the likelihood of subsequent hypoglycemia, creating a vicious cycle of recurrent hypoglycemia and progressive deterioration of counterregulatory defenses. This has led to the concept of ‘hypoglycemia-associated autonomic failure’ (HAAF) in diabetes that postulates that recent antecedent (i.e. previous periods of) hypoglycemia causes both defective glucose counterregulation and IAH (39). A schematic representation of this concept is given in figure 2.

Semantically, HAAF may not be the best term, since there is adaptation rather than complete failure of the counterregulatory system. Indeed, meticulous avoidance of hypoglycemia at least partly restores the counterregulatory deficits induced by recurrent hypoglycemia (31; 40). In addition, not only antecedent hypoglycemia, but other situations where the autonomic nervous system is activated, such as antecedent low- and moderate-intensity exercise (41; 42), are also able to attenuate the sympathoadrenal response to hypoglycemia, as is sleep (43). The effects of more intensive exercise types, such as the more and more popular high-intensity interval training (HIIT), on awareness of hypoglycemia have not yet been investigated.

Since hypoglycemia plays such a pivotal role in the development of impaired awareness, clinical risk factors for IAH relate primarily to prior exposure to hypoglycemia, and include increased duration of diabetes, absent endogenous insulin secretion, a history of recurrent (severe) hypoglycemia, counterregulatory failure, and strict glycemic control (44-46). While many people with type 1 diabetes experience recurrent hypoglycemia, less than half of them develop IAH. The reason for the inter-individual susceptibility to develop IAH is unknown, but may involve genetic factors (46).
The role of the brain

Although the role of antecedent hypoglycemia in the development of IAH is undisputed, the mechanisms by which hypoglycemia reduces responses to a subsequent episode of hypoglycemia are still poorly understood and warrant further investigation. There is agreement that the cause should reside in the brain (19), given its dependence on glucose and its critical role in hypoglycemia sensing and in coordinating responses to restore euglycemia.

Most hypotheses regarding IAH involve adaptations in brain fuel transport, cerebral blood flow or metabolism (19; 24), and both global changes and local adaptations in specific brain regions essential for glucose sensing have been

Figure 2. Schematic diagram of the concept of hypoglycemia-associated autonomic failure in type 1 diabetes. The mechanisms contributing to the development of IAH and impaired counterregulation, including the potential role of adaptations in brain metabolism. Figure adapted from (19).
suggested to play a role. A full discussion of these theories and the methods to study cerebral metabolism will be provided in Chapter 2 of this thesis. Importantly, previous studies from our study group have shown that brain metabolism is relatively preserved during hypoglycemia, hence does not decrease. Remarkably, brain metabolism during hypoglycemia was maintained at a higher level in patients with type 1 diabetes than in non-diabetic individuals, despite a similar fall in brain glucose levels (2; 3). This suggests that metabolism of a non-glucose, alternative fuel is involved. We posit that this alternative fuel is lactate.

**The role of lactate**

Lactate or lactic acid is a hydroxycarboxylic acid that exists in two isomers: L-lactate and D-lactate, the former being the dominant isoform in humans. At a physiological pH, lactic acid is more than 99% dissociated into lactate anions and protons (47). Lactate is continuously produced by most tissues in the body, with the highest production in skeletal muscle and adipose tissue (48; 49). It is predominantly derived from glucose via the glycolytic pathway after its conversion into pyruvate, and a small amount of lactate is derived from alanine. The enzyme lactate dehydrogenase catalyzes the formation and removal of lactate through a reversible oxidation-reduction (redox) reaction. After conversion to pyruvate, lactate may proceed to the oxidative pathway in various tissues or to the gluconeogenesis route in the liver (49).

Under resting conditions, plasma lactate concentrations vary between 0.5 and 1.5 mmol/L. Lactate accumulation and acidosis occur when the production of lactate exceeds its clearance, as can be seen in septic shock, during seizures or due to regional ischemia (50). Plasma lactate levels also markedly increase in response to high-intensity work-outs, with lactate values rising up to 12-20mM during the first minutes following ‘all-out’ maximal exercise bouts. Although lactate was traditionally considered a harmful waste product of glycolysis that accumulated as a result of hypoxia, this dogma has dramatically changed since the early 1970s. It is now widely accepted that lactate is produced all the time in fully oxygenated tissue and is not just a noxious waste product of glycolytic metabolism (47; 51). In fact, lactate is considered a crucial intermediary in numerous metabolic processes, an important metabolic regulator, and a mobile fuel for aerobic metabolism that can be rapidly exchanged between cells and tissues (47), including the brain (52).
Under resting conditions, lactate is present in the brain at tissue levels of approximately 0.5 mM and in cerebrospinal fluid at about 1-2.0 mM. It can be measured non-invasively by proton magnetic resonance spectroscopy (1H-MRS) (53), a technique that is further explained in Chapter 2. Lactate is a valuable energy source for the brain during euglycemia (54; 55) and may spare cerebral glucose utilization, especially when plasma lactate levels are elevated (54). Administration of lactate may improve neuroenergetics in patients suffering from traumatic brain injury (56), has neuroprotective effects during severe hypoglycemia in rodents (57) and is able to suppress counterregulatory hormone responses to and symptoms of hypoglycemia in humans (58; 59). More recently, it has been reported that patients with IAH have increased capacity for brain lactate transport during hypoglycemia (60; 61). One could then hypothesize that increased use of lactate by the brain during hypoglycemia may prevent cognitive dysfunction in patients with IAH, since the brain is no longer deprived of fuel. At the same time this maintenance of brain metabolism may interfere with the brain’s ability to detect hypoglycemia, thus explaining the clinical picture of IAH. Alternatively, lactate may affect awareness of hypoglycemia by acting as a signaling molecular or metabolic regulator, instead of, or in addition to being a metabolic substrate (62).

Real life experience with IAH

Clinical consequences of impaired awareness of hypoglycemia

The morbidity associated with IAH is mostly related to the increased risk of severe hypoglycemia, with all its accompanying physical, psychological and social consequences.

Hypoglycemia accounts for 7 to as much as 10% of deaths in young adults with type 1 diabetes. Most of these deaths are not directly related to brain death, but rather due to trauma (e.g. hypoglycemia while driving) and cardiovascular effects, such as fatal arrhythmia (63). Severe hypoglycemia has also been associated with increased mortality from cardiovascular events, particularly in patients with type 2 diabetes (64; 65). Evidence for an association between severe hypoglycemia and cardiovascular disease is less consistent in type 1 diabetes. A recent prospective cohort study found that neither exposure to severe hypoglycemia, nor the presence of IAH increased all-cause or cardiovascular mortality in patients with type 1 diabetes (66).
Patients are often concerned that recurrent severe hypoglycemia may lead to persistent cognitive decline, obviously because cognitive functioning is extremely sensitive to acute changes in plasma glucose levels. Most studies in adults with type 1 diabetes have reported the absence of such an association (67), although a recent study did detect subtle deficits in cognitive function in patients with IAH that may reflect impaired functioning of the hippocampus, a brain region that is most vulnerable to damage from hypoglycemia (68).

Regarding psychological consequences, severe hypoglycemia has a negative impact on patients’ well-being at various dimensions and is associated with increased fear of hypoglycemia and diabetes-related distress, reduced diabetes-related self-efficacy, and a reduction in quality of life (69; 70). Patients with IAH report imposed and self-imposed changes to their lives following the onset of IAH that include having to leave employment, reducing physical activity, spending more time being supervised by others, and extensively planning time spent away from home (71). Clearly, the effects of IAH extend beyond the person with the condition, as IAH is also a significant burden for family members of patients, who have to assist in detecting and treating hypoglycemia and are often concerned about the safety of the person with IAH (72). In addition to the psychological consequences, severe hypoglycemia represents a substantial economic burden, not only due to direct costs for medical treatment, but also because of indirect cost such as those related to absence from work resulting in lost productivity (73).

Interestingly, a substantial part of patients with IAH is not appropriately concerned about their condition (74). These patients are generally more worried about being high than low and place excessive emphasis on avoiding hyperglycemia and its associated complications. In contrast, they underestimate the consequences of hypoglycemia, despite previous severe, potentially life threatening, episodes. It is true that most young people with type 1 diabetes recover uneventfully from even severe acute hypoglycemia, but there is much controversy about the long-term effects of hypoglycemia. Increasing our knowledge of long-term effects of hypoglycemia is crucial for patients and care providers in order to provide a more balanced view on potentially harmful effects of hypoglycemia.

Several studies have shown that acute hypoglycemia induces pro-inflammatory effects such as an increase in circulating leukocyte numbers, and an elevation of plasma proatherothrombotic and proinflammatory factors (75-77). The systemic inflammatory response thus induced by hypoglycemia may provide
a mechanistic link between hypoglycemia and long-term cardiovascular complications. However, the mechanisms by which hypoglycemia increases circulating proinflammatory cytokines and leukocytes have not been investigated. In addition, it is unknown whether repeated hypoglycemia or IAH affects inflammatory responses to hypoglycemia or whether hypoglycemia alters the function of inflammatory cells.

**Treatment of IAH**

Although it has been demonstrated that IAH can be reversed, at least in part, by meticulous avoidance of further hypoglycemia for several weeks to months (31; 40), achieving this in clinical practice is extremely difficult, hard to sustain, and frequently accompanied by deterioration of glycemic control (78). Strategies used to reduce the incidence of hypoglycemia include (temporary) relaxation of glycemic control, structured educational approaches with or without behavioral interventions, technological interventions such as continuous subcutaneous insulin infusion (CSII), continuous glucose monitoring (CGM) and sensor-augmented insulin pumps with low-glucose suspend features. Despite the application of such treatment strategies, a substantial number of patients continues to experience disabling hypoglycemia (79). In some of these cases, islet cell transplantation has been used as a last-resort therapeutic option (80). Clearly, islet cell transplantation, or ideally, beta-cell replacement, has important limitations, including limited availability of donors, costs and severe side effects of immune suppression.

So far, treatment of IAH has focused primarily on avoidance of hypoglycemia, and although a few studies have taken a more mechanistic approach and investigated pharmacological treatments that improve counterregulatory responses, none of these are currently clinically used (81-83). Novel, targeted treatment strategies for IAH are urgently needed. To identify such new treatment options, more detailed knowledge regarding the underlying mechanisms of IAH is needed.
Outline of the thesis

As discussed earlier, recurrent hypoglycemia causes the syndrome of impaired awareness of hypoglycemia (IAH) in one out of three patients with type 1 diabetes, and results in a high risk for severe hypoglycemia. Hence, IAH contributes to substantial morbidity and mortality. Despite extensive studies, the underlying mechanisms by which recurrent hypoglycemia causes IAH are insufficiently clarified, but adaptations in the brain’s handling of energy substrates are most likely involved. The central hypothesis in our studies is that the brain of patients with IAH has adapted to recurrent hypoglycemia and is better able to use alternative fuels when glucose supply is low. The overall aim of this thesis is to investigate the role of lactate as a non-glucose alternative fuel for the brain in the development of IAH.

Much of the work presented in this thesis has been established in close collaboration with the radiology department, using a proton magnetic resonance spectroscopy (\(^\text{1}\text{H-}\text{MRS}\)) method which was optimized for lactate detection. \(^\text{1}\text{H-}\text{MRS}\) was our technique of choice, since it is non-invasive and allows the quantification of brain lactate concentrations in vivo. In a series of experiments, described in chapter 3, 4 and 6, we combine \(^\text{1}\text{H-}\text{MRS}\) with the glucose clamp technique, a method that allows plasma glucose levels to be held at specific predetermined targets by using a constant insulin infusion and a variable glucose infusion.

First, the available literature about brain metabolism during and in response to hypoglycemia was critically reviewed. Chapter 2 describes the different metabolic and functional neuroimaging techniques that can be used to study brain metabolism in vivo, summarizes and discusses their most important findings in dept, and highlights remaining gaps in the literature.

In Chapter 3 we attempt to fill the first of these gaps and investigated what actually happens with brain lactate levels during hypoglycemia under ‘physiological conditions’, i.e. when no additional lactate is infused or otherwise increased. Brain lactate concentrations were assessed by \(^\text{1}\text{H-}\text{MRS}\) during a hyperinsulinemic euglycemic-hypoglycemic clamp. Results obtained in patients with type 1 diabetes and IAH were compared with results of patients with type 1 diabetes and normal awareness of hypoglycemia (NAH), and healthy individuals. Next, the effects of elevated plasma lactate levels on brain lactate concentrations during hypoglycemia were investigated. We examined both
the effect of exogenous and endogenous plasma lactate elevations with $^1$H-MRS. Previous studies have shown that exogenous administration of lactate during hypoglycemia suppresses counterregulatory hormone responses and hypoglycemic symptoms, presumably because lactate can substitute for glucose as a cerebral fuel. Whether elevated plasma lactate levels lead to an accumulation of lactate in the brain is subject of debate. In Chapter 4, we examined the effects of stable plasma lactate elevations on brain lactate concentrations during euglycemia and hypoglycemia. We hypothesized that exogenously administered lactate, resulting in high physiological plasma levels, sufficient to impair awareness of hypoglycemia in patients with NAH, is taken up and used by the brain. Second, to detect and confirm adaptations in brain handling in IAH, we compared the effect of lactate administration on brain lactate concentrations between patients with NAH and IAH.

When elevation of endogenous plasma lactate levels, as frequently occurs in response to high-intensity (interval) exercise, increases brain lactate, this may negatively affect awareness of subsequent hypoglycemia in patients with diabetes. In Chapter 5, we used a single bout of high-intensity interval training (HIIT) to raise endogenous plasma lactate levels and studied the effect on cerebral lactate levels with $^1$H-MRS during subsequent hypoglycemia in patients with type 1 diabetes with and without IAH, and in healthy individuals.

Following on the results obtained in chapter 5, we set out to investigate the clinical consequences of a single bout of HIIT on counterregulatory responses to hypoglycemia. We hypothesized that elevated levels of endogenous lactate induced by HIIT have similar suppressive effects on awareness of hypoglycemia as exogenous lactate infusion. In this context, we performed a randomized crossover trial in which patients with NAH, patients with IAH and healthy individuals underwent a hypoglycemic clamp, either after a short high-intensity interval training or after seated rest, and we measured counterregulatory responses to, symptoms of and cognitive functioning during hypoglycemia. The results of this study are presented in Chapter 6.

Hypoglycemia does not only affect the brain, but has broader, systemic effects that have gained renewed interest in recent years. Hypoglycemia increases circulating pro-inflammatory cytokines and has been suggested to contribute to systemic inflammation in patients with diabetes and therefore to cardiovascular complications. However, the mechanisms that lead to increased cytokine production and the effect of (recurrent) hypoglycemia on the inflammatory
function of immune cells have received limited attention. In Chapter 7 we provide the results of ex vivo stimulations of peripheral blood mononuclear cells obtained during a euglycemic-hypoglycemic clamp in patients with and without IAH, and healthy volunteers, to determine whether hypoglycemia altered the composition and inflammatory function of immune cells.

Finally, the results of the presented studies are summarized and put into perspective in Chapter 8.
Introduction


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Introduction

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Chapter 2

Brain glucose metabolism during hypoglycemia in type 1 diabetes: insights from functional and metabolic neuroimaging studies

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Brain glucose metabolism during hypoglycemia

Abstract

Hypoglycemia is the most frequent complication of insulin therapy in patients with type 1 diabetes. Since the brain is reliant on circulating glucose as its main source of energy, hypoglycemia poses a threat for normal brain function. Paradoxically, although hypoglycemia commonly induces immediate decline in cognitive function, long-lasting changes in brain structure and cognitive function are uncommon in patients with type 1 diabetes. In fact, recurrent hypoglycemia initiates a process of habituation that suppresses hormonal responses to and impairs awareness of subsequent hypoglycemia, which has been attributed to adaptations in the brain. These observations sparked great scientific interest into the brain’s handling of glucose during (recurrent) hypoglycemia. Various neuroimaging techniques have been employed to study brain (glucose) metabolism, including PET, fMRI, MRS and ASL. This review discusses what is currently known about cerebral metabolism during hypoglycemia, and how findings obtained by functional and metabolic neuroimaging techniques contributed to this knowledge.
Introduction

The brain is one of the most metabolically active organs in the body and it consumes energy disproportionate to its size. In humans, the brain represents only about 2% of total body weight, yet it accounts for approximately 20% of the body’s oxygen use and 25% of the body’s use of glucose. Glucose is the primary fuel for the adult brain. In young adults, the ‘resting’ brain consumes approximately 110 grams of glucose per day, i.e. 5.5 mg glucose per 100 g of brain tissue per minute (1). Since the brain’s energy stores are small, normal brain function depends on a continuous supply of glucose from the bloodstream. Under normal conditions, the human body takes great effort and is very efficient in avoiding hypoglycemia in almost all circumstances to maintain sufficient glucose delivery to the brain.

Type 1 diabetes mellitus is an autoimmune-mediated disease, characterized by destruction of most, if not all, of the insulin-producing capacity of pancreatic beta-cells. As a consequence, supplemental insulin treatment is required to maintain glucose control and decrease the risk of complications resulting from hyperglycemia (2). Unfortunately, therapeutic insulin is still poor at mimicking the pharmacology of endogenous insulin. As a consequence, people with type 1 diabetes – in particular those aiming for strict glycemic control – are at continuous risk of hypoglycemia, the average frequency of which has been estimated at two non-severe, symptomatic episodes per week (3-5) and one severe, potentially hazardous event, per year (4; 6; 7). Although there is substantial variation in both the rate and the severity of hypoglycemia, both between and within persons (4; 8), this estimation means that the brains of people with type 1 diabetes are exposed to many thousands of hypoglycemic episodes over a lifetime of diabetes.

Studying brain metabolism during hypoglycemia may reveal the potential harmful effects of (recurrent) hypoglycemia on the brain and may increase our understanding of metabolic adaptations that might underlie impairments in the defenses against hypoglycemia (9). Modern neuroimaging techniques have enabled the study of cerebral metabolism in vivo in a relatively non-invasive manner. This review will focus on the effect of hypoglycemia on brain (glucose) metabolism, with a particular emphasis on recent findings from functional and metabolic neuroimaging studies. Basic mechanisms of brain energy metabolism and neuroimaging techniques will be discussed briefly.
Glucose counterregulation

In healthy, non-diabetic humans, hypoglycemia is unlikely to ever occur due to a hierarchically coordinated system that integrates insulin secretion and counterregulatory hormone and symptom responses (10; 11). When glucose levels in the low-physiological range (e.g. late post-absorptive or fasting state) tend to fall, insulin secretion is suppressed to such an extent that true hypoglycemia can almost always be prevented. When insulin is given to experimentally induce hypoglycemia in people without diabetes, glucose levels at or below ~3.8 mmol/L will induce a glucagon response, the secretion of which by pancreatic alpha-cells is probably controlled by the neighboring beta-cells (10; 11). Such a glucose level also stimulates the secretion of adrenaline, whereas slightly lower levels are needed to elicit autonomic warning symptoms, such as sweating, palpitations, trembling and feeling hungry (10; 11). These symptoms are aimed at initiating a behavioral response (i.e. ingesting carbohydrates). Further falls in plasma glucose values result in neuroglycopenic symptoms, which range from mild cognitive impairment, such as difficulty in concentrating, to overt confusion and even coma or seizures in its most severe form (10; 11).

In patients with type 1 diabetes, hypoglycemia typically results from the interplay between therapeutic peripheral hyperinsulinemia and impaired defenses against falling plasma glucose levels (10). These impairments first include the inability to decrease insulin and to increase glucagon in response to hypoglycemia. The latter is not a structural defect, but specific for hypoglycemia and probably secondary to loss of control by non-functioning beta-cells (12). In patients with longer diabetes duration and more frequent exposure to hypoglycemia, adrenaline responses to hypoglycemia become attenuated, in part due to a shift of these responses to lower glucose values (13). The defective adrenaline responses are associated with, although not necessarily the cause of, similar defects in the emergence of autonomic symptom responses (14; 15). Disappearance of these symptoms interferes with the ability to timely and accurately perceive, interpret and respond to falling plasma glucose levels. This inability is known as the clinical syndrome of impaired awareness of hypoglycemia and increases the risk of particularly severe hypoglycemia, defined as those events requiring assistance from another person (16), by a factor of six or more (17; 18). Both the attenuated adrenaline response and impaired awareness of hypoglycemia are usually the result of (recurrent) antecedent hypoglycemia rather than of autonomic neuropathy, for which the term ‘hypoglycemia-associated autonomic failure’ (HAAF) has been introduced (19). HAAF can be effectively treated by several weeks to months of scrupulous avoidance of hypoglycemia (15; 20; 21), although
it appears that the symptomatic component responds earlier and better than the hormonal component (15). The underlying mechanism(s) explaining the attenuating effect of prior hypoglycemia on responses to subsequent events have not been fully elucidated. However, there is agreement that alterations in the brain play a pivotal role.

**Morbidity associated with hypoglycemia**

The glucose level at which cognitive function declines is subject to substantial variation; in some people cognitive dysfunction already occurs at plasma glucose levels between 3.0 and 4.0 mmol/L, whereas others continue to function well at levels below 2.5 mmol/L (8; 22). Almost all domains of cognitive function are potentially at risk during acute hypoglycemia, with complex tasks (e.g. car driving) being affected earlier than simple tasks (23; 24). Prolonged and/or profoundly severe hypoglycemia may eventually cause neuronal death (25; 26). The cerebral cortex and hippocampus are the most vulnerable regions in the brain to be injured by severe hypoglycemia, while the brain stem and cerebellum are most resistant (27). However, although persistent vegetative states or brain death resulting from hypoglycemia have been described (28-30), most patients with type 1 diabetes recover uneventfully from even severe hypoglycemia complicated by seizures or coma, especially when they are young and in good clinical condition. In addition, evidence for an association between multiple episodes of severe hypoglycemia and long-term cognitive decline in people with type 1 diabetes is lacking (31; 32). Finally, although 4 to 10% of all deaths in patients with type 1 diabetes have been attributed to hypoglycemia, most of these deaths are thought to be either accidental (e.g. in traffic) or cardiovascular (e.g. arrhythmia) rather than the direct consequence of brain death (33; 34).

It should be noted that both the developing brain of young children with type 1 diabetes (35; 36) and the brain of the elderly, in particular in patients with type 2 diabetes (37; 38), seem more susceptible to harm from hypoglycemia. Children with type 1 diabetes performed worse on cognitive tests when they had a history of severe hypoglycemia below the age of 5 years, compared to patients without such a history and non-diabetic controls (35). In prospective cohorts of people with type 2 diabetes, a history of severe hypoglycemia has been associated with cognitive decline and frank dementia (38), as well as with greater risks of cardiovascular events and death (39; 40). On the cellular level, there are now indications that glucose deprivation may accelerate apoptosis of neurons, which could underlie neuronal cell death and predispose for cognitive decline (41). It has also been speculated that the acute, physiological changes
Brain glucose metabolism during hypoglycemia may be particularly damaging when the vasculature has already been injured (42; 43), possibly explaining the discrepancy between type 1 and type 2 diabetes (31; 44). Another factor explaining this discrepancy may lie in the concept of hypoglycemic preconditioning. Rodents exposed to recurrent hypoglycemic events of moderate severity were less likely to develop neuronal damage or cognitive impairments or die during subsequent severe hypoglycemia than age-matched littermates who were not pre-exposed to hypoglycemia (45; 46). These data may help to explain recent observations that patients with type 1 diabetes and impaired awareness of hypoglycemia, as a reflection of recurrent hypoglycemia, appeared not at greater risk of dying than patients with intact awareness (47).

The role of the brain in glucose counterregulation

The brain is not just at the receiving end of hypoglycemia, but it plays an important role in both the detection of hypoglycemia and in the subsequent initiation and coordination of counterregulatory responses to restore euglycemia, as described above. This system maintains glucose homeostasis through a classic sensory-motor integrative pathway in which a decrease in plasma glucose levels is detected by an extended network of glucose sensing neurons located within the brain and the periphery (48). Specialized glucose-sensing cells are located in the hepatic portal/mesenteric vein, gut, carotid body and oral cavity. In the brain, glucose-sensing neurons are found at a number of locations, but particularly in the ventromedial nucleus of the hypothalamus (VMH) and areas that originate from the hindbrain. Integrative networks receive projections from these sensing neurons and subsequently assimilate their input with signals from other brain regions, such as information about circadian rhythm and arousal state. This information is relayed to motor neurons, which generate an output that drives the counterregulatory response and subsequently restores plasma glucose levels. Conversely, glucose sensing may influence other neural processes that have no role in glucose counterregulatory function (49).

Although the VMH is only one of a number of regions involved in the detection of hypoglycemia, it is thought to be the most important. The VMH serves as the central rely station for signals from many other regions and plays a crucial role in the coordination of the counterregulatory responses to falling glucose levels. Animal studies have shown that both destruction of the VMH and local perfusion of the VMH with glucose, disrupt counterregulatory hormone responses to systemic hypoglycemia (50; 51). Conversely, local glucopenia in
the VMH stimulates these responses in the absence of hypoglycemia (52). In analogy, glucose counterregulation was also found defective in a patient with lesions from sarcoidosis in the hypothalamus, presumably due to destruction of the glucose-sensing neurons in the VMH (53).

The mechanism of glucose-sensing by the brain, in particular in the VMH, has not been fully clarified. Two main types of glucose-sensing neurons have been identified: glucose-excited neurons, whose activity increases as glucose levels rise, and glucose-inhibited neurons, which become more active as glucose levels fall and less active when they rise (54). These neurons ‘sense’ a fall in glucose probably as a result of alterations in ATP/ADP and AMP/ATP ratios, respectively, following a reduction in glucose metabolism. This could explain why fuelling the VMH with an alternative source of energy, such as lactate, suppresses glucose counterregulation (55; 56). The subsequent intracellular actions that may ultimately lead to a counterregulatory response probably involve activation of AMP-activated protein kinase, formation of nitric oxide and release of glutamate in glucose-inhibited neurons. Other potential mediators involved in these responses include (a decrease in) gamma-aminobutyric acid (GABA) release from glucose-excited neurons, noradrenaline, serotonin and corticotrophin-releasing hormone (57). For further reading on this subject, we refer to recent reviews by McCrimmon (54) and Chan and Sherwin (57).

Cerebral glucose delivery, uptake and metabolism
Glucose is transported across the blood-brain barrier into extracellular fluid (ECF) by facilitated diffusion, mediated via glucose transporter protein 1 (GLUT1) (58). The predominant transporters involved in subsequent glucose uptake from the ECF in neurons and in astrocytes are GLUT3 and GLUT1, respectively (59), both insulin-independent glucose transporters. Once intracellular, glucose is phosphorylated by hexokinase as the initial step of glucose metabolism. The glucose-6-phosphate (Glc-6-P) thus produced can enter several metabolic pathways in the brain (60).

In 1945, Kety and Schmidt developed the first method to quantitatively assess brain glucose uptake in humans in vivo and to derive data on its subsequent metabolism (61). This highly invasive technique required the use of arterial and internal jugular vein catheterizations to determine arteriovenous concentration differences for glucose, which together with measurement of global cerebral blood flow (CBF) were then used to calculate the global cerebral metabolic rate of glucose (61-64). In humans, the Kety-Schmidt method was used to show that
Brain glucose uptake falls during hypoglycemia and that this coincides with the appearance of counterregulatory hormone responses and autonomic warning symptoms (65; 66). However, whether these data can be used to reliably assess brain glucose metabolism is a matter of debate. Indeed, the calculations rely solely on the disappearance rate of glucose from the circulation. Therefore, this technique cannot discriminate between specific metabolic steps and ignores the potential contribution of other metabolites. Moreover, the highly invasive nature of the Kety-Schmidt technique is a considerable limitation for research in humans.

The past forty years have shown rapid advances in modern metabolic and functional neuroimaging techniques to study brain (glucose) metabolism vis-à-vis CBF during hypoglycemia, including positron emission tomography (PET), functional magnetic resonance imaging (fMRI) and magnetic resonance spectroscopy (MRS). It is important to note that the distribution of CBF and the cerebral metabolic rate of glucose (CMR$_{\text{glc}}$) are closely linked to local brain activity. Brain activation causes proportionate increases in both local CBF and CMR$_{\text{glc}}$. These processes are being referred to as neurovascular coupling or neurometabolic coupling, respectively, and hypothesized to be mediated by neurotransmitter release and vasoactive metabolic products (67). Many functional neuroimaging techniques, including fMRI, rely on neurovascular coupling. The principles of the various imaging techniques will be briefly discussed (Fig.1).

**PET**

Positron emission tomography can be used to measure emissions from a variety of radioactively labeled tracers in the brain to quantify CBF, glucose uptake and phosphorylation, oxygen consumption and brain receptors for major neurotransmitters (by binding to the radioligand), depending on the type of radiotracer used (68). $^{15}$O-labeled water PET, for example, has been commonly applied to quantify regional CBF (69; 70). For the study of brain glucose metabolism, $[^{18}\text{F}]$fluoro-2-deoxy-D-glucose (FDG) is the most widely used tracer. FDG is taken up by the brain in a similar manner as glucose, but unlike native glucose, once phosphorylated (FDG-6-P), it cannot be metabolized further, resulting in accumulation of the tracer in the cell. Under steady-state conditions, in which total influx of metabolites into a pathway equals the outflow, the rate of tracer accumulation in the brain can be used to estimate global and regional rates of glucose transport and metabolism (58). PET has been particularly valuable in studying the effect of hypoglycemia on CBF, brain...
Fig. 1 A simplified illustration of the multiple metabolic pathways of glucose in the brain and the metabolic signals used in different neuroimaging techniques. The initial step of glucose metabolism is phosphorylation of glucose to glucose-6-phosphate (Glc-6-P) by hexokinase. Glc-6-P can enter several metabolic pathways in the brain. It can be metabolized to produce energy via glycolysis or the TCA cycle. Glycolytic and TCA cycle intermediates are also used for the synthesis of amino acids and neurotransmitters. In addition, Glc-6-P is a precursor for glycogen. Lastly, metabolism of Glc-6-P via the pentose phosphate pathway (PPP) provides pentose for nucleotide synthesis and NADPH, required for reductive reactions, such as lipid synthesis and for protection against oxidative stress. Arteriovenous concentration differences (AV dif) can be used to estimate global cerebral metabolic rate from the disappearance of metabolites from the circulation. PET (depicted in orange) uses radiolabeled glucose analogues (such as FDG), which are trapped early in metabolism (for example fluorodeoxyglucose-6-phosphate/FDG-6-P), to estimate rates of glucose uptake and metabolism. $^{31}$P MRS (depicted in blue) provides information about ATP production and thus brain energy metabolism. $^{13}$C-MRS (depicted in green) is useful for estimating TCA cycle fluxes and CMR$_{glc}$, derived from $^{13}$C label incorporation into specific metabolites (Glu, Gln). Both ALS and BOLD fMRI provide estimates of CBF.
glucose uptake and cerebral metabolic rate in humans with and without type 1 diabetes (71-74). However, this technique cannot be used to study glucose metabolism downstream of its conversion to glucose-6-phosphate (75). Also, animal studies suggest that the lumped constant, a correction factor that relates the metabolic rate of FDG to that of native glucose (76), may increase during hypoglycemia (77; 78). The tracer $[^{11}\text{C}][3\text{-O}-\text{methyl-D-glucose}]$ (3-OMG) may provide more robust information about cerebral glucose uptake at varying glucose concentrations, as it is not phosphorylated (79), but its relative short half-life time (~20 minutes) and complex preparation limits the use of this compound in a clinical setting (80).

**fMRI**

Functional magnetic resonance imaging is primarily used to study regional neuronal activation (patterns) by the detection of changes in oxygen demand by the brain (81), based on the concept of neurovascular coupling described above. Blood oxygenation level dependent (BOLD) contrast is one of the primary contrast mechanisms for fMRI, which exploits the differences in magnetic properties between deoxygenated and oxygenated hemoglobin (82). Regional brain activation increases local oxygen demands, but because the consequent increase in CBF exceeds these demands, the balance between deoxygenated and oxygenated hemoglobin changes towards the latter. This change in hemoglobin oxygenation can be probed and detected, so that a brain map of regions with increased or decreased activation can be constructed (81). fMRI has been especially useful in detecting brain activation patterns in response to specific cognitive tasks or visual stimulation. Hypoglycemia has been reported to reduce regional BOLD activation in response to these tasks, but less so in patients with type 1 diabetes (83) than in non-diabetic subjects (84; 85). These reductions in BOLD responses are commonly attributed to decreased neuronal activity, yet the potential impact of hypoglycemia on (global or regional) CBF, neurovascular coupling or oxidative metabolism, remains to be determined.

**ASL**

Arterial spin labeling (ASL) is an MRI method that provides non-invasive quantification of global and regional CBF. ASL does not require an exogenous contrast agent, but uses magnetically labeled arterial blood water as a diffusible tracer. Arterial blood water is first labeled magnetically using radiofrequency (RF) pulses. Subsequently, this labeled arterial blood flows into the brain where it exchanges with tissue water, after which an image is taken. The experiment is then repeated without labeling the arterial blood to create a control image.
The signal difference between control and labeled images reflects local CBF (86; 87). While the signal to noise (SNR) ratio in BOLD fMRI is higher, ASL measures brain perfusion more directly, enables quantification of CBF, and is suitable for studying variations in CBF over a longer period of time due to stable noise characteristics (88). ASL thus allows the detection of changes in CBF during hypoglycemia and is, in contrast to fMRI, less dependent on other metabolic parameters, such as oxygenation or glucose concentrations that might change during hypoglycemia. A high magnetic field (e.g. 3 Tesla) is usually recommended to improve SNR when performing ASL.

**MRS**

MRS is a non-invasive technique, closely related to MRI. Both techniques make use of the spin properties of certain nuclei when brought into a magnetic field. For MRI, the proton nucleus is used to construct a highly detailed anatomical image based on the different water concentrations in various tissues. For MRS, these spin properties are used to determine the concentration of specific metabolites in the tissue examined. These concentrations are derived from the peaks in a spectrum (89). MRS is feasible on any nucleus possessing a magnetic moment, but is most frequently performed on the high natural abundant and MR sensitive proton nucleus (\(^{1}H\)), providing steady-state information on concentrations of proton-containing brain metabolites at a single time point (90). However, because water contains most of the proton nuclei, the water signal needs to be suppressed to allow reliable measurements of metabolite concentrations. As a consequence, the sensitivity of MRS is manifold lower than that of MRI, even at high magnetic fields. Nevertheless, since nearly all metabolites contain protons, \(^{1}H\)-MRS is a powerful technique to identify and quantify a large number of metabolites relevant for glucose metabolism (e.g. lactate, glutamate, glutamine) at *in vivo* concentrations typically above 0.5 mM.

The use of carbon-13 (\(^{13}C\)) in MRS is specifically relevant for the study of brain glucose metabolism. Carbon exists in the human body in two isotopes, of which carbon-12 (\(^{12}C\)) is dominant with a natural abundance of 98.9%. \(^{12}C\) does not possess a net nuclear spin and consequently cannot be detected by MRS. In contrast, \(^{13}C\) does possess a magnetic moment, but has a very low natural abundance of 1.1%. However, the intravenous infusion of \(^{13}C\)-enriched substrates, such as [\(^{1-13}C\)]glucose, [\(^{3-13}C\)]lactate or [\(^{3-13}C\)]acetate, offers the possibility to study fluxes of these substrates in the brain through important metabolic pathways (Fig. 2).
Fig. 2 Time series of $^{13}$C-MR spectra, acquired from a ~125 mL voxel, placed in the occipital cortex. Spectra are averaged over 20 minutes, after administration of [1-$^{13}$C]glucose during a hypoglycemic clamp in one healthy subject. Once the infused [1-$^{13}$C]glucose is taken up by the brain and incorporated into various glucose metabolites, an increase in signal over time is observed. Numbers indicate the position of the $^{13}$C label, as explained in more detail in Fig. 3. Asp; aspartate; Gln, glutamine; Glu, glutamate; Lac, lactate (from: (143), with permission from Elsevier).
**Fig. 3** One-compartment metabolic model describing the incorporation of $^{13}$C label from (infused) [1-$^{13}$C]glucose into the TCA cycle and its metabolites. When taken up by the brain, the $^{13}$C-label is first incorporated into the C3 position of pyruvate and subsequently into the C3 position of lactate. Once the $^{13}$C-label continues through the TCA cycle, it is incorporated into the C4 position of αKG, glutamate and glutamine. In the second turn of the cycle, the label is equally distributed over the C2 and C3 positions of these metabolites. The TCA cycle flux can be estimated using a metabolic model where the time courses of the uptake of the $^{13}$C-label in glutamate and glutamine in the different carbon positions, measured with $^{13}$C-MRS, are used as input. Filled circles represent the carbon position that is labeled with $^{13}$C, white circles represent unlabeled carbons. αKG, α-ketoglutarate; BBB, blood-brain-barrier; Glc, glucose; Gln, glutamine; Glu, glutamate; Lac, lactate; LDH, lactate dehydrogenase.; Pyr, pyruvate; $V_{\text{gln}}$, exchange rate between glutamate and glutamine; $V_{\text{TCA}}$, TCA cycle rate; $V_{\text{αKG}}$, exchange rate between α-ketoglutarate and glutamate.
Brain glucose metabolism during hypoglycemia

For the study of brain glucose metabolism, it is important to note that [1-13C] glucose is taken up and metabolized by the brain similar to native (i.e. unlabeled) glucose. Following transport across the blood-brain barrier and phosphorylation by hexokinase, glucose is the main substrate for the production of energy (by formation of ATP) via glycolysis and the tricarboxylic acid (TCA) cycle (82). As such, 13C-MRS allows the fate of the 13C-labeled glucose to be followed as it flows into glycolysis. The 13C-label is transferred from glucose on the C-1 position to pyruvate on the C-3 position during glycolysis and, subsequently, passes through all metabolites of the TCA cycle. In this process, the 13C-label is incorporated into the MRS-detectable metabolites glutamate, glutamine and aspartate, all at specific carbon positions. Because these positions change during the second time the isotope flows in the TCA cycle (Fig. 3), the time-course of 13C-label incorporation into these metabolites can be used as input for a metabolic model to calculate the TCA cycle flux and CMRglc (91). However, although the fates of individual carbon atoms can be tracked in the TCA cycle, cerebral 13C-MRS provides no information about the loss of label in diffusible metabolites, such as glutamine and lactate which may exchange between brain and blood plasma (82; 92).

13C-MRS has been proven to be a valuable imaging technique to study brain (glucose) metabolism via specific pathways in humans in vivo, under various conditions, including hyperglycemia (93) and hypoglycemia (94). In addition, 13C labeled compounds other than glucose, such as 13C-acetate and 13C-lactate, can be used to provide a more complete picture of the very complex metabolic processes in the brain. Indeed, 13C-acetate, which is metabolized almost exclusively in astroglia (95), has been used to distinguish astroglial and neuronal metabolism more directly, and to study transport and metabolism of non-glucose fuels during hypoglycemia (96; 97).

Phosphorus-31 (31P) is another naturally abundant nucleus with a relatively high sensitivity for MRS. 31P-MRS of the brain can be used to detect metabolites that play a key role in brain energy metabolism and provides information on flux through the creatine kinase reaction (e.g. ATP, phosphocreatine, inorganic phosphate), intracellular pH and magnesium concentrations (98). Thus far, this technique has been seldom used to study brain metabolism during hypoglycemia (99).
Glucose uptake

As mentioned above, glucose uptake into the brain occurs through facilitated transport independent of insulin. As a consequence, there is a linear relationship between plasma glucose concentrations and brain glucose content over a range of plasma glucose values up to ~30 mmol/L (100-103). This linear relationship also extends well into the hypoglycemic range, although data below plasma levels of ~2.5mmol/L are missing in humans (Fig.4)(104). To explain HAAF, it has been hypothesized that chronic or repeated hypoglycemia increases glucose transport capacity over the blood-brain barrier to compensate for the fall in glucose availability to the brain during subsequent hypoglycemia. Indeed, several animal studies have shown that days to weeks of chronic hypoglycemia cause upregulation of brain glucose transporters, including both GLUT-3 on neuronal membranes (105; 106), and GLUT-1 on the vascular endothelium at the blood-brain barrier (107; 108). In accordance, Boyle and co-workers applied the Kety and Schmidt technique to show preservation of brain glucose transport rather than a fall during hypoglycemia in healthy volunteers after prior exposure to hypoglycemia, whereas it fell when such exposure had not taken place (65).

**Fig. 4** Linear relationship between plasma and brain glucose levels under normo- and hypoglycemic conditions in healthy subjects (open squares) and patients with type 1 diabetes(closed circles). Brain glucose levels were measured with 13C-MRS. The plasma versus brain glucose relation was fitted with linear regression analysis to determine reversible Michaelis-Menten kinetics to show the best fit of the data with 95% confidence intervals. R²=0.59. P<0.001. Assuming continuation of this linear relationship between plasma and brain glucose levels, brain glucose approaches zero at a plasma glucose level of approximately 1.2 mmol/L (from: (104), with permission from the American Diabetes Association).

**Cerebral nutrient transport capacity and hypoglycemia**

**Glucose uptake**

As mentioned above, glucose uptake into the brain occurs through facilitated transport independent of insulin. As a consequence, there is a linear relationship between plasma glucose concentrations and brain glucose content over a range of plasma glucose values up to ~30 mmol/L (100-103). This linear relationship also extends well into the hypoglycemic range, although data below plasma levels of ~2.5mmol/L are missing in humans (Fig.4)(104). To explain HAAF, it has been hypothesized that chronic or repeated hypoglycemia increases glucose transport capacity over the blood-brain barrier to compensate for the fall in glucose availability to the brain during subsequent hypoglycemia. Indeed, several animal studies have shown that days to weeks of chronic hypoglycemia cause upregulation of brain glucose transporters, including both GLUT-3 on neuronal membranes (105; 106), and GLUT-1 on the vascular endothelium at the blood-brain barrier (107; 108). In accordance, Boyle and co-workers applied the Kety and Schmidt technique to show preservation of brain glucose transport rather than a fall during hypoglycemia in healthy volunteers after prior exposure to hypoglycemia, whereas it fell when such exposure had not taken place (65).
investigators went on to report similar findings of preserved glucose transport in patients with type 1 diabetes and near-normal glycosylated hemoglobin (HbA\textsubscript{1c}), possibly reflecting high hypoglycemic burden, as they also had reduced awareness of hypoglycemia (66).

In mice and in rats, very low plasma glucose values, typically well below 2.0 mmol/L, have been found to proportionally increase brain glucose uptake as a function of increased cerebral perfusion (102; 109). Various neuroimaging studies investigating glucose transport over the blood-brain barrier in humans have produced conflicting results. A \textsuperscript{1}H-MRS study performed under hyperglycemic conditions showed greater brain glucose concentrations in patients with type 1 diabetes and impaired awareness of hypoglycemia than in people without diabetes (110). However, a similar study found no evidence of altered brain glucose transport in healthy volunteers subjected to antecedent repeated hypoglycemia, despite clearly attenuated hormone responses to hypoglycemia (111). In accordance, global blood-to-brain glucose transport, as measured with \textsuperscript{[1-11C]}-glucose PET, remained unaltered in healthy volunteers after exposure to 24 hours of moderate hypoglycemia, albeit interspaced with transient glucose normalizations during meals (112). Finally, a 3-OMG-PET study also showed no differences in global brain glucose transport during hypoglycemia between patients with normal and those with impaired awareness of hypoglycemia (72).

**Monocarboxylic acid (MCA) uptake**

Although glucose is its principal source of energy, the brain may resort to alternative non-glucose fuel substrates under glucopenic conditions. These alternative substrates include foremost ketones, and lactate, which enter the TCA cycle after conversion to pyruvate or acetyl coenzyme A, and can be metabolized in a similar way as glucose to sustain brain metabolism, and spare glucose.

Ketones such as beta-hydroxybutyrate and acetoacetate are synthesized in the liver from fatty acids during prolonged fasting, starvation and severe carbohydrate restriction. Under such conditions, up to 60% of brain energy requirements may be derived from ketone metabolism (113), whereas ketogenic diets can more or less restore brain energy metabolism and prevent epileptic seizures in patients with GLUT1 deficiency who are unable to transport glucose into the brain (114; 115). However, because insulin suppresses the production of ketones, the brain is usually unable to use this source of energy during insulin-induced hypoglycemia (116). PET studies with the use of both ketone
and glucose tracers may help to unravel the complex interaction between the metabolism of ketones and glucose by the brain under different circumstances, including hypoglycemia (113).

In recent years, it has gradually been recognized that lactate plays an important role in the energy metabolism in the brain, particularly during hypoglycemia since both hypoglycemia and insulin increase plasma levels of lactate, at least in healthy subjects (94; 117; 118). Under basal, euglycemic, conditions, the contribution of systemic lactate to cerebral energy metabolism is approximately 8-10%. However, the proportional contribution of lactate has been reported to increase during strenuous exercise, when plasma lactate levels rise substantially (119; 120). The role of lactate in specific areas of the brain includes its involvement in or interference with hypoglycemia detection in the VMH as stated above. Lactate has also been found to be a crucial monitored variable in the detection of energy imbalance in the caudal hindbrain (121).

The importance of lactate for the brain was first highlighted when Pellerin and Magistretti published their astrocyte-neuron lactate shuttle (ANLS) hypothesis. This hypothesis posits that glucose is taken up by and metabolized in astrocytes to form lactate, after which lactate is exported to neighboring neurons where it is oxidized, especially during activation (122). This concept, which bears analogy to the cell-cell lactate shuttle, through which skeletal muscle can transport a non-glucose energy source to other organs (123), thus suggests that astrocytes play the primary role in brain glucose metabolism. Simpson et al. later came to a different conclusion and developed a model that basically adopts the opposite view, in which neurons are the principal site of glucose uptake and metabolism, and the chief exporter of lactate. This hypothesis was therefore termed the neuron-astrocyte lactate shuttle (NALS) (59) and fuelled a heavy debate (124; 125). The debate on the direction of the lactate shuttle is ongoing with studies identifying the neuron as the principal locus of glucose uptake (126), and other studies indicating that neurons rather than astrocytes are the primary sites for oxidation of exogenous lactate (119; 127).

Monocarboxylic acid transporters (MCTs) facilitate the uptake of lactate as well as that of acetate and ketone bodies into the brain, the expression of which may increase following sustained hyperketonemia or recurrent hypoglycemia. A recent study in rats demonstrated a two-fold increase in the expression of MCTs 1 and 2 in the cerebral cortex after the induction of diabetes by streptozotocin. After 8 weeks of frequent, prolonged endurance training and concomitant exposure to hypoglycemia after and between exercise sessions, the expression of
both transporters increased even further (128). Such greater transport capacity may explain recent observations in which recurrent exposure to hypoglycemia increased the uptake of $^{13}$C-labeled lactate into the rat brain under hypoglycemic conditions (129). During hypoglycemia, the uptake of both acetate and lactate into the human brain, as measured by $^{13}$C-MRS during infusion of $^{13}$C-labeled acetate or lactate, respectively, was found to be considerably greater in patients with well-controlled type 1 diabetes than in healthy controls (97; 130).

**Transport and uptake of other substrates**

Oral intake of amino acids has been reported to enhance the glucagon response to hypoglycemia and to improve some aspects of cognitive function during hypoglycemia in non-diabetic and diabetic subjects (131; 132). Amino acids might also serve as a non-glucose substrate that could be used by the brain as an alternative fuel and to sustain cognitive function during hypoglycemia. Early studies showing utilization of amino acids by the rat brain during prolonged hypoglycemia and of amino acids contributing to glycogen synthesis in brain cell cultures, supported this theory (133; 134). However, data obtained in humans using arteriovenous concentration differences found no evidence that greater availability of amino acids increased its net brain uptake during hypoglycemia (135) or was able to offset energy deficit due to reduced glucose supply (136).

A few studies have investigated whether the human brain can use lipid substrates to support cerebral metabolism and brain function during hypoglycemia. Fatty acids can readily cross the blood-brain barrier to be oxidized by the brain, as demonstrated by a $^{13}$C-MRS study in rats (137). In healthy humans, elevated plasma levels of non-esterified fatty acids and glycerol were found to reduce hormonal and symptom responses to hypoglycemia, but could not protect against the fall in cognitive function (138). Conversely, in a more recent study, ingestion of medium-chain triglycerides maintained cognitive function during hypoglycemia without affecting adrenergic or symptomatic responses to hypoglycemia in intensively treated subjects with type 1 diabetes (139). It should be acknowledged, however, that the inferences made with respect to the uptake of lipid substrates in the brain were indirect and that no neuroimaging studies have been performed that evaluated the effects of these substances on cerebral metabolism more directly.
Brain metabolism during hypoglycemia

*Glucose metabolism*

Both PET and MRS have been used to investigate the effect of hypoglycemia on brain glucose metabolism. PET has been particularly useful in detecting regional differences in tracer accumulation in the brain, both during hypoglycemia (71), and after restoration to euglycemia (74). However, rather than focusing on glucose uptake or metabolism, the close link with neuronal activation is then exploited to use the data as input factors for mapping regional brain activity. Thus, the observation that CMR$_{\text{glc}}$ relatively increased during hypoglycemia in patients with type 1 diabetes and normal awareness of hypoglycemia, and relatively fell in patients with impaired awareness of hypoglycemia, was interpreted as an increase in brain activation and absence of such a response (72). When this increased activation would occur in brain areas involved in the perception of and the generation of responses to hypoglycemia, the lack of increased activation could then underlie loss of hypoglycemic awareness (72; 140). Support for this hypothesis came from another FDG-PET study (141) and a subsequent analysis of these data (73), as tracer uptake in areas that engage appetite control and food-seeking behavior was reduced in patients with impaired compared to patients with intact awareness of hypoglycemia.

As outlined above, $^{13}$C-MRS in combination with infusion of $^{13}$C-labeled glucose has the unique property that it enables the investigation of cerebral glucose metabolism in humans *in vivo*. Since the SNR is relatively low, most studies employing this technique used large doses of isotopically enriched glucose at high enrichment percentages. Measurements have consequently generally been performed under hyperglycemic conditions with glucose levels up to 17 mmol/L and plasma C-13 enrichment values exceeding 60%. Under such conditions, Henry et al. (93) reported no differences in the TCA cycle rate between patients with type 1 diabetes with impaired awareness of hypoglycemia and healthy controls. More recently, an improved sensitivity of the $^{13}$C-MRS method (142) in combination with an optimized $^{13}$C-glucose infusion protocol enabled us to study glucose metabolism in the human brain during hypoglycemia at lower enrichment values (143). With this optimized technique, no differences were observed in cerebral glucose metabolism between hypoglycemia and euglycemia, neither in healthy controls (94), nor in patients with type 1 diabetes (144). Under hypoglycemic conditions, however, the TCA cycle rate was approximately 45% higher in patients than in healthy subjects, and inversely related to HbA$_{1c}$. Appreciating a low HbA$_{1c}$ as a proxy for a high hypoglycemic burden, these data suggested a role for prior hypoglycemic exposure in the higher TCA cycle rate in
patients with type 1 diabetes. Differences in brain glucose levels did not explain the preservation of brain metabolism and the higher TCA cycle rate in the patients, which suggested influx of a non-glucose carbohydrate source (104). In an animal study by Herzog et al. (129), brain glucose transport capacity during hypoglycemia became rate limiting for TCA cycle activity in control animals, but not in rats exposed to antecedent recurrent hypoglycemia. Explanations for the discrepancy between the human and rodent data include the different species and the fact that the hypoglycemic condition was more profound in the animals. Indeed, studies in mice suggest that intracellular brain glucose concentrations approach depletion at plasma glucose values between 2 and 3 mmol/L (109).

**Glycogen metabolism**

The brain is able to store glycogen and to use this compound when plasma glucose levels are low, although its capacity to do so is very limited compared to other tissues such as skeletal muscle and the liver. It was long assumed that this presence of glycogen was restricted to astrocytes. However, a recent study showed that neurons contain a low but measurable amount of glycogen, the use of which was found to protect against hypoxic stress, at least in neuronal cell cultures and animal models (145). Both in rodents (146) and in humans (147), it was shown that brain glycogen was used during hypoglycemia, and that its stores were replenished above baseline levels after restoration of euglycemia, a phenomenon termed glycogen supercompensation. It has been speculated that this expanded source of glucose within the brain could contribute to the development of impaired awareness of hypoglycemia by fuelling the brain or at least those areas involved in glucose-sensing during subsequent hypoglycemia (146). However, prior exposure to recurrent hypoglycemia neither facilitated nor impaired access to glucose from glycogen in the rat brain during subsequent hypoglycemia (148). Additionally, brain glycogen content, as measured by $^{13}$C-MRS in conjunction with $^{13}$C-glucose administration, was lower rather than higher in patients with type 1 diabetes and hypoglycemia unawareness (149).

**Glutamate metabolism**

Glutamate is the major excitatory neurotransmitter in the brain, but has many other metabolic fates, including the formation of glutamine, GABA and glutathione (150). In addition, a new concept has been introduced by Sonnewald (151), who proposed that glutamate degradation in astrocytes contributes to most of the lactate that is released from the brain under resting conditions, offering a novel explanation for the concept of aerobic glycolysis in the resting state (151; 152). Lastly, glutamate can be oxidized for the production of energy
(150). To this end, glutamate production in the brain is tightly coupled to TCA cycle activity (153). Using 1H-MRS, Bischof et al. reported that hypoglycemia reduced the cerebral glutamate to creatine ratio in healthy controls, but not in patients with type 1 diabetes (154). Similar results were reported by a more recent 1H-MRS study, where hypoglycemia reduced brain glutamate levels in healthy controls and in patients with type 1 diabetes with normal hypoglycemic awareness, but not in patients with impaired awareness of hypoglycemia (155). The authors concluded that the preservation of brain glutamate during hypoglycemia in the latter group reflected a metabolic adaptation that eliminated the need to oxidize glutamate. They speculated that this adaptation could be augmented transport of glucose or of alternative fuels to the brain.

**Metabolism of monocarboxylic acids**

As discussed earlier, MRS studies using 13C-labelled acetate and lactate have clearly suggested that the capacity to transport MCAs over the blood-brain barrier during hypoglycemia is increased in patients with well-controlled type 1 diabetes. Indeed, a study during which 13C-acetate was infused under hypoglycemic conditions showed more than twofold higher brain acetate concentrations in subjects with type 1 diabetes compared to healthy controls. This greater acetate availability translated into a fraction of oxidative metabolism that resulted from acetate to be similarly increased (97). In accordance, the relative contribution of acetate to brain metabolism in rats exposed to recurrent antecedent hypoglycemia was also increased during next-day hypoglycemia, indicating that brain substrate preferences may change rapidly from glucose to alternative substrates if needed (156). To delineate whether this effect was a function of diabetes, prior hypoglycemia or both, the investigators repeated their 13C-acetate study in patients with type 1 diabetes with normal or impaired awareness of hypoglycemia and in healthy controls. They found that absolute rates of acetate metabolism during hypoglycemia were only higher in the patients with impaired awareness of hypoglycemic, suggesting that changes in acetate metabolism are the consequence of prior exposure to hypoglycemia rather than of diabetes per se (96).

Lactate uses the same MCT as acetate to cross the blood-brain barrier. Since plasma levels of lactate are approximately 10-fold higher than those of acetate, and hypoglycemia stimulates the production of lactate (117), it seems plausible that lactate is the more likely substrate for brain metabolism when glucose levels are low. Studies dating back to the 1990s have shown that exogenous administration of lactate attenuates counterregulatory responses
to and preserves cognitive function during hypoglycemia, presumably because lactate is used as an alternative source of energy by the brain (157-160). In agreement, brain lactate concentrations during hypoglycemia, derived from the cerebral uptake of $^{13}$C-labelled lactate, were several fold higher in patients with type 1 diabetes with a history of frequent hypoglycemic episodes than in non-diabetic subjects (130) and in rats exposed to recurrent hypoglycemia versus those not exposed (129). Surprisingly, the authors found no indication of greater lactate oxidation, as reflected by unchanged $^{13}$C fractional enrichments of brain glutamate and glutamine (130). Data from the rodent study, in which lower glucose levels were achieved than in the human study, suggested that prior hypoglycemic exposure increased both the uptake and the oxidation of glucose by the brain, despite the higher lactate levels (129). However, when the animal brain was stimulated during hypoglycemia, animals exposed to recurrent hypoglycemia had a partial loss of their functional cortical response, which was only normalized after the administration of lactate. This suggests that the higher capacity for lactate transport only becomes critical when the brain is activated during (deep) hypoglycemia.

**Cerebral blood flow and hypoglycemia**

There is uncertainty as to whether hypoglycemia affects global CBF and in what direction. Previous research in both patients with type 1 diabetes and healthy controls has reported either no change in global CBF during hypoglycemia (65; 66; 136), a modest increase (161-163), or even a slight decrease (71). Differences in the plasma glucose levels achieved during hypoglycemia and, more importantly, in imaging techniques probably explain many of the discrepancies. Studies that investigated the effect of hypoglycemia on regional relative changes in CBF seem to have produced more consistent data. Both in healthy controls (161) and in patients with type 1 diabetes (164), hypoglycemia was found to increase blood flow to the frontal lobes. This relative redistribution of regional CBF was already observed under euglycemic conditions in patients with type 1 diabetes, and was more pronounced in patients who had experienced frequent hypoglycemia (165). Since the frontal lobes are among the most vulnerable brain areas to suffer structural damage, this may be an adaptive response to prevent such damage by maintaining fuel supply during subsequent hypoglycemia.

Hypoglycemia has also been found to increase CBF in the thalamus (71; 166; 167) and hypothalamus (168; 169). Mild or moderate hypoglycemia caused a rise in CBF in the hypothalamus in healthy non-diabetic subjects, as assessed by fMRI (168) or ASL (169), which preceded the rise in counterregulatory
hormone responses seen during hypoglycemia (169). Interestingly, Mangia et al. found blunting of this increase in thalamic perfusion during hypoglycemia in patients with type 1 diabetes with hypoglycemia unawareness, and a correlation between thalamic perfusion and the adrenaline response to hypoglycemia (167). In contrast, recurrent hypoglycemia enhanced, rather than decreased, thalamic perfusion during subsequent hypoglycemia in healthy controls (170), so that the role of this brain region in the adaptation to hypoglycemia remains uncertain.

**Discussion**

The scientific field of metabolic and functional neuroimaging techniques for the brain has tremendously progressed over the past couple of decades. The application of these techniques to hypoglycemia research has considerably advanced our understanding of the brain’s responses to hypoglycemia. As plasma glucose falls below levels that can be reversed by responses at the level of pancreatic islets, i.e. suppression of insulin release and stimulation of that of glucagon, the brain’s sensing abilities are activated to allow timely detection of hypoglycemia. Data from functional and metabolic neuroimaging techniques now suggest that such moderate hypoglycemia neither affects the perfusion to nor the uptake of glucose into the brain, at least not globally, unless much deeper levels of glucose are achieved (102; 109). In accordance, cerebral glucose metabolism appears largely maintained during moderate hypoglycemia (94; 129; 144). However, on the regional level, moderate hypoglycemia causes redistribution of CBF to various brain areas involved in the detection of hypoglycemia, particularly the (hypo)thalamus (71; 166-169), where enhanced neuronal activation stimulates glucose uptake and metabolism. Such enhanced neuronal activation has also been found to occur in brain areas involved in appetitive motivational networks (73), thus linking the detection of hypoglycemia to a behavioral response.

Modern neuroimaging studies have revealed that recurrent hypoglycemia, which typically affects people with type 1 diabetes and underlies the clinical syndrome of impaired awareness of hypoglycemia, may initiate cerebral adaptations at many different levels. First, there is interference with the accurate detection of hypoglycemia, probably occurring at the level of the VMH. Brain areas that control appetite and induce fear and anxiety may not become activated during hypoglycemia. Whether or not locally increased glucose uptake in or reduced neuronal activation of the hypothalamic area (or both) form the underlying
mechanism remains to be revealed. Importantly, it should be acknowledged that neurovascular coupling may be altered as a consequence of diabetes per se (171), chronic hyperglycemia (172) or microangiopathy (173), thus limiting the interpretation of studies relying on this concept. There is conflicting evidence as to whether recurrent hypoglycemia can stimulate brain glucose uptake during hypoglycemia (65; 66), although most studies employing neuroimaging techniques found no evidence for this suggestion (72; 111; 112). Nevertheless, patients with type 1 diabetes, particularly those with impaired awareness of hypoglycemia (155), seem better able in maintaining brain (glucose) metabolism during hypoglycemia than healthy controls (94; 144; 154; 155), probably as a consequence of prior hypoglycemia (144). Since profound hypoglycemia will eventually cause brain glucose metabolism to deteriorate (129), such an adaptation may shift the threshold for deterioration of the metabolic rate to lower plasma glucose levels.

Several mechanisms have been proposed that could explain the discrepancy between hypoglycemia-induced preservation of cerebral glucose metabolism and the fall in glucose availability during hypoglycemia. It seems likely that influx of a non-glucose energy substrate plays a role. Recent (neuroimaging) studies found little evidence to support enhanced blood to brain transport of amino acids (135; 136) or lipid substrates transport (138; 139) and ketones are unlikely candidates because its production is suppressed by insulin. Also, the lower brain glycogen content in patients with type 1 diabetes and impaired awareness of hypoglycemia compared to controls (149) argues strongly against the glycogen supercompensation hypothesis. Several arguments suggest a major role for lactate in preserving brain glucose metabolism during hypoglycemia. These include: 1) lactate can be used by the brain and may even be preferred over glucose under non-hypoglycemic conditions (174; 175); 2) the capacity for lactate transport over the blood-brain barrier is increased in patients with impaired awareness of hypoglycemia and in rats after exposure to hypoglycemia (97; 129); 3) the use of lactate by glucose-sensing neurons in the VMH may interfere with hypoglycemia sensing (54; 56; 57). However, there are data that suggest that lactate is not used as major energy source for the brain during moderate hypoglycemia, despite greater availability (129; 130). Also, it is not yet known whether brain uptake or metabolism of endogenously produced lactate is increased during hypoglycemia in patients with type 1 diabetes and impaired awareness of hypoglycemia. Finally, it has been suggested that lactate may serve as a metabolic regulator or intercellular signaling molecule rather than a fuel, modulating brain glucose metabolism, oxygen delivery and CBF
Mechanisms by which lactate might exert this effect (178) include modulation of prostaglandin action (and thus CBF) (177; 179), adjustment of the NADH/NAD⁺ redox ratio (123), and the regulation of neuronal cAMP formation via the lactate receptor G-protein-coupled receptor 81 (GPR81)(180).

Conclusion
Hypoglycemia is the principal barrier for achieving optimal, let alone normal, glycemic control for indefinite periods of time in patients with type 1 diabetes and advanced insulin-requiring type 2 diabetes (181). Recurrent hypoglycemia forms the basis of HAAF and the clinical syndrome of impaired awareness of hypoglycemia by attenuating physiological defenses against subsequent hypoglycemia, consequently increasing the risk for severe hypoglycemia. Paradoxically, the mechanism(s) underlying these glucose counterregulatory impairments may be related to, or even caused by, processes that are seemingly aimed at protecting the brain against harm from severe hypoglycemia. The progress in metabolic and functional neuroimaging techniques has revealed that recurrent hypoglycemia causes cerebral adaptations to occur on many different levels. These adaptations include those in the regional delivery (blood flow) and transport of glucose to the brain, the handling of glucose by the brain and that of non-glucose alternative fuels, as well as activation or de-activation of brain areas involved in behavioral responses. It remains to be elucidated whether, and if so under which circumstances and in which brain areas, the brain uses non-glucose alternative sources of energy, particularly lactate, and whether this contributes to the emergence of impaired awareness of hypoglycemia. Such information is needed first to foster personalized decision-making with respect to glycemic targets, but should eventually lead to treatments that eliminate hypoglycemia from the lives of people with type 1 diabetes without compromising glucose control.

Acknowledgements
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Chapter 3

Brain lactate concentration falls in response to hypoglycemia in patients with type 1 diabetes and impaired awareness of hypoglycemia


*: contributed equally

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Abstract

Brain lactate may be involved in the development of impaired awareness of hypoglycemia (IAH), a condition that affects approximately 25% of patients with type 1 diabetes and increases the risk of severe hypoglycemia. The aim of this study was to investigate the effect of acute hypoglycemia on brain lactate concentration in patients with IAH as compared with those with normal awareness of hypoglycemia (NAH) and healthy control subjects ($n = 7$ per group). After an overnight fast, all subjects underwent a two-step hyperinsulinemic euglycemic (5.0 mmol/L)-hypoglycemic (2.8 mmol/L) glucose clamp. Brain lactate concentrations were measured continuously with 1H-MRS, using a specific lactate detection method. Hypoglycemia generated symptoms in patients with NAH and healthy control subjects, but not in patients with IAH. Brain lactate fell significantly by ~20% in response to hypoglycemia in patients with type 1 diabetes with IAH but remained stable in both healthy control subjects and in patients with NAH. The fall in brain lactate is compatible with increased brain lactate oxidation providing an alternative fuel source during hypoglycemia, which may contribute to the impaired detection of hypoglycemia.
Introduction

Approximately 25% of patients with type 1 diabetes have lost the capacity to timely detect hypoglycemia, a condition referred to as impaired awareness of hypoglycemia (IAH) (1). IAH increases the risk for severe, potentially hazardous hypoglycemia up to sixfold (2) and is usually the end result of a process of habituation to recurrent hypoglycemia (1).

Although the precise mechanisms underlying IAH remain to be revealed, there may be a pivotal role for the alteration in the brain’s handling of energy substrates other than glucose (3). Indeed, using $^{13}$C magnetic resonance spectroscopy (MRS), we found that brain metabolism was largely preserved during hypoglycemia in both subjects without diabetes and subjects with type 1 diabetes, despite a similar fall in brain glucose availability (4-6). These observations indicate that metabolism of a non-glucose carbohydrate energy source may be involved.

Several observations suggest that this nonglucose energy source is lactate. Lactate is a valuable energy source for the brain during euglycemia (7-9) and may be critical to maintaining brain function during severe hypoglycemia (10). Administration of lactate during hypoglycemia impairs hypoglycemic symptoms, attenuates counterregulatory hormone responses, and preserves cognitive function, mirroring the changes seen in subjects with IAH (11; 12). Finally, brain lactate transport capacity through monocarboxylic acid transporters was found to be increased during hypoglycemia in patients with IAH (13; 14).

The brain of patients with type 1 diabetes and IAH may have been conditioned to use lactate under glucopenic conditions to maintain brain function, thereby simultaneously impairing hypoglycemia sensing. We therefore hypothesized that brain lactate levels would fall during hypoglycemia in people with type 1 diabetes and IAH. To test this hypothesis, we measured brain lactate under hypoglycemic conditions with a dedicated $^1$H-MRS method optimized for lactate detection (15).

Research Design and Methods

Subjects

We recruited seven patients with type 1 diabetes and IAH, seven patients with normal awareness of hypoglycemia (NAH), and seven healthy subjects without diabetes. Awareness state was based on the Dutch modified version of the
Cox questionnaire, where scores of 0-1 out of 5 indicate normal awareness and scores ≥3 impaired awareness (16; 17). Patients were eligible if they had an HbA$_{1c}$ <9.0% (75 mmol/mol) and were free from microvascular complications, except for background retinopathy. Exclusion criteria were contraindications for MRI examinations, a history of brain injury or cardiovascular events, and the use of drugs other than insulin interfering with glucose metabolism. The institutional review board of the Radboud university medical center approved the study, and all subjects gave written informed consent.

Hyperinsulinemic glucose clamps
All participants presented at 8:00 A.M. after an overnight fast having abstained from caffeine, alcohol, and smoking for 24 h and from strenuous exercise for three days. Subjects with diabetes were instructed to adjust their basal insulin dose the evening before the clamp to prevent nocturnal hypoglycemia and to omit their morning prandial insulin dose. The brachial artery of the nondominant arm was cannulated under local anesthesia for frequent blood sampling. An intravenous catheter was inserted into the antecubital vein of the contralateral arm to administer glucose 20% (Baxter, Deerfield, IL) and insulin (insulin aspart; Novo Nordisk, Bagsvaerd, Denmark). After cannulation and baseline measurements, the subjects were positioned in the MR scanner, and a two-step hyperinsulinemic (60 mU/m$^2$/min) euglycemic (5.0 mmol/L)-hypoglycemic (2.8 mmol/L) glucose clamp was initiated. During the clamp, arterial plasma glucose and lactate levels were determined every 5 min (Biosen C-Line, EKF Diagnostics, Cardiff, U.K.). Counterregulatory hormone and insulin levels were determined at the end of each glycemic phase. Insulin levels were also measured at baseline. Subjects completed an 18-item semiquantitative symptom questionnaire just prior to initiating the glucose clamp and at the end of the hypoglycemic phase in which symptoms were scored from 0 (none) to 6 (severe).

Analytical methods
Plasma insulin was assessed by an in-house radioimmunoassay (RIA) (18). Plasma adrenaline was measured by high performance liquid chromatography combined with fluorometric detection (19).

MRS protocol
MR measurements were performed at 3T (Tim MAGNETOM Trio; Siemens, Erlangen, Germany) using a 12-channel receive-only head coil. First, an anatomical image was acquired (T1-weighted MPRAGE; 256x256 mm$^2$ field of view, 256 slices, 1 mm$^3$ isotropic voxels). Subsequently, $^1$H-MRS data were
acquired from a 25 cm$^3$ voxel (Fig. 1A), in data blocks consisting of two consecutive acquisitions to determine tissue concentrations of brain lactate and of the other major brain metabolites, respectively. Brain lactate concentrations were determined using an interleaved J-editing semi-LASER sequence (20) optimized for lactate detection (15) (echo time (TE) 144 ms; repetition time (TR) 3,000 ms; 32 averages; total duration of acquisition (TA) 1.40 min). J-editing was performed with MEGA-pulses with a bandwidth of 75 Hz. Spectra with a shorter TE with water suppression were acquired to determine the tissue concentrations of other major brain metabolites (sLASER, TE 30 ms, TR 3,000 ms, 32 averages; TA 1.40 min). Lastly, spectra acquired without water suppression were used for quantification of the metabolite concentrations (TE 30 ms; TR 5,000 ms; 8 averages).

**Figure 1**: Representative MR data from one healthy subject. A: T1-weighted anatomical image with typical location of the voxel (2.0 x 5.0 x 2.5 cm) for the acquisition of the MRS data B: MEGA off, MEGA on, and difference spectra of one subject. J-editing was performed with MEGA-pulses centered on the lactate quartet at 4.1 ppm (MEGA on) and subsequently at -3 ppm (MEGA off). As a consequence, the lactate doublet at 1.3 ppm is inverted in the MEGA off spectrum and upright in the MEGA on spectrum. Subtracting the MEGA on spectrum from the MEGA off spectrum results in the difference spectrum, which contains only the positive lactate doublet, removing the signals from all other metabolites in the spectrum. C: MR spectrum recorded with a TE of 30 ms. Glu, glutamate; ml, myo-inositol; tCre, total creatine; tCho, total choline; tNAA, total N-acetylaspartate; Lac, lactate.

**Analysis of MRS data**

After zero-filling (from 1,024 to 2,048 points) and Fourier Transformation, all J-edited spectra from each subject were phase and frequency aligned with the first spectrum recorded by maximizing the scalar product between this so-called reference spectrum and the other spectra. Difference spectra were apodized with a 5 Hz Lorentzian, and moving averaging with a sliding window of three scans was performed. In the final difference spectra, the lactate doublet was fitted with the AMARES algorithm in jMRUI (21).
Brain lactate concentration during hypoglycemia

The spectra acquired with a TE of 30 ms were analyzed with the LCModel software to quantify the other major brain metabolites, including total N-acetylaspartate, total choline, total creatine, myo-inositol, aspartate, glutamine, glutamate, scyllo-inositol and taurine. Only metabolites with a Cramér-Rao lower bound <20% were considered to be reliably quantified and included in further analyses (22). All metabolite concentrations were calculated taking voxel composition (determined by segmenting the T1-weighted anatomical images using SPM8) and differences in T2 relaxation of metabolite spins into account.

**Statistical analysis**

Within group differences were compared with two-sided Student t tests. Between group differences were analyzed by ANOVA followed by pairwise Bonferroni post hoc tests between all groups. All data are expressed as mean ± SEM unless otherwise indicated. A P value <0.05 was considered statistically significant. Statistical analyses were performed with IBM SPSS Statistics 20.

**Results**

The groups were well matched for relevant parameters (Table 1). Baseline plasma glucose values were elevated to a similar extent in both diabetes groups (Fig. 2A). During the clamp, plasma glucose levels (mean ± SD) were sequentially clamped at 5.0 ± 0.1 and 2.8 ± 0.1 mmol/L without differences between the groups (Fig. 2A). Insulin levels were also comparable during the clamps (data not shown).

Hypoglycemic symptom scores increased significantly in response to hypoglycemia in both healthy volunteers and in patients with NAH but not in patients with IAH (mean increase: 2.0 ± 0.9, 12.9 ± 3.9 and 17.4 ± 3.7 for patients with IAH, patients with NAH and healthy control subjects, respectively). Adrenaline responses to hypoglycemia were lower in patients than in healthy volunteers (P < 0.05), particularly in those with IAH, although the difference between the two patient groups was not statistically significant (P = 0.88) (Supplementary Table 1).

Baseline plasma lactate levels were similar across the three groups, but time courses during the clamp were different (Fig. 2B). During the hypoglycemic phase of the clamp, mean plasma lactate levels were significantly higher in healthy subjects than in subjects with diabetes (P < 0.01).
**Table 1: Subject characteristics**

<table>
<thead>
<tr>
<th></th>
<th>T1DM IAH (n = 7)</th>
<th>T1DM NAH (n = 7)</th>
<th>Healthy subjects (n = 7)</th>
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</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>24.7±8.1</td>
<td>26.2±5.8</td>
<td>27.6±6.9</td>
</tr>
<tr>
<td>Sex, M/F</td>
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<td>4/3</td>
<td>3/4</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
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<td>23.5±1.7</td>
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<tr>
<td>HbA₁c, % (mmol/mol)</td>
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<td>7.3±0.4 (56.6±3.8)</td>
<td>-</td>
</tr>
<tr>
<td>Duration of diabetes, years</td>
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<td>10.0 (6.0-14.0)</td>
<td>-</td>
</tr>
<tr>
<td>Score on modified Cox questionnaire (range)</td>
<td>3.7±0.8 (3-5)</td>
<td>0.4±0.5 (0-1)</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are presented as n, mean ± SD, or median (interquartile range). F, female; M, male; T1DM, type 1 diabetes mellitus.

**Figure 2:** Time courses of plasma glucose (A) and plasma lactate (B). The dashed lines represent the beginning of the euglycemic phase, the end of the euglycemic phase, and the beginning of the hypoglycemic phase, respectively. Baseline values represent the sample obtained upon arrival at the research facility. Black triangles, patients with type 1 diabetes (T1DM) and IAH; black squares, patients with type 1 diabetes and NAH; open circles, healthy subjects.
In one patient with NAH, the $^1$H-MR spectral quality was insufficient for analysis because of head movement during data acquisition. The J-edited difference spectra of all other subjects showed a clear lactate doublet at 1.3 ppm (Fig. 1B). The MR voxel contained 65.5 ± 2.9% white matter, 31.2 ± 2.8% gray matter and 3.2 ± 0.5% cerebral spinal fluid, with no differences between groups (data not shown).

Brain lactate concentration dropped from 0.52 ± 0.02 to 0.41 ± 0.02 µmol/g wet weight (ww) in response to hypoglycemia in patients with IAH ($P < 0.001$), corresponding with a fall of ~20% (Fig. 3). In contrast, brain lactate concentrations remained stable during euglycemia and hypoglycemia in both healthy subjects (0.49 ± 0.02 versus 0.46 ± 0.01 µmol/g ww, $P = 0.12$) and patients with NAH (0.46 ± 0.03 versus 0.45 ± 0.03 µmol/g ww, $P = 0.73$). There were no differences between groups in absolute brain lactate concentrations during euglycemia ($P = 0.17$) or during hypoglycemia ($P = 0.36$).

$^1$H-MR spectra without editing and a TE of 30 ms (Fig. 1C) revealed a significant drop in brain glutamate concentrations in response to hypoglycemia in healthy subjects (from 6.0 ± 0.3 to 5.7 ± 0.3 µmol/g ww $P < 0.01$) but not in patients with NAH (6.6 ± 0.3 vs. 6.4 ± 0.3 µmol/g ww, $P = 0.13$) or in patients with IAH (7.3 ± 0.3 versus 7.1 ± 0.3 µmol/g ww, $P = 0.11$). There were no significant changes in response to hypoglycemia regarding other major brain metabolites.

**Figure 3:** Hypoglycemia-induced changes in brain lactate. Mean (with SEM) group differences (horizontal bars) as well as individual changes (black squares) between average euglycemic and hypoglycemic brain lactate concentrations (percent change from euglycemic value) are depicted. *$P < 0.001$ for euglycemia vs. hypoglycemia and *$P < 0.05$ vs. T1DM NAH and healthy subjects.
Discussion

The major finding of this study is that brain lactate concentrations decrease by ~20% in response to hypoglycemia in patients with type 1 diabetes and IAH but not in patients with type 1 diabetes and NAH or in healthy control subjects. This finding suggests that adaptations in cerebral lactate handling are involved in the etiology of IAH.

A recent \(^1\)H-MRS study also reported decreased brain lactate concentrations in response to hypoglycemia, albeit this change was only significant in patients with diabetes and normal adrenaline responses to hypoglycemia (23). However, the MR methods in that study were focused on glutamate detection, and patients were stratified according to the observed adrenaline response to hypoglycemia rather than according to the awareness of hypoglycemic symptoms.

A change in brain lactate concentration reflects a change in the balance between uptake, export, production (through glycolysis), and oxidation of cerebral lactate (24). The hypoglycemia-induced reduction in brain lactate in patients with IAH most likely resulted from increased lactate oxidation as an adaptation to recurrent exposure to hypoglycemia to preserve brain metabolism when glucose supply is low. Our observation that plasma lactate levels fell in the IAH group argues against increased brain lactate export. Furthermore, it is unlikely that the lower brain lactate levels were the result of decreased cerebral lactate uptake, given that plasma lactate levels fell to a similar extent in both patient groups and that lactate transport capacity has been reported to be increased in patients with IAH (14). We cannot completely exclude that the fall in lactate reflected a decrease in glycolysis due to reduced neuronal activation (25).

In a recent \(^{13}\)C-MRS study, De Feyter et al. (13) showed that the human brain oxidizes \(^{13}\)C-labeled lactate that was infused during hypoglycemia. Somewhat surprisingly, they found no differences in lactate oxidation between patients with diabetes and healthy control subjects, despite a higher calculated brain lactate concentration in the patients with diabetes, which seems at odds with our findings. However, inherit to their study design, infusion of \(^{13}\)C-lactate may have resulted in greater brain lactate availability. Therefore, the physiological context (blood lactate levels and its source, pH, etc.) may be different, which renders comparison with our data difficult.
The strengths of our study include the ability to detect and quantify brain lactate concentrations in vivo in humans in a direct and optimized manner without the use of exogenous lactate and the three distinctly different groups of subjects, which enabled us to differentiate between the impact of diabetes and IAH. Although MR spectra were recorded continuously, 1H-MRS does not provide information about lactate fluxes or consumption, which is a limitation of our study.

In conclusion, we found that brain lactate concentration dropped in response to acute hypoglycemia in patients with type 1 diabetes and IAH, but not in the other two groups. The fall in brain lactate is compatible with increased brain lactate oxidation during hypoglycemia in patients with IAH, and hence, the need for glucose by the brain and the consequent initiation of hypoglycemic symptoms are suppressed. Together our findings indicate that changes in brain lactate levels play an important role in the pathophysiology of IAH.

Acknowledgments
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Contributors
EW, HR, BdG and MvdG designed the study with input from CT and AH. HR recruited the participants and performed the glucose clamps. EW and HR collected the data. EW and MvdG analysed the MR data, HR was responsible for all other data analysis. All authors discussed the results and implications and commented on the manuscript at all stages. BdG and MvdG are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
References

Brain lactate concentration during hypoglycemia


Supplementary Material

Supplementary Table 1. Counterregulatory hormone levels

<table>
<thead>
<tr>
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<th>T1DM IAH</th>
<th>T1DM NAH</th>
<th>Healthy subjects</th>
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<tr>
<td></td>
<td>Euglycemia</td>
<td>Hypoglycemia</td>
<td>Euglycemia</td>
</tr>
<tr>
<td>Glucagon (pmol/L)</td>
<td>9.57 ± 2.61†</td>
<td>22.71 ± 8.24*</td>
<td>11.29 ± 1.52</td>
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<td>Adrenaline (nmol/L)</td>
<td>0.30 ± 0.07</td>
<td>1.50 ± 0.25*</td>
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<tr>
<td>Noradrenaline (nmol/L)</td>
<td>1.29 ± 0.14</td>
<td>1.48 ± 0.18</td>
<td>1.01 ± 0.13</td>
</tr>
<tr>
<td>Cortisol (µmol/L)</td>
<td>0.44 ± 0.10</td>
<td>0.62 ± 0.09*</td>
<td>0.54 ± 0.11</td>
</tr>
<tr>
<td>hGH (mU/L)</td>
<td>3.80 ± 2.47</td>
<td>73.97 ± 11.62*</td>
<td>10.35 ± 6.01</td>
</tr>
</tbody>
</table>

Data is presented as mean ± SEM. *P <0.05 for euglycemia versus hypoglycemia and †P <0.05 versus healthy subjects.

Plasma glucagon was measured by RIA, with a commercially available kit (Eurodiagnostica, Malmö, Sweden). Plasma growth hormone and cortisol were determined using a routine analysis method with an Electrochemiluminescent Immunoassay on a Modular Analytics E170 (Roche Diagnostics, GmbH, Manheim, Germany). Plasma adrenaline and noradrenaline were measured by HPLC combined with fluorometric detection (1).

Reference

Chapter 4

Effect of lactate administration on brain lactate levels during hypoglycemia in patients with type 1 diabetes


*: contributed equally

Abstract

Administration of lactate during hypoglycemia suppresses symptoms and counterregulatory responses, as seen in patients with type 1 diabetes and impaired awareness of hypoglycemia (IAH), presumably because lactate can substitute for glucose as a brain fuel. Here, we examined whether lactate administration, in a dose sufficient to impair awareness of hypoglycemia, affects brain lactate levels in patients with normal awareness of hypoglycemia (NAH). Patients with NAH ($n = 6$) underwent two euglycemic-hypoglycemic clamps (2.8 mmol/L), once with sodium lactate infusion (NAH w|lac) and once with saline infusion (NAH w|placebo). Results were compared to those obtained during lactate administration in patients with IAH ($n = 7$) (IAH w|lac). Brain lactate levels were determined continuously with J-difference editing $^1$H-MRS. During lactate infusion, symptom and adrenaline responses to hypoglycemia were considerably suppressed in NAH. Infusion of lactate increased brain lactate levels modestly, but comparably, in both groups (mean increase in NAH w|lac: $0.12 \pm 0.05 \mu$mol/g and in IAH w|lac: $0.06 \pm 0.04 \mu$mol/g). The modest increase in brain lactate may suggest that the excess of lactate is immediately metabolized by the brain, which in turn may explain the suppressive effects of lactate on awareness of hypoglycemia observed in patients with NAH.
Introduction

Hypoglycemia is the most frequent side-effect of insulin therapy in patients with type 1 diabetes. When blood glucose levels drop, this normally initiates a hierarchically organized counterregulatory response, which includes release of counterregulatory hormones and the appearance of hypoglycemic symptoms. It has been demonstrated that the administration of lactate considerably diminishes symptomatic and hormonal responses to hypoglycemia and simultaneously mitigates cognitive dysfunction during hypoglycemia (1-3). These effects are suggested to be the result of lactate acting as an alternative fuel for the brain, when glucose supply is low (2). Clinically, the administration of lactate mimics the situation seen in patients with type 1 diabetes and impaired awareness of hypoglycemia (IAH). In IAH, the threshold for the onset of hypoglycemic symptoms and counterregulatory hormone responses is shifted to lower glucose values, which substantially increases the risk for severe hypoglycemia (4).

Most studies that investigated the effects of lactate administration during hypoglycemia have focused on clinical effects (i.e., symptoms, hormonal responses and cognitive function), and did not examine physiological changes in the brain. Whether elevated plasma lactate levels lead to an accumulation of lactate in the brain is subject of debate. In a 13C-MR study, performed under euglycemic conditions in non-diabetic subjects, it was calculated that brain lactate levels and lactate oxidation increase almost linearly with the increase in plasma lactate levels (5). In contrast, other studies have reported direct oxidation of lactate after its transport across the blood-brain barrier, with limited accumulation in the brain (6; 7). Thus, lactate oxidation may even spare glucose as a cerebral fuel under euglycemic conditions (8).

The only study that has investigated the effect of exogenous lactate on human brain lactate concentrations during hypoglycemia, calculated increased brain lactate concentrations without increased oxidation, in patients with type 1 diabetes compared to healthy volunteers (9). Such elevated brain lactate levels are in line with studies that demonstrated an enhanced capacity to transport lactate into the brain in patients with diabetes and IAH (10; 11). The lack of a euglycemic control and a placebo arm (i.e. no lactate infusion) precluded conclusions about lactate accumulation during euglycemia versus hypoglycemia, or about the effect of lactate infusion on awareness of hypoglycemia.
In the current study, we aimed to show that infusion of lactate, in a dose sufficient to impair awareness of hypoglycemia in patients with type 1 diabetes and normal awareness of hypoglycemia (NAH), increases brain lactate levels. Secondly, we aimed to compare the effect of lactate administration on brain lactate levels during euglycemia and hypoglycemia between patients with NAH and those with IAH.

Material and Methods

Participants
We enrolled seven patients with type 1 diabetes and NAH and seven patients with type 1 diabetes and IAH. The classification of awareness state was based on the Dutch modified version of the Cox questionnaire (12; 13). Patients with type 1 diabetes were eligible if they were younger than 50 years, had an HbA₁c below 9.0% (75 mmol/mol) and were free from microvascular complications, except for background retinopathy. Exclusion criteria included contraindications to the MRI examination, a history of brain injury, epilepsy, liver or cardiovascular diseases, anxiety disorders, and the use of drugs other than insulin interfering with glucose metabolism. One participant with NAH moved too much during data acquisition, which hampered MR data quality and was consequently excluded from data analysis. The study was approved by and studied in accordance with the ethical standards of the institutional review board of the Radboud university medical center (Commissie Mensgebonden Onderzoek Arnhem-Nijmegen) and with the Helsinki declaration of 1975/1983. All participants gave written informed consent.

Study protocol
Patients with NAH underwent two hyperinsulinemic euglycemic-hypoglycemic clamp studies, once with sodium lactate infusion (NAH w|lac) and once with saline infusion (NAH w|placebo), as described below. Experiments were carried out in a single-blind fashion (i.e. participants were blinded for the infusions) and in randomized order, scheduled at least two weeks apart. In female participants, both experiments were performed during equal phases of the menstrual cycle. Patients with IAH participated only in the sodium lactate infusion study arm (IAH w|lac).
Participants came to the research facility in the morning after an overnight fast, having abstained from smoking, alcohol and caffeine containing substances for 24 hours prior to the experiment, and from strenuous exercise for at least two days before the experiment. In addition, participants were instructed to prevent hypoglycemia and to check their blood glucose levels regularly in the 24 hours before the experiment.

The brachial artery of the non-dominant arm was cannulated under local anesthesia (Xylocaine 2%) for frequent blood sampling. Blood was sampled every 5 min for the determination of plasma glucose and plasma lactate levels (Biosen C-line; EKF Diagnostics). An intravenous catheter was inserted in the antecubital vein of the contralateral arm for infusion of glucose 20% (Baxter B.V., Deerfield, IL), insulin (insulin aspart; Novo Nordisk, Bagsvaerd, Denmark) and sodium lactate (600 mmol/L; Spruyt Hillen, Ijsselstijn, The Netherlands and prepared by the Department of Pharmacy, Radboud university medical center, Nijmegen, The Netherlands) or normal saline (sodium chloride; 0.9%).

After cannulations, a two-step hyperinsulinemic (60 mU/m²/min) euglycemic (5.0 mmol/L)-hypoglycemic (2.8 mmol/L) glucose clamp was initiated, and participants were placed in the MR scanner for baseline MR spectroscopy (MRS) data acquisition. At 15 min after the start of the euglycemic clamp, infusion of sodium lactate, or an equivalent volume of saline, was started while MRS data acquisition continued. We aimed for plasma lactate levels of approximately 3.5 mmol/L and initially used a primed (50 µmol/kg/min for 15 min) continuous (30 µmol/kg/min) infusion of sodium lactate. Since achieved plasma lactate levels were slightly above target levels, the dose was lowered after the first two participants (both patients with NAH) to 40 µmol/kg/min for 15 min and 25 µmol/kg/min for the remainder of the experiment. After ~25 min, blood glucose levels were allowed to drop to 2.8 mmol/L and were maintained at that level for 45 min. Hypoglycemic symptoms were quantified with a validated questionnaire just prior to positioning the participant in the MR scanner and at the end of the stable hypoglycemic phase. Participants were asked to score from 0 to 6 (none to most severe) for each of 18 symptoms which included six autonomic symptoms, six neuroglycopenic symptoms, four general and two dummy symptoms. Additional blood samples were taken for determination of plasma insulin, pH, catecholamines, cortisol and growth hormone prior to the start of the sodium lactate or saline infusion (baseline), at the end of the euglycemic phase and at the end of the hypoglycemic phase. During hypoglycemia, counterregulatory hormone responses were determined at 15 min intervals (see Figure 1(a) for a...
Effect of lactate administration on brain lactate

schematic overview of the study protocol). After completing both experimental days, patients with NAH were asked on which day the hypoglycemic phase was most evident or felt more intense.

**MRS protocol and data analysis**

To measure brain lactate, we performed MR spectroscopy (MRS) on a 3T MR system (MAGNETOM Prisma, Siemens, Erlangen) using a body coil for excitation and a 12-channel receive-only head coil. A $T_1$-weighted anatomical image (MPRAGE, echo time (TE) 2.4 ms, repetition time (TR) 1900 ms, and a field of view of 256x256 mm$^2$) was acquired, and a 22.5 cm$^3$ voxel for MRS data acquisition was placed in the periventricular and supraventricular region (Figure 2(a)). Brain lactate levels were then determined continuously with an interleaved J-difference editing semi-LASER (14) spectroscopy sequence (TE 144 ms, TR 3000 ms and 8 averages). J-difference editing was performed with frequency selective inversion MEGA pulses (15) (30 ms, 75 Hz bandwidth). Unsuppressed water spectra (TE 33 ms, TR 5000 ms and 8 averages) were acquired from the same voxel for signal quantification.

Additionally, at the end of euglycemia and at the end of hypoglycemia we acquired MR spectra without J-difference editing from the same voxel (sLASER, TE 33 ms, TR 3000 ms, 32 averages) to explore differences in other major brain metabolites.

The J-difference edited spectra were zero-filled from 1024 to 2048 data points and phase and frequency aligned (16). Motion-corrupted spectra, identified as a spectrum which deviated more than 2.6 standard deviations from the average spectrum of that participant (17), were discarded. Subsequently, spectra were subtracted pairwise. The difference spectra were averaged within participants across six different phases: baseline (i.e. before the start of lactate or placebo infusion), euglycemia, transition to hypoglycemia, and each 15 min block of hypoglycemia. From these final difference spectra, the cerebral lactate doublet at 1.3 ppm was quantified using the AMARES algorithm in jMRUI (18). Visual inspection of the difference spectra showed co-edited macromolecules at ~1.2 ppm, which were included in the fitting routine in AMARES to avoid overestimation of the lactate signal (19). The unsuppressed water signal was used as an internal standard for absolute quantification and we took the voxel composition and contribution of plasma lactate into account (20). We assumed a $T_2$ of 240 ms for cerebral lactate (21), and a $T_2$ of 80 ms and 110 ms for water in gray and white matter, respectively (22). The voxel composition
Figure 1: Study protocol, plasma glucose and plasma lactate levels. (a) Schematic overview of the study protocol. MRS measurements were performed continuously during euglycemia and hypoglycemia. Counterregulatory hormone responses (CH) and hypoglycemic symptoms (Symp) were determined at several time points during euglycemia and hypoglycemia, as indicated. (b) Time courses of plasma glucose and (c) plasma lactate levels. The dashed lines represent the start of the lactate or saline infusion (T = 0 min), the end of euglycemia, and the start of hypoglycemia. Open circles: patients with type 1 diabetes and NAH with saline infusion (NAH w|placebo); black circles: patients with type 1 diabetes and NAH with lactate infusion (NAH w|lac); black triangles: patients with type 1 diabetes and IAH with lactate infusion (IAH w|lac).
Effect of lactate administration on brain lactate (i.e., percentages white matter, grey matter, and cerebrospinal fluid) was determined by segmenting the $T_1$-weigthed anatomical images using SPM8 (Wellcome Trust Centre for Neuroimaging, UCL, London, UK) and subsequently co-registering the segmented image with the MRS voxel location. As the voxel mainly contained white matter, we assumed a vessel volume of 2% (23; 24).

Figure 2: Example of difference spectra. (a) Typical location of the MRS voxel on a $T_1$-weighted anatomical image. (b) Representative examples of difference spectra of one subject with NAH (NAH w|placebo and NAH w|lac) and one subject with IAH (IAH w|lac). The baseline difference spectra were recorded before the start of lactate or placebo infusion. Lac, lactate; MM, macromolecules.

Spectra acquired without J-difference editing were analyzed using the LCModel software (25). A basis set was created in Bruker TopSpin, and extended with a measured macromolecular baseline. Metabolites quantified with Cramér-Rao lower bounds >50% were not included in the analysis (as recommended by the LCModel manual (26)). The unsuppressed water signal was used as a reference for absolute quantification of aspartate (Asp), total choline (tCho), total creatine (tCre), glutamate (Glu), glutamine (Gln), myo-inositol (mI), total N-acetylaspartate (tNAA), scyllo-inositol (scyllo) and taurine (Tau).

Analytical methods
Plasma insulin was determined by an in-house radioimmunoassay (RIA) (27). Plasma adrenaline and noradrenaline were analyzed by high-performance liquid chromatography combined with fluorometric detection (28). Plasma growth hormone and cortisol were determined using a routine analysis method with an Electrochemiluminescent immunoassay on a Modular Analytics E170 (Roche...
Diagnostics GmbH, Mannheim, Germany). pH was measured by routine arterial blood gas analysis on the RapidPoint 500 (Siemens Nederland B.V., Den Haag, the Netherlands).

**Statistical analysis**
Differences in means or medians within the NAH group (NAH w|lac vs. NAH w|placebo) and differences within groups between euglycemia and hypoglycemia were statistically tested with paired Student \( t \) tests or Wilcoxon signed rank tests when data were not normally distributed. Differences between NAH w|lac and IAH w|lac were compared with two-sided Student \( t \) tests or Mann-Whitney \( U \) tests, when appropriate. Serial data were compared between NAH w|lac and NAH w|placebo with a two-way repeated measures ANOVA. To analyze effects over time in patients with IAH, a one-way repeated measures ANOVA was performed. Since patients with IAH were only studied with lactate infusion, comparison to a placebo condition is not possible. Thus, to determine whether there was an effect of lactate infusion on brain lactate levels in patients with IAH, the area under the curve (AUC) of the baseline-corrected brain lactate levels was calculated by trapezoidal numerical integration, and a one-sample \( t \) test was performed. Differences in means at baseline across the three groups were analyzed by one-way ANOVA followed by pairwise Bonferroni post hoc tests. All data are expressed as mean ± SD unless otherwise indicated. Significance levels of Student \( t \) tests were not corrected for multiple testing. A \( P \) value < 0.05 was considered statistically significant. Statistical analyses were performed with IBM SPSS Statistics 20.

**Results**

Participants were well-matched for relevant clinical parameters, including age, duration of diabetes and glycemic control (Table 1). Upon arrival at the research facility, plasma glucose levels were slightly elevated with no significant differences between groups (9.4 ± 3.4, 7.4 ± 1.7 and 10.3 ± 4.9 mmol/L in NAH w|lac, NAH w|placebo and IAH w|lac, respectively). Stable euglycemia was achieved at a glucose level of 5.1 ± 0.2 mmol/L and stable hypoglycemia at 2.8 ± 0.1 mmol/L, again with no differences between groups or intervention (Figure 1(b)). Plasma insulin levels during the clamp were similar for all groups and conditions (data not shown). Plasma lactate levels increased similarly in NAH w|lac and IAH w|lac by ~3.5-fold to 3.6 ± 0.5 mmol/L within ~15 min after starting the lactate infusion, and remained at that level for the remainder of
the experiment (Figure 1(c)). During the control experiment, i.e. when saline was infused, plasma lactate levels did not change significantly from baseline levels in NAH w/ placebo \((P = 0.58)\). Sodium lactate infusion caused the pH to increase in both groups to similar extent (from \(7.41 \pm 0.02\) at baseline to \(7.48 \pm 0.01\) at the end of hypoglycemia, \(P < 0.001\)), whereas saline infusion did not affect the pH \((7.40 \pm 0.02\) versus \(7.42 \pm 0.03\)). Glucose infusion rates (GIR) during hypoglycemia (Supplementary Figure 1) were not significantly different across groups and intervention (mean GIR: \(3.1 \pm 1.3\), \(2.7 \pm 1.0\) and \(3.3 \pm 1.3\) mg/kg/min for NAH w/ lac, NAH w/ placebo, and IAH w/ lac, respectively).

### Table 1: Participant characteristics

<table>
<thead>
<tr>
<th></th>
<th>T1DM-NAH</th>
<th>T1DM-IAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>28.0 ± 10.3</td>
<td>25.9 ± 6.0</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>3/3</td>
<td>3/4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.0 ± 2.5</td>
<td>24.0 ± 2.1</td>
</tr>
<tr>
<td>Duration of T1DM (years)</td>
<td>11.6 ± 5.9</td>
<td>13.2 ± 5.8</td>
</tr>
<tr>
<td>HbA₁c (mmol/mol ([%]))</td>
<td>54.7 ± 9.1 ([7.2 ± 0.8])</td>
<td>55.0 ± 5.0 ([7.2 ± 0.5])</td>
</tr>
<tr>
<td>Self reported exercise (hours/week)</td>
<td>3.5 ± 0.9</td>
<td>2.9 ± 1.5</td>
</tr>
</tbody>
</table>

Data are presented as number or means ± SD. M: male; F: female; BMI: body mass index; T1DM NAH: type 1 diabetes with normal awareness of hypoglycemia; T1DM IAH: type 1 diabetes with impaired awareness of hypoglycemia.

### Table 2: Hypoglycemia-induced changes in symptom subscores (median \([\text{interquartile range}]\))

<table>
<thead>
<tr>
<th></th>
<th>T1DM-NAH</th>
<th>T1DM-IAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autonomic</td>
<td>7.5 ([2.5 ; 15.5]^{a})</td>
<td>2.5 ([-0.5 ; 4.5])</td>
</tr>
<tr>
<td>Neuroglycopenic</td>
<td>3.5 ([0.75 ; 7])^{b}</td>
<td>2 ([-0.25 ; 3.5])</td>
</tr>
<tr>
<td>General</td>
<td>3.5 ([3 ; 4.75]^{b,c})</td>
<td>2 ([0.75 ; 3.25]^{a})</td>
</tr>
</tbody>
</table>

\(^{*}P < 0.05\) vs. NAH w/ lac, \(^{#}P < 0.05\) vs euglycemia. T1DM NAH: type 1 diabetes with normal awareness of hypoglycemia; T1DM IAH: type 1 diabetes with impaired awareness of hypoglycemia.
Hypoglycemic symptoms and counterregulatory hormones

Lactate infusion clearly suppressed total symptom scores during hypoglycemia in patients with NAH, as compared to placebo ($P = 0.03$, Figure 3), and resembled symptom score of patients with IAH when lactate was infused ($P = 0.18$ between groups). Symptom subcategories are depicted in Table 2. Five out of six patients with NAH indicated that hypoglycemia was less severe on the day of the lactate infusion, one patient experienced no difference between both study days. In patients with IAH neither the total nor the subcategory symptom score changed significantly in response to hypoglycemia. Lactate infusion considerably suppressed the adrenaline response to hypoglycemia in patients with NAH as compared to placebo infusion ($P < 0.01$; Figure 4). Lactate infusion also suppressed cortisol and hGH responses to hypoglycemia in patients with NAH (both $P < 0.01$), but did not significantly reduce noradrenaline responses ($P = 0.18$) (Supplementary Figure 2).

Figure 3: Difference in symptom scores. Total symptoms scores in response to hypoglycemia in patients with type 1 diabetes and NAH with saline infusion (NAH w|placebo), patients with NAH with lactate infusion (NAH w|lac) and patients with IAH with lactate infusion (IAH w|lac). *$P < 0.05$, **$P < 0.01$.

Brain lactate

The tissue content of the MRS voxels was not different between groups and contained on average $64.1 \pm 11.1\%$ white matter, $32.8 \pm 10.5\%$ grey matter and $3.2 \pm 1.6\%$ cerebrospinal fluid. Baseline brain lactate levels (i.e., before the infusion of lactate or placebo) were similar across groups ($0.58 \pm 0.08$, $0.64 \pm 0.1$ and
0.59 ± 0.05 µmol/g in NAH w|lac, NAH w|placebo, and IAH w|lac, respectively; 
\( P = 0.42 \). J-edited difference MR spectra, recorded during infusion of lactate, 
showed an increase of the lactate signal in the brain (Figure 2(b)). In patients 
with NAH, brain lactate levels were higher after infusion of lactate than after 
infusing saline (Figure 5(a); mean difference: 0.12 ± 0.05 µmol/g, equivalent to 
an increase of 20.7 ± 6.6%; \( P = 0.048 \)). There was no significant interaction effect 
(time x infusion) or main effect of time. Although brain lactate levels in NAH 
w|lac tended to be elevated compared to placebo values at each time point, with 
post hoc analysis revealing that the difference in brain lactate levels reached 
significance during the final 30 min of the experiment.

**Figure 4**: Adrenaline responses. Adrenaline levels were determined at baseline (Bsln; i.e. 
before the infusion of lactate or saline), during euglycemia (Eu) and at 15-min intervals during 
hypoglycemia. Open circles: patients with type 1 diabetes and NAH with saline infusion (NAH 
w|placebo); black circles: patients with type 1 diabetes and NAH with lactate infusion (NAH 
w|lac); black triangles: patients with type 1 diabetes and IAH with lactate infusion (IAH w|lac).

In patients with IAH, lactate infusion resulted in an increase in brain lactate 
levels compared to baseline values (Figure 5(b); mean increase: 0.06 ± 0.04 
µmol/g, equivalent to an increase of 10.2 ± 4.2%; AUC of baseline-corrected 
brain lactate levels: 4.4 ± 4.3 µmol·min/g; \( P = 0.03 \)). In these patients, brain 
lactate levels showed a trend towards an increase at each time point compared 
to baseline values, but the trend was stronger at the beginning of lactate 
infusion and disappeared at the end of hypoglycemia. When comparing brain 
lactate time curves with lactate infusion between patients with IAH and NAH, 
the increase in brain lactate levels in IAH w|lac seemed less pronounced.
compared to NAH w|lac, but this difference was not statistically significant ($P = 0.36$). Brain lactate levels did not change in patients with NAH in response to hypoglycemia when saline was infused. Notably, there was no correlation, whatsoever, between brain lactate levels and plasma lactate levels ($R^2 = 0.01$, $P = 0.27$).

**Figure 5:** Brain lactate levels. Mean (± SEM) baseline corrected and percentage change in cerebral lactate levels in (a) patients with type 1 diabetes and NAH (NAH w|placebo and NAH w|lac) and (b) patients with type 1 diabetes and IAH (IAH w|lac). The dashed line represent the start of hypoglycemia. $P$ values indicate significance of time-series analysis.
Other brain metabolites

Brain glutamate levels decreased in response to hypoglycemia in patients with NAH, both during lactate infusion (from 7.5 ± 0.7 to 7.1 ± 1.0 µmol/g, \( P = 0.03 \)) and while infusing placebo (from 7.6 ± 0.6 to 7.0 ± 0.8 µmol/g, \( P = 0.01 \)). In contrast, hypoglycemia did not change brain glutamate levels in patients with IAH (8.0 ± 0.7 versus 7.8 ± 0.9 µmol/g, \( P = 0.59 \)). Additionally, we found an increase in myo-inositol in IAH w|lac and a slight decrease in myo-inositol in NAH w|lac. None of the other major brain metabolites (Asp, tCho, tCre, Gln, tNAA, scyollo and Tau) was significantly altered by the hypoglycemic condition in any of the groups (Supplementary Figure 3).

Discussion

The results of the present study confirm that lactate infusion considerably suppresses counterregulatory hormone responses to and symptomatic awareness of hypoglycemia in patients with NAH. While lactate infusion did increase brain lactate content, the increase was modest and not clearly different between patients with NAH and IAH. The lack of pronounced brain lactate accumulation suggests that the excess of lactate is immediately oxidized by the brain which may explain the suppressive effects of lactate on awareness of hypoglycemia in patients with NAH.

Our results are in line with those of several other studies reporting that elevated plasma lactate levels result in an increase in cerebral lactate transport and oxidation, preventing substantial accumulation of brain lactate upon infusion (6-8). However, an earlier study reported fivefold higher brain lactate concentrations in the brain of patients with type 1 diabetes who were exposed to frequent hypoglycemia, as compared to healthy controls, when \(^{13}\)C-labelled lactate was administered during hypoglycemia (9). Another \(^{13}\)C study that used a similar methodology in healthy volunteers, found a linear correlation between plasma and brain lactate concentrations during euglycemia (5). According to these calculations, brain lactate levels should have been as high as ~2 µmol/g rather than ~0.7 µmol/g with the plasma lactate levels of 3.5 mmol/L that we achieved. It should be noted that \(^{13}\)C-MRS calculates brain lactate levels indirectly. These calculations require a number of critical assumptions, which may have resulted in an overestimation of brain lactate levels. Here we used \(^{1}\)H-MRS to measure the signal intensity of brain lactate, which is directly proportional to the tissue concentration of lactate, thus providing a direct measurement of brain lactate levels.
Lactate is taken up by the brain through proton-linked facilitated diffusion by monocarboxylate transporters (MCTs), and its uptake is driven by a concentration gradient from blood to brain and further enhanced by neuronal activation (29; 30). Therefore, it is likely that elevated plasma lactate levels result in an increased uptake of lactate by the brain. Furthermore, it has repeatedly been shown that lactate can serve as a metabolic substrate for the brain (5; 7-9; 31), especially when plasma lactate levels are elevated (7; 32; 33). Lactate taken up by the brain needs to be converted to pyruvate before it enters the citric acid cycle, a process catalyzed by lactate dehydrogenase (LDH). Even relatively small increases in local brain lactate concentration can drive lactate utilization by shifting the LDH reaction to pyruvate formation and to subsequent oxidation (34). As we found only a small increase in brain lactate levels, we posit that lactate taken up by the brain is largely oxidized, both during euglycemia and hypoglycemia. Increased brain lactate concentrations during hypoglycemia are likely responsible for the suppressive effects on counterregulatory responses in patients with NAH, presumably because of increased brain lactate oxidation. Alternatively, lactate may exert its suppressive effects via alterations in cerebral blood flow, redox signaling or neuronal activity (35; 36).

Since brain lactate transport capacity is reported to be upregulated in patients with IAH (10; 11), one can expect higher brain lactate levels in patients with IAH than in patients with NAH. Brain lactate concentrations are the result of a balance between its uptake, production, oxidation and export. The absence of a difference in brain lactate concentrations between NAH w|lac and IAH w|lac at the currently elevated plasma lactate levels either indicates comparable brain lactate uptake in both groups, or upregulation of both lactate uptake and lactate oxidation in patients with IAH. Without the use of exogenous lactate infusion, we previously showed that brain lactate levels fall in response to hypoglycemia in patients with IAH, but not in those with NAH (37). This may indeed indicate upregulated capacity to oxidize lactate in patients with IAH. Analogously, the trend towards increased brain lactate levels in the current study disappeared at the end of hypoglycemia in patients with IAH, again hinting at upregulated capacity to oxidize lactate. When we used intense exercise to raise endogenous plasma lactate to much higher levels, we observed higher brain lactate levels in patients with IAH than in those with NAH (38). Cerebral lactate uptake is not only dependent on plasma lactate concentrations but also on pH levels in plasma and in brain (30). The lactate-induced increase in plasma pH value (i.e., a decrease in H⁺ concentration) observed in the current study may have limited brain lactate uptake in comparison to an exercise-induced decrease in plasma
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pH. However, these effects are generally small when pH values remain within the physiological range.

We found brain glutamate levels to fall in response to hypoglycemia in patients with NAH, which is in line with previous observations (39) and has been attributed to glutamate oxidation (39-41). This decrease in brain glutamate was also present in patients with NAH when lactate was infused, which suppressed adrenaline responses to hypoglycemia. This is in line with the absence of a relation between the hypoglycemia-induced decrease in glutamate and the adrenaline response to hypoglycemia, as reported by Terpstra et al. (39), and may indicate that even though lactate is abundantly available, it is not sufficient to compensate for the decrease in glucose supply to the brain in patients with NAH, or alternatively, that lactate oxidation is already saturated in these patients. In contrast, but again in line with previous data without lactate infusion (39), brain glutamate did not decrease in patients with IAH, possibly because of their greater capacity to use (the excess of) lactate, thus eliminating the need to oxidize glutamate.

Our study has a small sample size, though based on power calculations. However, the study population was homogenous in terms of age, HbA1c and diabetes duration, and plasma glucose and lactate levels during the clamps were stable and almost identical in all groups. The homogeneity of the study population enables efficient comparison, but on the other hand, may limit the generalisability of the results. Importantly, to quantify the cerebral lactate content, we took the contribution of plasma lactate (ranging from ~0.8 mmol/L to ~3.5 mmol/L) to the MR signal of brain lactate into account. Since the voxel consisted of mainly white matter we estimated a 2% vessel volume, based on literature (23; 24). Deviations from this estimation (within the physiological range) may affect the magnitude of the outcome, but will not affect the main conclusion of the study.

In conclusion, we show that intravenous lactate administration reduces awareness of hypoglycemia and translates into a small increase in brain lactate levels, with no differences between patients with NAH and IAH. The rather limited increase in brain lactate suggests that the excess of lactate is immediately used by the brain both under euglycemic and hypoglycemic conditions. An increase in brain lactate oxidation during hypoglycemia may explain the suppressive effects of lactate on awareness of hypoglycemia observed in patients with NAH.
Acknowledgments

We thank all the volunteers for their participation in this work. We are indebted to Karin Saini and Adrianne Hofboer-Kapteijns (research nurses, Radboud university medical center) for assistance during the glucose clamps, and to Sjaak van Asten for his assistance with the LCModel analysis.

Author Contributions
EW, HR, BdG and MvdG designed the study with input from CT, AH and BP. HR recruited the participants and performed the glucose clamps. BP and EW developed/implemented the MR methods and MRS sequence. EW and HR collected the data. EW analyzed the MR data, HR and EW were both responsible for all other data analysis. All authors discussed the results and implications and commented on the manuscript at all stages. All authors approved the final version of the manuscript.

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Supplementary material

Fig S1: Glucose infusion rates during hypoglycemia (GIR). Black triangles: patients with type 1 diabetes and IAH with lactate infusion (IAH w|lac); black circles: T1DM NAH with lactate infusion (NAH w|lac); Open circles: T1DM NAH with saline infusion (NAH w|placebo).

Fig S2: Counterregulatory hormone responses to hypoglycemia. (A) Noradrenaline, (B) cortisol, and (C) growth hormone (hGH) responses to hypoglycemia. Black triangles: patients with type 1 diabetes and IAH with lactate infusion (IAH w|lac); black circles: T1DM NAH with lactate infusion (NAH w|lac); Open circles: T1DM NAH with saline infusion (NAH w|placebo).
**Fig S3:** Concentrations of major brain metabolites quantified during euglycemia (white bars) and hypoglycemia (black bars). *P* values indicate significant differences for euglycemia vs. hypoglycemia within a group. Asp, aspartate; tCho, total choline; tCre, total creatine; Glu, glutamate; Gln, glutamine; ml, myo-inositol; tNAA, total N-acetylaspartate; scyllo, scyllo-inositol; Tau, taurine.
Chapter 5

Effect of exercise-induced lactate elevation on brain lactate levels during hypoglycemia in patients with type 1 diabetes and impaired awareness of hypoglycemia


*: contributed equally

Diabetes 2017 Dec; 66(12):3105-3110
Abstract

Since altered brain lactate handling has been implicated in the development of impaired awareness of hypoglycemia (IAH) in type 1 diabetes, the capacity to transport lactate into the brain during hypoglycemia may be relevant in its pathogenesis. High-intensity interval training (HIIT) increases plasma lactate levels. We compared the effect of HIIT-induced hyperlacticacidemia on brain lactate during hypoglycemia between 1) patients with type 1 diabetes and IAH, 2) patients with type 1 diabetes and normal awareness of hypoglycemia (NAH), and 3) healthy participants without diabetes (n = 6 per group). All participants underwent a hypoglycemic (2.8 mmol/L) clamp after performing a bout of HIIT on a cycle ergometer. Before HIIT (baseline) and during hypoglycemia, brain lactate levels were determined continuously with J-difference-editing 1H-MRS, and time curves were analyzed using nonlinear mixed effects modeling. At the beginning of hypoglycemia (after HIIT), brain lactate levels were elevated in all groups, but most pronounced in patients with IAH. During hypoglycemia, brain lactate decreased ~30% below baseline in patients with IAH but returned to baseline levels and remained there in the other two groups. Our results support the concept of enhanced lactate transport as well as increased lactate oxidation in patients with type 1 diabetes and IAH.
Introduction

Impaired awareness of hypoglycemia (IAH) affects 25-30% of patients with type 1 diabetes, is characterized by the suppression of symptoms during hypoglycemia (1; 2), and considerably increases the risk for severe hypoglycemia (1). IAH results from habituation to prior hypoglycemia (3), but the underlying mediators have yet to be fully revealed.

One likely mediator is brain lactate (4-6). We have previously shown that brain lactate levels fall in response to hypoglycemia in patients with type 1 diabetes and IAH, whereas lactate levels remain unaltered in patients with normal awareness of hypoglycemia (NAH) and in healthy participants (6). This fall in brain lactate likely reflects increased cerebral lactate oxidation, which may preserve brain metabolism and interferes with the brain’s capacity to detect hypoglycemia.

This concept is supported by the finding that intravenous administration of lactate has brain glucose-sparing effects under euglycemic conditions (7), suppresses counterregulatory hormone and symptom responses to hypoglycemia, and preserves cognitive function (8; 9). We observed similar, albeit less pronounced, effects when plasma lactate levels were endogenously raised during hypoglycemia after a bout of high-intensity interval training (HIIT) in patients with type 1 diabetes and NAH (10).

Using isotopically enriched lactate infusions, de Feyter et al. (5) observed increased brain lactate concentrations during hypoglycemia in patients with IAH compared with volunteers without diabetes but no increase in cerebral lactate oxidation. This finding contrasts with observations that endogenous hyperlacticacidemia after exercise enhances both brain lactate uptake (7; 11; 12) and oxidation (7; 13; 14), at least under euglycemic conditions. The effect of endogenously elevated plasma lactate levels on brain lactate concentrations during hypoglycemia is currently not known. Therefore, we used a single bout of HIIT to raise endogenous plasma lactate levels, and used ³¹H-MRS to assess cerebral lactate levels during subsequent hypoglycemia in patients with type 1 diabetes with and without IAH and healthy participants.
Research Design and Methods

Participants

We enrolled six patients with type 1 diabetes and IAH, six patients with type 1 diabetes and NAH, and six healthy participants. Patient assignment was initially based on the Dutch modified version of the Cox questionnaire (15), but one patient initially classified as having IAH switched groups because of intact hormonal and symptomatic responses to hypoglycemia. Patients with type 1 diabetes were excluded from participation if their HbA₁c levels exceeded 75 mmol/mol (9.0%) or if they had microvascular complications, except for background retinopathy. Other exclusion criteria were as follows: age >40 years, BMI >30 kg/m², cardiopulmonary disease, contraindications for MRI examination, and a history of brain injury. The study was approved by the institutional review board of the Radboud university medical center, and all participants gave written informed consent.

Study protocol

All participants presented in the morning (8:00 A.M.) after an overnight fast, having abstained from caffeine, alcohol and smoking for 24 h and from strenuous exercise for 2 days. Patients with type 1 diabetes were instructed to check their plasma glucose levels regularly in the 24 h before the experimental day to prevent hypoglycemia and to omit their morning prandial insulin dose. An intravenous catheter was inserted in the antecubital vein to administer insulin (insulin aspart; Novo Nordisk, Bagsvaerd, Denmark) and glucose 20% (Baxter, Deerfield, IL). The brachial artery of the contralateral arm was cannulated under local anesthesia for frequent blood sampling to determine plasma glucose and plasma lactate levels every 5 minutes (Biosen C-line; EKF Diagnostics).

After cannulations, a hyperinsulinemic (60 mU/m²/min)-euglycemic (5.0 mmol/L) glucose clamp was initiated, and baseline magnetic resonance (MR) measurements were acquired. Participants were subsequently taken out of the scanner and performed a HIIT session on a cycle ergometer, consisting of three 30-s all-out sprints interspersed with 4 min of active recovery, as described previously (10). After the HIIT session, participants were placed in the MR scanner at the earliest opportunity, and meanwhile plasma glucose levels were gradually decreased to 2.8 mmol/L and maintained there for 50-60 min.

Before the HIIT session and at the end of hypoglycemia, participants completed an 18-item questionnaire in which hypoglycemic symptoms were scored from 0 (none) to 6 (most severe), and blood samples were drawn to determine counterregulatory hormone responses and insulin levels (see Fig. 1A for a schematic overview of the study protocol).
**MRS protocol and data analysis**

MR data were acquired using a 3T MR system (TIM Magnetom Trio; Siemens, Erlangen, Germany). Each MR session, i.e., at baseline and postexercise, started with the acquisition of an anatomical image (T1-weighted MPRAGE, 256 x 256 mm² field of view, 256 slices) for voxel localization and for later voxel content determination. The 20 x 45 x 25 mm voxel was placed in the periventricular region of the brain (Fig. 2A). During both MR sessions, brain lactate levels were determined continuously with a time resolution of ~4 min, using a J-difference-editing MEGA-semi-LASER sequence (TE 144 ms, TR 3,000 ms, and 32 averages), as described previously (6).

The J-difference-edited spectra were zero-filled from 1,024 to 2,048 points and Fourier transformed. Thereafter, spectra were phase and frequency aligned (16), subtracted pairwise, and apodized in the time-domain with a 5-Hz Lorentzian. A three-point moving average filter over time was applied. The lactate doublets at 1.3 ppm in the final difference spectra were fitted with the AMARES algorithm in jMRUI (17). Cerebral lactate was quantified using the unsuppressed water signal as a reference, taking voxel composition, differences in T2 relaxation, and the contribution of plasma lactate into account, assuming a vessel volume in the (mainly white matter voxel) of 2% (18). Baseline cerebral lactate levels (before HIIT) were averaged; cerebral lactate levels acquired after HIIT were analyzed over time.

**Analytical methods**

Plasma insulin was determined by an in-house radioimmunoassay (RIA) (19) and plasma glucagon by a commercially available RIA (Eurdiagnostica, Malmö, Sweden). Plasma adrenaline and noradrenaline were analyzed by high-performance liquid chromatography combined with fluorometric detection (20).

**Statistical analysis**

Within-group differences were compared with two-sided Student t tests or Wilcoxon signed rank test for nonparametric data, and between-group differences with ANOVA followed by Bonferroni post hoc tests or with the Kruskal-Wallis test and post hoc Mann-Whitney U tests. The change in brain lactate from baseline to the start of hypoglycemia ($\Delta L_\text{ac}_{\text{startHypo}}$), the difference in steady state lactate levels between hypoglycemia and baseline ($\Delta L_\text{ac}_{\text{Plateau}}$), and the decay rate ($k$) were estimated per group using a nonlinear mixed effects (NLME) model. The following exponential decay function was fitted to the data:
Effect of HIIT on brain lactate during hypoglycemia

\[
\text{Lac}(t) = (\Delta \text{Lac}_{\text{startHypo}} - \Delta \text{Lac}_{\text{Plateau}})e^{-kt} + \Delta \text{Lac}_{\text{Plateau}}
\]

Differences in model-parameters (i.e., \(\Delta \text{Lac}_{\text{startHypo}}\), \(\Delta \text{Lac}_{\text{Plateau}}\) and \((k)\) were compared between groups and within groups. All data are expressed as mean ± SD, unless otherwise stated. \(P < 0.05\) was considered statistically significant. Statistical analyses were performed in SAS 9.2 (NLMIXED procedure) or with IBM SPSS Statistics 20.

**Results**

The study participants were well matched for relevant parameters (Table 1). Upon arrival, plasma glucose levels were elevated to a similar extent in both patient groups (7.9 ± 3.1 and 9.5 ± 2.2 mmol/L for type 1 diabetes and IAH and type 1 diabetes and NAH, respectively). Insulin levels at baseline and during hypoglycemia did not differ between groups (data not shown). During the clamp, plasma glucose levels were maintained at 5.1 ± 0.1 mmol/L and 2.8 ± 0.1 mmol/L, without differences between study groups (Fig. 1B). Plasma lactate levels for the three groups were comparable at baseline and increased markedly and to a similar extent in response to HIIT. During hypoglycemia, plasma lactate fell gradually but remained above baseline levels (Fig. 1C).
Figure 1: Study protocol and plasma glucose and plasma lactate levels. A: Schematic overview of the study protocol. MRS measurements were performed prior to HIIT (baseline) and during hypoglycemia. Counterregulatory hormone responses and hypoglycemic symptom score were assessed prior to the HIIT session and at the end of hypoglycemia. Time courses of plasma glucose (B) and plasma lactate (C) levels. The HIIT session is indicated by the gray area. The dashed lines represent the beginning and the end of the hypoglycemic phase. Open circles, patients with type 1 diabetes (T1DM) and IAH; open triangles, patients with T1DM and NAH; open squares, healthy participants.
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Figure 2: Example of difference spectra. A: Typical location of the MRS voxel, indicated by the white rectangles, (20 x 45 x 25 mm) on a T1-weighted anatomical image of one subject with type 1 diabetes and IAH. B: Representative difference spectra (i.e., MEGA on – MEGA off) of one subject with type 1 diabetes and IAH, depicting the lactate doublet at 1.3 ppm over time. The baseline difference spectra was recorded before HIIT. After HIIT, hypoglycemia was induced and brain lactate concentrations were measured continuously.

Table 1: Participant characteristics

<table>
<thead>
<tr>
<th></th>
<th>T1DM IAH</th>
<th>T1DM NAH</th>
<th>Healthy participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>23.5 ± 6.1</td>
<td>21.5 ± 2.5</td>
<td>23.8 ± 3.0</td>
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<tr>
<td>Sex (male/female)</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
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<tr>
<td>BMI (kg/m²)</td>
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<td>22.7 ± 2.4</td>
<td>23.5 ± 1.6</td>
</tr>
<tr>
<td>Duration of T1DM (years)</td>
<td>12.0 ± 8.8</td>
<td>10.2 ± 4.8</td>
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</tr>
<tr>
<td>HbA₁c (mmol/mol (%))</td>
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<td>60.7 ± 8.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>[6.9 ± 0.91]</td>
<td>[7.7 ± 0.74]</td>
<td></td>
</tr>
<tr>
<td>Self reported exercise (h/week)</td>
<td>4.9 ± 3.0</td>
<td>4.8 ± 3.1</td>
<td>3.3 ± 2.0</td>
</tr>
</tbody>
</table>

Data are presented as number or mean ± SD. T1DM, type 1 diabetes.
Figure 3: Brain lactate levels. Mean cerebral lactate levels before (baseline) and after HIIT during hypoglycemia. For illustration purpose only, mean (± SEM) brain lactate levels after the HIIT training were calculated on a 4 minute time-interval grid (±SD). Dashed lines represent the NLME model fit. Open circles, patients with type 1 diabetes (T1DM) and IAH; open triangles, patients with T1DM and NAH; open squares, healthy participants.

As expected, total symptom scores did not change in response to hypoglycemia in patients with IAH (mean increase: 0.67 ± 1.26) but increased considerably in patients with NAH and in healthy participants (14.83 ± 3.61 and 10.83 ± 2.06, respectively). The plasma adrenaline response to hypoglycemia was reduced in patients with IAH compared with patients with NAH and healthy participants (Supplementary Table 1).

Baseline brain lactate levels were similar across the three groups (Fig. 3). The first MR spectra were acquired on average 38.5 ± 5.5 min after the HIIT session, when plasma glucose levels were <3.6 mmol/L. Figure 2B shows a typical example of difference spectra with the evolution of the lactate doublet over time. According to the NLME model, brain lactate levels at the beginning of hypoglycemia were elevated compared to baseline levels ($\Delta L_{\text{startHypo}}$ in Eq. 1) by 0.20 ± 0.05 µmol/g ($P = 0.01$) in patients with IAH, by 0.11 ± 0.05 µmol/g ($P = 0.03$) in patients with NAH, and by 0.06 ± 0.02 ($P = 0.01$) in healthy participants. The increase in brain lactate after HIIT was higher in patients with IAH compared with healthy participants ($P = 0.04$). During hypoglycemia, brain lactate levels returned to and remained at baseline levels in patients with NAH and healthy participants (0.00 ± 0.04 and -0.07 ± 0.04 µmol/g), whereas a further -0.20 ± 0.06 µmol/g decrease below
baseline ($P = 0.02$) (Fig. 3) was observed in patients with type 1 diabetes and IAH during hypoglycemia ($\Delta Lac_{\text{Plateau}}$ in Eq. 1). There was no difference in the estimated exponential decay rate of brain lactate between groups ($P = 0.52$).

**Discussion**

The current study shows that endogenously raised plasma lactate levels by a single bout of HIIT result in increased brain lactate concentrations at the start of hypoglycemia. This increase is most pronounced in patients with IAH, and this was the only group where brain lactate levels decreased below baseline values at the end of the hypoglycemic episode. These findings support altered brain lactate handling in IAH.

The HIIT-induced increase in brain lactate content is in line with previous studies using $^1$H-MRS in healthy participants in response to endogenously raised plasma lactate levels by vigorous exercise, albeit under euglycemic conditions (11; 12). Cerebral lactate uptake is driven by a concentration gradient from blood to brain. Interestingly, although plasma lactate levels were similar between groups, we found that the increase in brain lactate concentrations after HIIT was most pronounced in patients with type 1 diabetes and IAH, which supports enhanced brain lactate transport capacity in patients with IAH (21; 22). This difference is most visible at the beginning of hypoglycemia, when plasma lactate levels are highest.

During hypoglycemia, we showed that brain lactate levels returned to baseline values in healthy volunteers and patients with NAH but dropped to levels below baseline in patients with IAH, which could not be explained by differences in plasma lactate levels between the study groups. This decrease matches the hypoglycemia-induced fall in brain lactate in patients with IAH that we observed in a previous study, in which plasma lactate levels were not elevated (6), and may reflect enhanced brain lactate oxidation. Previous studies have found that lactate contributes substantially to cerebral energy metabolism, when its availability is high (7; 23-25). Increased lactate oxidation may protect the brain by maintaining metabolism when glucose supply is low, while at the same time it could impede the brain’s capacity for hypoglycemia sensing. This is in line with the observation that a prior bout of HIIT attenuated awareness of and cognitive deterioration during subsequent hypoglycemia, particularly in patients with NAH (10), which may have been mediated by brain lactate.
HIIT not only increases plasma lactate levels but also has an impact on (stress) hormones, brain activation, and physical parameters, which may have influenced our results. Because the HIIT session was performed outside the MR system, we were unable to capture early changes in brain lactate after exercise. Brain lactate levels may have been higher in the time gap between HIIT and the post-HIIT scans. All participants were young, healthy, and fit, which should be kept in mind when interpreting the results. A strength of our study is the inclusion of three clinically distinct, but well-matched, groups of participants, allowing the differentiation between the effect of type 1 diabetes and the effect of IAH. Furthermore, plasma glucose and lactate levels were almost identical among the groups.

In conclusion, we found that brain lactate concentrations increase after a bout of HIIT, particularly in patients with diabetes and IAH. In addition, brain lactate levels fall below baseline during the subsequent hypoglycemic period in patients with IAH but not in those with NAH or in healthy participants. These results support upregulation of lactate transport capacity and increased cerebral lactate oxidation during hypoglycemia in patients with IAH. Both adaptations in cerebral lactate handling may contribute to IAH by lactate serving as an alternative nonglucose fuel for the brain during hypoglycemia.

**Acknowledgments**
We thank all the volunteers for their participation in this work. We are indebted to Karin Saini and Adrianne Hofboer-Kapteijns (research nurses, Radboud university medical center) for assistance during the glucose clamps.

**Author Contributions**
EW, HR, BdG and MvdG designed the study with input from CT, AH and HG. HR recruited the participants and performed the glucose clamps. EW and HR collected the data. EW analyzed the MR data, HR was responsible for all other data analysis. HG was responsible for the statistical analysis. All authors discussed the results and implications and commented on the manuscript at all stages.

**Funding**
Research support from the Dutch Diabetes Research Foundation (DFN 2012.00.1542) and the European Foundation for the Study of Diabetes is gratefully acknowledged.
Effect of HIIT on brain lactate during hypoglycemia

Prior Presentation
This study has been presented as oral presentation at the 77th Scientific Sessions of the American Diabetes Association (ADA), San Diego, June 9-13, 2017.
References

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Supplementary material

Table S1: Hypoglycemia-induced changes in symptom scores and counterregulatory hormones

<table>
<thead>
<tr>
<th></th>
<th>T1DM IAH</th>
<th>T1DM NAH</th>
<th>Healthy participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total symptom scores</td>
<td>0.67 ± 1.26</td>
<td>14.83 ± 3.61*#</td>
<td>10.83 ± 2.06*#</td>
</tr>
<tr>
<td>Adrenaline (nmol/L)</td>
<td>0.60 ± 0.11*</td>
<td>2.81 ± 1.22*#</td>
<td>2.47 ± 0.33*#</td>
</tr>
<tr>
<td>Noradrenaline (nmol/L)</td>
<td>0.41 ± 0.17</td>
<td>0.71 ± 0.34</td>
<td>0.26 ± 0.08</td>
</tr>
<tr>
<td>Glucagon (pmol/L)</td>
<td>-1.70 ± 1.00</td>
<td>2.68 ± 1.43</td>
<td>29.02 ± 7.28*#</td>
</tr>
</tbody>
</table>

Data are presented as mean (± SEM). * $P < 0.05$ for euglycemia vs. hypoglycemia; # $P < 0.05$ vs. T1DM IAH; + $P < 0.05$ vs. T1DM NAH.
A single bout of high-intensity interval training reduces awareness of subsequent hypoglycemia in patients with type 1 diabetes

Hanne M. Rooijackers, Evita C. Wiegers, Marinette van der Graaf, Dick H Thijssen, Roy P.C. Kessels, Cees J. Tack, Bastiaan E. de Galan

Abstract

High-intensity interval training (HIIT) has gained increasing popularity in patients with diabetes. HIIT acutely increases plasma lactate levels. This may be important, since administration of lactate during hypoglycemia suppresses symptoms and counterregulation while preserving cognitive function. We tested the hypothesis that, in the short term, HIIT reduces awareness of hypoglycemia and attenuates hypoglycemia-induced cognitive dysfunction. In a randomized crossover trial, patients with type 1 diabetes and normal awareness of hypoglycemia (NAH), patients with impaired awareness of hypoglycemia (IAH), and healthy participants (n = 10 per group) underwent a hyperinsulinemic-hypoglycemic (2.6 mmol/L) clamp, either after a HIIT session or after seated rest. Compared with rest, HIIT reduced symptoms of hypoglycemia in patients with NAH but not in healthy participants or patients with IAH. HIIT attenuated hypoglycemia-induced cognitive dysfunction, which was mainly driven by changes in the NAH subgroup. HIIT suppressed cortisol and growth hormone responses, but not catecholamine responses to hypoglycemia. The present findings demonstrate that a single HIIT session rapidly reduces awareness of subsequent hypoglycemia in patients with type 1 diabetes and NAH, but does not in patients with IAH, and attenuates hypoglycemia-induced cognitive dysfunction. The role of exercise-induced lactate in mediating these effects, potentially serving as an alternative fuel for the brain, should be further explored.
Introduction

Regular exercise is recommended for patients with type 1 diabetes, as it improves physical fitness, insulin sensitivity, and general well-being, while reducing cardiovascular risk factors (1; 2). High-intensity interval training (HIIT) is a relatively new training modality that consists of repeated, brief bouts of exercise at high-intensity (i.e. ≥85% peak oxygen uptake), interspersed by periods of rest or low-intensity exercise (3; 4). HIIT is becoming increasingly popular because it has similar or superior training effects compared with endurance training, despite having a lower time commitment (3). In patients with type 1 diabetes, high-intensity (interval) training has been reported to exert a short-term glucose-stabilizing effect when compared with endurance exercise (5-7). Notwithstanding this short-term effect, the risk of late nocturnal hypoglycemia after HIIT may be increased (8). It is currently unknown whether HIIT affects awareness of subsequent hypoglycemic episodes.

Performance of HIIT markedly increases plasma lactate levels (9). This is highly relevant since previous studies have demonstrated that the administration of lactate during hypoglycemia suppresses counterregulatory hormone responses and hypoglycemic symptoms (10-13). In addition, the infusion of lactate attenuates the decline in cognitive function that normally occurs during hypoglycemia (10-13). These effects are presumed to result from increased use of lactate by the brain (12; 13), which can substitute for glucose and act as an alternative energy source under hypoglycemic conditions. Elevated levels of endogenous lactate induced by HIIT may thus be expected to have similar effects as exogenous lactate and to suppress normal physiological responses to subsequent hypoglycemia (i.e., reduced counterregulatory hormones, reduced hypoglycemic symptoms and attenuated cognitive dysfunction).

Such a suppressive effect would be particularly worrisome for the ~25% of patients with type 1 diabetes and impaired awareness of hypoglycemia (IAH) who already have compromised symptomatic and hormonal responses to hypoglycemia (14) and are at high risk of severe hypoglycemia (15). In these patients, high levels of endogenous lactate after HIIT might even further impair counterregulation. Therefore, the aim of the present study was to investigate the effect of HIIT on hypoglycemic symptoms, counterregulatory hormone responses, and cognitive function during subsequent hypoglycemia in patients with type 1 diabetes and normal awareness of hypoglycemia (NAH) and IAH but also in healthy participants.
Research design and methods

Participants
We recruited 10 patients without diabetes, 10 patients with type 1 diabetes and NAH, and 10 patients with type 1 diabetes and IAH. Patients with type 1 diabetes were eligible if they had an HbA₁c level <9.0% (75 mmol/mol) and were free from macrovascular and microvascular complications, except for background retinopathy. Exclusion criteria included a history of cardiopulmonary disease, anxiety disorders, brain injury, age > 40 years, and the use of drugs other than insulin interfering with glucose metabolism. Awareness state was first assessed by a Dutch version of the Clarke questionnaire (16; 17), but final stratification was based on adrenaline and symptomatic responses to the hypoglycemic clamps. Eighteen out of 20 patients were rightly characterized as having IAH or NAH by the Clarke questionnaire. One patient initially classified as having intact awareness (i.e., NAH) had very low adrenaline and symptom responses to the hypoglycemic clamp, whereas another patient, presumably with impaired awareness, displayed normal responses to the clamp. The latter two patients switched groups. All participants were recreationally active. The institutional review board of the Radboud university medical center approved the study, and all study participants gave written informed consent before participation.

Experimental design
In a random order, all enrolled participants underwent two hyperinsulinemic-hypoglycemic glucose clamp (nadir, 2.6 mmol/L) experiments, one that was preceded by a HIIT session and one that was preceded by seated rest (duration equivalent of HIIT session). The two experiments were scheduled at least two weeks apart, except in the women participating, in whom experiments were conducted during similar phases of the menstrual cycle.

Participants presented between 8.00 and 8.30 A.M. at the clinical research facility after an overnight fast, having abstained from caffeine, alcohol and smoking for 24h and from strenuous exercise for two days. Participants with diabetes received specific instructions to avoid (nocturnal) hypoglycemia the day before the clamp. Experiments were rescheduled in cases of hypoglycemia in the 24h before the clamp. Upon arrival, intravenous cannulae were inserted into the antecubital veins of both forearms. One forearm was placed in a heated box (55°C), so that arterialized venous blood could be obtained for frequent blood sampling. The cannula in the contralateral arm was used for infusion of glucose 20% (Baxter B.V., Deerfield, IL) and insulin (insulin aspart; Novo Nordisk, Bagsvaerd, Denmark). Baseline plasma glucose and lactate levels were
determined (Biosen C-Line, EKF Diagnostics, Cardiff, U.K.), and hyperglycemia in patients with diabetes was corrected as needed with a small bolus of insulin. Subsequently, a two-step hyperinsulinemic (60 mU/m²/min) euglycemic (5.0 mmol/L)-hypoglycemic (2.6mmol/L) glucose clamp was initiated. Plasma glucose and lactate values were determined every five minutes. Blood samples for measurement of catecholamines and cortisol were taken at euglycemia, 5 min after exercise, and at 20-min intervals during hypoglycemia. Insulin, glucagon, growth hormone (GH) and pH were measured at euglycemia, after exercise (only GH and pH) and at the end of hypoglycemia. Under clamped, euglycemic conditions, participants performed HIIT or rested for the same period of time. Subsequently, plasma glucose levels were allowed to fall to 2.6 mmol/L over ~35 min and were maintained there for another 60 min.

**Exercise protocol**

The HIIT session was performed on a cycle ergometer (Lode Corival; Procare, Groningen, The Netherlands) and had a total duration of ~15 min. Prior to the start, a short 10-s test sprint was performed to determine optimal resistance of the ergometer (equaling ~0.1 kg/kg body mass, depending on the participant’s physical activity level and cycling experience). The exercise protocol consisted of a 4-min warm-up at 50 W, followed by three 30-s all-out sprints during which participants had to cycle as fast as possible, interspersed with 4 min active recovery (at 50W). The resistance applied during the all-out sprints was adjusted depending on the perceived intensity of the previous sprint, measured with the Borg scale (18), aiming at a Borg score of >15 (running from 6, indicating no exertion, to 20, indicating maximal exertion). The scale was displayed in front of the participants while exercising, and after each all-out sprint participants selected the number that best described their perceived level of exertion.

**Symptom scores**

Participants were asked to complete a semiquantitative symptom questionnaire at euglycemia, and at 20, 40 and 60 min of hypoglycemia. Symptoms were scored from 0 (none) to 6 (severe) and included six autonomic symptoms (trembling, palpitations, anxiety, sweating, hunger, and tingling), six neuroglycopenic symptoms (blurred vision, difficulty speaking, feeling faint, difficulty thinking, fatigue, and confusion), four general symptoms (dry mouth, weakness, nausea, and headache) and two dummy symptoms (yellow vision and pain in the legs). Total symptom scores (i.e., scores including all symptom subcategories) and subscores were averaged over the three time points (after 20, 40 and 60 min of hypoglycemia). Peak symptom scores were defined as the highest total
symptom score during hypoglycemia, irrespective of the time point.

**Cognitive function tests**
The following cognitive function tests were applied during the screening visit and during hypoglycemia, starting 15 min after hypoglycemia was reached. In cases of hypoglycemia (n=2) or hyperglycemia (n=1) during the screening visit, the first battery of cognitive function tests was applied during the euglycemic phase of the first test day. Non-specific practice effects were controlled for by counterbalancing the order of the rest and HIIT intervention, and parallel forms were used to avoid material-specific practice effects if applicable.

**Digit span forward and backward.** A subtest of the Dutch version of the Wechsler Adult Intelligence Scale-III that measures attention and working memory was conducted (19). Sequences of digits of increasing lengths were presented verbally, and participants were asked to recall the digits in the presented order and in the reverse order.

**Verbal fluency test.** A Dutch version of the Controlled Oral Word Association Test (COWAT) that serves as an index of executive function was conducted (20). Participants were given 1 min to name as many words as possible starting with a given letter. Parallel sets of letter triads (D-A-T, K-O-M, and P-G-R) were used for baseline, HIIT and rest measurements.

**Paced Auditory Serial Addition Test (PASAT).** The Paced Auditory Serial Addition Test evaluates the speed of information processing and divided and sustained attention (21). Continuous sequences of 61 digits are auditorily presented, and participants are asked to add each new digit to the one immediately prior to it and say the answer out loud. Sequences were presented at three rates of presentation (i.e., every 2.4, 2.0 or 1.6 s).

In order to provide a composite score of cognitive function, raw scores of each test were transformed into z scores, based on the distribution of test scores at baseline (including all three study groups), and were averaged (22).

**Analytical methods**
Plasma insulin was assessed by an in-house radioimmunoassay (23). After extraction (24), plasma glucagon was measured by radioimmunoassay with a commercially available kit (Eurodiagnostica, Malmö, Sweden). Plasma GH and cortisol were determined using a routine analysis method with an
Electrochemiluminescent Immunoassay on a Modular Analytics E170 (Roche Diagnostics, GmbH, Manheim, Germany). Plasma adrenaline and noradrenaline were analyzed by high-performance liquid chromatography combined with fluorometric detection (25). pH was measured by routine venous blood gas analysis in lithium heparin-anticoagulated blood immediately after withdrawal, on the RapidPoint 500 (Siemens Nederland B.V., Den Haag, the Netherlands).

**Statistical analysis**
Data were tested for normality using the Shapiro-Wilk test and QQ-plots. Differences in means within groups were analyzed with paired Student t tests or Wilcoxon signed rank test when data were not normally distributed. Differences in means between groups were analyzed by ANOVA followed by Bonferroni post hoc tests to delineate statistical significance, and for non-parametric data with the Kruskal-Wallis test and post hoc Mann-Whitney U tests. Serial data (hormone responses and glucose infusion rates) were analyzed by two-way repeated measures ANOVA, in which missing data were replaced with the last observed value. The cognitive tests were analyzed with a repeated-measures general linear model ANOVA with study group (healthy control subjects, patients with NAH, or patients with IAH) as a between-subject factor and intervention (HIIT or rest) as a within-subject factor, followed by post hoc paired Student t tests. All data are expressed as the mean ± SEM, unless otherwise specified. Alpha was set at 0.05 throughout. Statistical analyses were performed with IBM SPSS Statistics 20.

**Results**
The three groups of participants were well matched for age, sex and BMI (Table 1). Diabetes duration did not differ significantly between the two diabetes groups, and the HbA1c level was borderline significantly lower in patients with IAH compared with those with NAH (P = 0.05). Glucose levels during the clamps are shown in Fig. 1A and B.

**Physiological effects of exercise**
Average workload during all-out sprints for the whole study population was 437 ± 17 W and mean perceived exertion was 16 ± 0.4 on the Borg scale, with no difference between groups. HIIT markedly increased plasma lactate levels from 1.2 ± 0.1 to 13.1 ± 0.5 mmol/L (Fig. 1D) and decreased pH from 7.36 ± 0.00 to 7.19 ± 0.01 to a similar extent in all groups. After reaching peak levels, plasma lactate levels fell gradually but remained well above baseline levels
Effects of HIIT on awareness of hypoglycemia during the subsequent hypoglycemic clamp in both patient groups and for 40 minutes of hypoglycemia in healthy participants. Immediately after HIIT, plasma glucose levels increased slightly in all three groups, but remained within the normoglycemic range (Fig. 1B). HIIT increased plasma levels of adrenaline by 0.11 ± 0.04 nmol/L, noradrenaline by 4.1 ± 0.4 nmol/L, and GH by 14 ± 4.7 mU/L (all P values <0.05), whereas plasma cortisol levels tended to increase (mean increase 0.04 ± 0.02 µmol/L; P = 0.06). Hormonal responses to exercise were similar between all groups. All hormone levels returned to levels indistinguishable from those at rest days prior to the beginning of hypoglycemia (~40 min after exercise) with the exception of plasma cortisol, which remained slightly elevated in patients with diabetes and IAH.

### Table 1: Participant characteristics

<table>
<thead>
<tr>
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<th>Healthy controls</th>
<th>Patients with T1DM-NAH</th>
<th>Patients with T1DM-IAH</th>
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<tr>
<td>Age, years</td>
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<td>25.7±5.8 (19-35)</td>
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<tr>
<td>Sex, M:F</td>
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<td>4:6</td>
<td>5:5</td>
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<tr>
<td>BMI, kg/m²</td>
<td>22.5±1.8</td>
<td>23.0±2.3</td>
<td>23.4±1.4</td>
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<td>Exercise, h/week</td>
<td>3.7±2.2</td>
<td>4.3±2.9</td>
<td>4.9±3.7</td>
</tr>
<tr>
<td>Score on modified Cox questionnaire</td>
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<td>0.8±1.3 (0-4)</td>
<td>3.7±1.3 (1-5)</td>
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<tr>
<td>HbA₁c, % (mmol/mol)</td>
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<td>7.5±0.6 (59.0±6.9)</td>
<td>6.9±0.7 (52.1±7.7)</td>
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<tr>
<td>Duration of diabetes, years</td>
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<td>10.7±4.5</td>
<td>13.9±8.1</td>
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<tr>
<td>Insulin dose, units/kg/day</td>
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<td>0.7±0.4 (0.4-0.9)</td>
<td>0.6±0.4 (0.4-0.8)</td>
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<tr>
<td>Insulin regimen, n</td>
<td>-</td>
<td>Multi-injection therapy</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pump therapy</td>
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</tbody>
</table>

Data are presented as number or means ± SD (range). F, female; M, male; T1DM, type 1 diabetes mellitus.

### Responses to hypoglycemia after HIIT or rest

**Hypoglycemic symptoms.** Compared with seated rest, HIIT reduced the mean total hypoglycemic symptom scores (P = 0.01) (Fig. 2) and peak symptom scores in patients with type 1 diabetes and NAH (P = 0.04) (Table 2). These decreases were mainly due to reduced neurogenic and general symptom responses (P = 0.008 and P = 0.04) (Table 2). In healthy participants, HIIT numerically decreased all symptom categories, but this failed to reach statistical significance (P = 0.19) (Fig. 2, Table 2). On both test days, patients with diabetes and IAH had the lowest hypoglycemic symptom scores compared with the other two groups; their scores were not affected by prior HIIT (P = 0.80) (Fig.2, Table 2).
Figure 1: Time courses of plasma glucose (A and B) and plasma lactate (C and D) during the rest day (left panels) or HIIT day (right panels). Dashed lines represent the beginning and end of the euglycemic phase and the beginning of the hypoglycemic phase, respectively. During the euglycemic phase, participants performed HIIT or rested. Baseline values represent the first sample obtained upon arrival. Open circles, healthy controls; black squares, patients with type 1 diabetes (T1DM) and NAH (T1DM-NAH); black triangles, patients with IAH (T1DM-IAH).

Counterregulatory hormones and glucose infusion rate. HIIT did not affect plasma adrenaline or noradrenaline responses to hypoglycemia in either subgroup (Fig. 3A-F). Plasma levels of glucagon increased to a similar extent in response to hypoglycemia on both test days in healthy participants, but, as expected, did not change on either day in the two patient groups (data not shown). HIIT suppressed the plasma cortisol response to hypoglycemia in healthy participants and patients with NAH (Fig. 3G-I) and suppressed the plasma GH responses in all groups (Fig. 4A-C). Glucose infusion rates during hypoglycemia were not different between HIIT and rest days in any group (5.6 ± 0.5 versus 4.8 ± 1.0 mg · kg⁻¹ · min⁻¹, 4.4 ± 0.6 versus 3.7 ± 0.7 mg · kg⁻¹ · min⁻¹, and 4.4 ± 0.4 versus 4.6 ± 0.4 mg · kg⁻¹ · min⁻¹, respectively, for healthy participants, patients with
NAH, and patients with IAH). The plasma adrenaline response to hypoglycemia was significantly lower in patients with IAH compared with patients with NAH and healthy participants, on both study days ($P < 0.005$) (Fig. 3A-C).

**Figure 2:** Average total hypoglycemic symptom scores. Average individual (individual dots) and average group total symptom scores (gray bars) during hypoglycemia after prior HIIT or seated rest in healthy controls (A), type 1 diabetes mellitus (T1DM)-NAH (B) and T1DM-IAH (C). Symptom scores after 20, 40 and 60 minutes of hypoglycemia were averaged to provide one composite score.

**Table 2. Symptom scores during hypoglycemia**

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls</th>
<th>T1DM-NAH</th>
<th>T1DM-IAH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rest</td>
<td>HIIT</td>
<td>Rest</td>
</tr>
<tr>
<td>Peak</td>
<td>20.3 ± 3.5†</td>
<td>17.8 ± 2.7</td>
<td>31.1 ± 5.6†</td>
</tr>
<tr>
<td>Neurogenic</td>
<td>7.7 ± 1.4†</td>
<td>6.6 ± 0.9†</td>
<td>10.9 ± 2.0†</td>
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<tr>
<td>Neuroglycopenic</td>
<td>5.6 ± 1.4†</td>
<td>4.3 ± 1.1</td>
<td>9.2 ± 1.8†</td>
</tr>
<tr>
<td>General</td>
<td>3.5 ± 0.9†</td>
<td>3.3 ± 0.7</td>
<td>5.2 ± 0.9†</td>
</tr>
</tbody>
</table>

The peak scores (±SEM) represent the highest total symptom score during hypoglycemia, irrespective of the time point. All other symptom scores were averaged over the three time points (after 20, 40 and 60 minutes of hypoglycemia, mean ± SEM). T1DM, type 1 diabetes mellitus. *$P < 0.05$ for HIIT vs. Rest, †$P < 0.05$ versus T1DM-IAH.

**Cognitive function.** All three subgroups performed worse on cognitive tests during hypoglycemia compared with the screening visit, with mean decreases in z scores of 0.6 ± 0.1 and 0.9 ± 0.1 on the HIIT and rest day (all $P < 0.05$) (Fig. 5). Hypoglycemia-induced cognitive dysfunction was less pronounced after prior HIIT than after prior rest (main effect of intervention, $P = 0.01$). There was no significant interaction effect (timepoint x study group, $P = 0.27$). Post hoc
analysis revealed that the attenuating effect of HIIT on hypoglycemia-induced cognitive deterioration was significant only in patients with diabetes and NAH ($P < 0.05$), but did not reach significance in the other two subgroups ($P = 0.11$ for healthy participants and $P = 0.46$ for patients with IAH).

**Figure 3:** Adrenaline, noradrenaline, and cortisol responses to hypoglycemia after prior rest (open circles) or prior HIIT (black squares) in healthy controls (left) and patients with type 1 diabetes mellitus (T1DM)-NAH (middle) and T1DM-IAH (right). Eu, euglycemia; Hypo, hypoglycemia.
Effects of HIIT on awareness of hypoglycemia

Figure 4: GH responses to hypoglycemia after prior rest (open bars) or prior HIIT (black bars) in healthy control subjects (left) and patients with type 1 diabetes mellitus (T1DM)-NAH (middle) and T1DM-IAH (right). Eu, euglycemia; hGH, human GH; Hypo, hypoglycemia, * \( P < 0.05 \).

Figure 5: Hypoglycemia-induced change in cognitive function after seated rest (open bars) or HIIT (closed bars). Mean (with SEM) differences in z scores between the screening visit and hypoglycemia are depicted. * \( P < 0.05 \).
Discussion

The main finding of the current study is that a bout of HIIT suppresses symptoms of subsequent hypoglycemia in patients with type 1 diabetes and NAH. HIIT also causes less hypoglycemia-induced cognitive deterioration, an effect that was mainly driven by the NAH patient subgroup. HIIT did not affect hypoglycemic awareness in patients with IAH, likely because of a ‘floor’ effect in that symptom responses could not be further suppressed than they already were. In healthy participants, HIIT numerically decreased symptoms of hypoglycemia, but this failed to reach statistical significance. These data demonstrate that one short HIIT session is able to rapidly blunt hormonal and symptomatic defenses against hypoglycemia in patients with NAH, which may increase the risk of post-exercise hypoglycemia.

Our findings of the suppressive effect of antecedent HIIT on defenses against hypoglycemia extend those of other exercise studies (26-29). However, there is substantial variation in the specific counterregulatory responses that are affected by antecedent exercise. Antecedent low- to moderate-intensity exercise (at 30% and 50% peak oxygen uptake) has been shown to cause a universal suppression of hormonal and symptomatic responses to next-day hypoglycemia (28). Remarkably, two bouts of more vigorous exercise (~70% peak oxygen uptake) had limited effects, in that only adrenaline responses to subsequent hypoglycemia were attenuated (27). We now demonstrate that an even more intensive exercise test causes blunting of symptomatic, cortisol and GH responses to subsequent hypoglycemia but not of catecholamine responses. Similar defects in counterregulatory hormones were observed in antecedent hypoglycemia studies in which the interval between the stimulus and subsequent hypoglycemia was relatively short (30; 31). Taken together, it appears that antecedent exercise, similar to antecedent hypoglycemia, is able to induce a range of counterregulatory defects, the magnitude and components of which seem to depend on the particular exercise protocol used (e.g., exercise intensity and duration) and the time frame between exercise and subsequent hypoglycemia.

The mechanisms underlying exercise-induced attenuation of counterregulatory hormone and symptom responses are currently not known. We hypothesize that elevated lactate levels in response to HIIT mediate a suppressive effect. High plasma lactate levels lead to an increase in brain lactate use, both after intravenous administration (32) and after vigorous exercise (33; 34). An increase
in brain lactate oxidation during hypoglycemia has been suggested to preserve brain metabolism (10; 12). This may prevent hypoglycemia-induced cognitive dysfunction since the brain is no longer deprived of fuel. Maintenance of brain metabolism may simultaneously impede hypoglycemia sensing by the brain and thus suppress the consequent initiation of protective counterregulatory and symptom responses (35). Previous work found that administration of exogenous lactate during hypoglycemia suppresses symptoms and hormone responses, while preserving cognitive function, thus mimicking the situation seen in patients with IAH (10-13). In these patients, several cerebral adaptations have been observed during hypoglycemia, including increased capacity to use lactate (35-38). Alternatively, lactate may act as a metabolic regulator in the brain rather than as a fuel per se (39; 40).

Several arguments support a role for elevated lactate levels in the suppression of physiological responses to hypoglycemia after HIIT. First, we showed that the deterioration of cognitive function during hypoglycemia was less after HIIT than rest, which is in accordance with previous studies that infused lactate during hypoglycemia (10; 12). In addition, a recent exercise study found that higher lactate levels after HIIT were associated with better executive function during post-exercise recovery under euglycemic conditions (41). We also observed fast suppression of hypoglycemic symptoms and cortisol and GH responses after HIIT (i.e., < 1 h after the initial stimulus). Whereas central nervous system adaptations to hypoglycemia are thought to take hours to days to become manifest (42), the suppressive effects of lactate are known to occur rapidly and probably do not involve an adaptation process (13). Interestingly, the blunting of symptoms was most pronounced in participants with the highest symptom scores. Changes in symptoms were not correlated to changes in adrenaline responses. Increased capacity to transport lactate over the blood-brain barrier in patients with type 1 diabetes (as a result of prior exposure to hypoglycemia) may in part explain differential effects of HIIT in patients with NAH and healthy control subjects (43). However, quantitative comparisons with studies using exogenous lactate during hypoglycemia need to acknowledge the influence of exercise itself, pH differences, and the duration and stability of elevations in plasma lactate levels.

The habituation of the brain in response to recurrent hypoglycemia is thought to underlie the development of IAH (44), and restoration of IAH can be achieved by scrupulous avoidance of hypoglycemia (45; 46). It has recently been speculated that ‘resensitizing’ the brain to hypoglycemia might also be achieved by exposure to high-intensity exercise as a novel stimulus in an animal
model of IAH (47). In this model, antecedent hypoglycemia-induced defects in glucose counterregulation were restored 24 hours after a bout of high-intensity exercise. This differs from our findings, in that we observed no improvements in counterregulatory responses to hypoglycemia after HIIT in patients with type 1 diabetes and IAH. However, differences in species and study design, particularly the timeframe between exercise and hypoglycemia, should be acknowledged when explaining these seemingly contradictory results.

Our study design differed from most studies that addressed the impact of antecedent exercise on defenses against hypoglycemia since the timeframe between exercise and hypoglycemia was short. This approach was chosen because we wanted to assess the effect of high endogenous plasma lactate levels during hypoglycemia, in view of the known effects of exogenous lactate during hypoglycemia. Although we provide arguments for increased lactate levels as an explanation for the suppression of awareness of hypoglycemia and attenuation of cognitive dysfunction, our studies cannot prove a cause-and-effect relationship. Although less likely, mechanisms other than lactate may explain the acute beneficial effects of HIIT on cognition, including increased arousal (48; 49). Participants could not be blinded for the intervention (HIIT vs. rest): although unlikely, some influence of participant expectations on the results cannot be excluded. The strengths of our study include the randomized crossover design that allowed us to compare responses to HIIT and rest in the same participants under similar conditions.

In conclusion, a short bout of high-intensity interval exercise suppresses symptoms of subsequent hypoglycemia in patients with type 1 diabetes but does not affect awareness in patients with IAH. The role of exercise-induced lactate in mediating the suppressive effects of HIIT on hypoglycemic awareness should be further explored. Reduced symptomatic responses may increase the risk of hypoglycemia after intensive exercise.

Aknowledgements
We are indebted to Iris Knoester and Lisa Simons (medical students, Radboud university medical center) for assistance during the glucose clamps. We thank Bregina Kersten for assistance with the exercise protocol (Department of Physiology, Radboud university medical center), Sandra Vos and Ilja Klabbers-Helsper (Department of Medical Psychology, Radboud university medical center) for their advice on cognitive testing, and lastly, all the volunteers for their participation and enthusiasm.
Effects of HIIT on awareness of hypoglycemia

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Contributors
HR, EW and BdG designed the study with input from MvdG, DT, RK and CT. HR performed the experiments and collected all the data. EW assisted with the experiments and data collection. HR analyzed the data and wrote the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages. BdG is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
References

7. Guelfi KJ, Jones TW, Fournier PA: The decline in blood glucose levels is less with intermittent high-intensity compared with moderate exercise in individuals with type 1 diabetes. Diabetes Care 2005;28:1289-1294


Chapter 7

Proinflammatory effects of hypoglycemia in humans with or without diabetes


*: contributed equally

Diabetes 2017 Apr; 66(4):1052-1061
Pro-inflammatory effects of hypoglycemia

Abstract

Severe hypoglycemic events have been associated with increased cardiovascular mortality in patients with diabetes, which may be explained by hypoglycemia-induced inflammation. We used ex vivo stimulations of peripheral blood mononuclear cells (PBMCs) and monocytes obtained during hyperinsulinemic-euglycemic (5.0 mmol/L)-hypoglycemic (2.6 mmol/L) clamps in 11 healthy participants, 10 patients with type 1 diabetes and normal awareness of hypoglycemia (NAH), and 10 patients with type 1 diabetes and impaired awareness (IAH) to test whether the composition and inflammatory function of immune cells adapt to a more proinflammatory state after hypoglycemia. Hypoglycemia increased leukocyte numbers in healthy control participants and patients with NAH but not in patients with IAH. Leukocytosis strongly correlated with the adrenaline response to hypoglycemia. Ex vivo, PBMCs and monocytes displayed a more robust cytokine response to microbial stimulation after hypoglycemia compared with euglycemia, although it was less pronounced in patients with IAH. Of note, hypoglycemia increased the expression of markers of demargination and inflammation in PBMCs. We conclude that hypoglycemia promotes mobilization of specific leukocyte subsets from the marginal pool and induces proinflammatory functional changes in immune cells. Inflammatory responses were less pronounced in IAH, indicating that counterregulatory hormone responses are key modulators of hypoglycemia-induced proinflammatory effects. Hypoglycemia-induced proinflammatory changes may promote a sustained inflammatory state.
Introduction

Hypoglycemia is the most common complication of insulin therapy in people with type 1 diabetes (1). Patients with type 1 diabetes experience, on average, two hypoglycemic events per week and one severe event per year (2). The extent to which hypoglycemia contributes to cardiovascular disease risks in diabetes is debated: an association between severe hypoglycemia and increased mortality from cardiovascular events has been established in patients with type 2 diabetes (3-6) but is less consistent in type 1 diabetes (6-10), even though hypoglycemia occurs much more frequently in patients with type 1 than in those with type 2 diabetes (11).

An increase in circulating proatherothrombotic factors in response to acute insulin-induced hypoglycemia can link hypoglycemia to cardiovascular complications (12-14). In addition, hypoglycemia has been reported to increase leukocyte counts and circulating proinflammatory cytokines in both healthy individuals (15-18) and patients with type 1 diabetes (14; 19), supporting the concept that hypoglycemia-induced systemic inflammation contributes to cardiovascular complications (12; 13; 15).

Adrenaline, the main counterregulatory hormone response to hypoglycemia in patients with type 1 diabetes, may play a role in the hypoglycemia-induced proinflammatory response. When adrenaline is administered to healthy individuals (normoglycemic conditions), it specifically mobilizes leukocytes equipped with cytotoxic effector potential from the marginal pool (vascular epithelium) (20). However, knowledge about the role of adrenaline in hypoglycemia-induced changes related to inflammation is lacking.

Patients with impaired awareness of hypoglycemia (IAH) are at particularly high risk of hypoglycemia (21) because they lack hypoglycemia warning symptoms and have attenuated adrenaline responses (1; 22). If adrenaline contributes to hypoglycemia-induced proinflammatory responses, such effects may be altered in patients with type 1 diabetes and IAH. Under euglycemic conditions, patients with IAH were found to have higher leukocyte counts and a higher rate of endothelial dysfunction and preclinical atherosclerosis than sex- and age-matched patients without IAH (23). In accordance, Joy et al. (16) reported that antecedent hypoglycemia, which underlies the emergence of IAH, results in greater endothelial dysfunction, but inflammatory responses to hypoglycemia are not enhanced after prior hypoglycemia.
Thus, hypoglycemia has been shown to increase circulating proinflammatory cytokines, but the underlying mechanisms, the role of repeated hypoglycemia, and the relationship with counterregulatory hormone responses, particularly adrenaline, are incompletely understood. Because an enhanced proinflammatory state is not exclusively reflected in the levels of circulating cytokines, we studied the effects of acute hypoglycemia on the composition and inflammatory output of immune cells. We investigated these aspects using ex vivo stimulation of peripheral blood mononuclear cells (PBMCs) obtained at various time points during hyperinsulinemic-euglycemic-hypoglycemic clamps in healthy participants and patients with type 1 diabetes. To assess the role of sympathoadrenal responses to hypoglycemia in inducing potential proinflammatory effects, we also included patients with type 1 diabetes and IAH characterized by impaired counterregulatory hormone responses to hypoglycemia.

Research Design and Methods

Participants
We recruited 11 participants without diabetes, 10 patients with type 1 diabetes and normal awareness of hypoglycemia (NAH), and 10 patients type 1 diabetes and IAH. Patients were otherwise healthy and did not use drugs that interfered with glucose metabolism other than insulin. Hypoglycemia awareness state, initially assessed by a Dutch version of the Cox questionnaire in which a score of 0-1 of 5 indicates normal awareness and a score ≥ 3 indicates impaired awareness (24; 25), was determined on the basis of adrenaline and symptomatic responses to the hypoglycemic clamp. Eighteen out of the 20 patients were correctly characterized as having IAH or NAH through the Cox questionnaire. The institutional review board of the Radboud university medical center (Nijmegen, the Netherlands) approved the study, and all participants gave written informed consent before participation.

Experimental design
All participants presented between 8.00 and 8.30 A.M. at the clinical research facility after an overnight fast and having abstained from caffeine, alcohol, and smoking for 24h. Patients with diabetes received specific instructions to avoid (nocturnal) hypoglycemia the day before the clamp. Experiments were rescheduled in cases of hypoglycemia in the 24 h before the clamp. Upon arrival, two intravenous cannulae were inserted, one into the antecubital vein of each forearm. One forearm was placed in a heated box (55°C) so that arterialized
venous blood could be obtained for frequent blood sampling. The cannula in the contralateral arm was used for infusion of glucose 20% (Baxter B.V., Deerfield, IL) and insulin (insulin aspart; Novo Nordisk, Bagsvaerd, Denmark). Baseline plasma glucose levels were determined (Biosen C-Line; EKF Diagnostics, Cardiff, U.K.) and a two-step hyperinsulinemic (60 mU/m²/min)-euglycemic (5.0 ± 0.2 mmol/L)-hypoglycemic (2.6 ± 0.1 mmol/L) glucose clamp was initiated. Plasma glucose levels were determined every five min, and after a short euglycemic phase (~20 min), plasma glucose levels were gradually decreased to 2.6 mmol/l and were maintained there for 60 min. Blood samples for measurement of adrenaline were taken at euglycemia and every 20 min during hypoglycemia. Insulin and glucagon were determined at euglycemia and at 60 min of hypoglycemia.

**Analytical methods**

Plasma insulin was assessed by an in-house radioimmunoassay (RIA) (26). After extraction (27), plasma glucagon was measured with a commercially available radioimmunoassay kit (Eurodiagnostica, Malmö, Sweden). Plasma growth hormone and cortisol were determined by routine analysis with an electrochemiluminescent immunoassay on a Modular Analytics E170 (Roche Diagnostics, GmbH, Manheim, Germany). Plasma adrenaline and noradrenaline were analyzed by high-performance liquid chromatography combined with fluorometric detection (28). Peripheral total and differential white blood cell counts were determined by routine patient sample analyses (flow cytometric analysis on a Sysmex XE-5000).

**Isolation of PBMCs and CD14⁺ monocytes**

Blood samples were processed for isolation of cells immediately after being drawn to ensure equal quality of the samples because previous experiments showed that cytokine responses are altered when blood samples are processed for isolation at different time points after being drawn (data not shown). Isolation of PBMCs was performed by differential centrifugation over Ficoll-Paque PLUS (GE Healthcare). PBMCs were washed three times with PBS and counted with a Coulter counter (Coulter Electronics). CD14⁺ monocytes were purified from freshly isolated PBMCs by using MACS MicroBeads (Miltenyi Biotec, Germany) for positive selection according to the manufacturer’s instructions.

**Stimulation experiments**

For analysis of cytokine release, glucose consumption and lactate production, 5 x 10⁵ PBMCs or 1 x 10⁵ monocytes were used per well in a 96-well plate. Cells were cultured in RPMI medium (no glucose, Gibco) supplemented with 10 μg/mL
gentamicin (Gibco), 10 mM pyruvate (Gibco), 10 mmol/L HEPES (Sigma-Aldrich), 5.5 mmol/L glucose (Sigma-Aldrich) and stimulated with either RPMI medium, 10 ng/mL of the TLR4 agonist lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich), 10 μg/mL of the TLR2 agonist Pam3CysSK4 (Pam3Cys) (EMC Microcollections, Tübingen, Germany), 1 μg/mL *Mycobacterium tuberculosis* (H37Rv) lysate, or 1 x 10⁶ heat-killed organisms/mL *Candida albicans* conidia for 24 h. Cell culture supernatants were collected and stored at −20°C.

**Cytokine measurements**

The production of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) (R&D Systems), IL-10, IL-6 (Sanquin) and MCP-1 (eBioscience) was measured by ELISA. In the analysis of cytokine production by CD14⁺ monocytes, participants were excluded from analysis when cytokine production upon stimulation of cells after both euglycemia and hypoglycemia was below detection limits. The number of ‘non-responders’ was comparable between groups.

**Glucose consumption and lactate measurements**

Glucose and lactate concentrations were measured in cell culture supernatants. Measurements were based on an enzymatic reaction in which glucose or lactate is oxidized and the resulting H₂O₂ is coupled to the conversion of Amplex Red reagent to fluorescent resorufin by horseradish peroxidase. The fluorescence of resorufin (excitation/emission maxima 570/585 nm) was measured on a 96-well plate reader (BioTek). Glucose consumption was calculated by subtracting the glucose concentration measured in cell culture supernatants from that in culture medium incubated for 24 h without cells.

**RNA isolation and qRT-PCRs**

For mRNA expression analyses, PBMCs (1.5 x 10⁶ PBMCs/condition) were lysed in TRIzol reagent (Invitrogen) directly after isolation and stored at −80°C until RNA isolation was performed according to the manufacturer’s instructions. RNA was transcribed into cDNA by reverse transcription using the iScript cDNA synthesis kit (Bio-Rad). Primer sequences used for quantitative real-time PCR (qRT-PCR) are listed in Supplementary Table 2. Power SYBR Green PCR Master Mix (Applied Biosystems) was used for qRT-PCR in the CFX384 Real-Time PCR Detection System (Bio-Rad). Expression data were normalized to the housekeeping gene human β₂M.
**Statistical analysis**

Data were tested for normality using the Shapiro-Wilk test and Q-Q plots. Within-group differences were compared with paired Student *t* or Wilcoxon signed rank tests when data were not normally distributed. Between-group differences were analyzed by ANOVA followed by pair wise Bonferroni post hoc tests to delineate statistical significance and for nonparametric data with the Kruskal-Wallis test and post hoc Mann-Whitney *U* tests. For correlation analysis Pearson correlation coefficient was used for normally distributed variables and Spearman rank sum test for nonnormally distributed data. All data are expressed as mean ± SEM unless otherwise specified. *P* < 0.05 was considered statistically significant. Statistical analyses were performed with SPSS version 20 software (IBM Corporation).

**Results**

Study participants were well matched for age, sex, and BMI (Table 1). Duration of diabetes and HbA<sub>1c</sub> did not differ significantly between patient groups. Plasma glucose levels (Fig. 1A) and plasma insulin levels (data not shown) were similar in all groups during both the euglycemic and the hypoglycemic phase, whereas glucagon levels increased in response to hypoglycemia in healthy control participants but did not change in either patient group (Supplementary Table 1). Adrenaline levels during hypoglycemia were significantly lower in patients with IAH than in healthy control participants and patients with NAH (0.39 ± 0.07, 1.94 ± 0.29, and 1.90 ± 0.46 nmol/L, respectively).

<table>
<thead>
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<td><strong>Healthy control participants (n = 11)</strong></td>
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<td>BMI, kg/m²</td>
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<tr>
<td>HbA&lt;sub&gt;1c&lt;/sub&gt;, % (mmol/mol)</td>
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<td>Duration of diabetes (years)</td>
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<td>Total daily insulin dose (IU)</td>
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Data are mean ± SD or n.
Pro-inflammatory effects of hypoglycemia

Figure 1: Hypoglycemia induces leukocytosis in healthy control participants (HC) and patients with type 1 diabetes (T1DM) and NAH but not in T1DM and IAH who have attenuated adrenaline responses to hypoglycemia. A: Time courses of plasma glucose levels during the clamp. Dashed lines represent the end of the euglycemic phase and the beginning and end of the hypoglycemic phase. B-E and G: Number of circulating leukocytes (B), neutrophils (C), lymphocytes (D) and monocytes (E) and composition of leukocytes (G) measured with a routine patient sample analysis at euglycemia and after 1 h of hypoglycemia. F: Correlation between the difference in leukocyte numbers during hypoglycemia vs. euglycemia and the difference in adrenaline levels between hypoglycemia and euglycemia. *P < 0.05, **P < 0.01.
**Hypoglycemia increases total leukocyte count**

The total leukocyte count increased in response to hypoglycemia in healthy control participants and patients with NAH but not in patients with IAH (Fig. 1B). This increase was mainly due to an increase in the number of lymphocytes and, to a lesser extent, an increase in the number of monocytes (Fig. 1C-E). Consequently, neutrophil-to-lymphocyte ratios decreased in response to hypoglycemia (Fig. 1G). The change in total leukocyte count correlated positively with the adrenaline response to hypoglycemia (R² = 0.70, P < 0.001) (Fig. 1F). The positive correlation was strongest in lymphocytes (R² = 0.75, P < 0.001), but was also seen in monocytes (R² = 0.33, P = 0.003) and neutrophils (R² = 0.29, P = 0.007) (Supplementary Fig. 1). By looking at the separate groups, the positive correlation between the change in total leukocyte count and adrenaline response was significant in healthy control participants and patients with NAH, but not in patients with IAH.

**Hypoglycemia increases ex vivo proinflammatory cytokine production**

PBMCs from healthy control participants and patients with NAH isolated after 1 h of hypoglycemia and stimulated with the TLR4 agonist LPS produced more proinflammatory cytokines (IL-6, IL1β, and TNFα) than PBMCs isolated after euglycemia. Hypoglycemia had no effect on LPS-stimulated cytokine production of PBMCs isolated from patients with IAH (Fig. 2A-C). Hypoglycemia increased the production of the chemokine MCP-1 in healthy control participants (Fig. 2D) but did not affect levels of the anti-inflammatory cytokine IL-10 in any group (Fig. 2E).

Hypoglycemia enhanced the TNF-α response of PBMCs stimulated with Pam3Cys, *M. Tuberculosis*, and *C. albicans* in all three groups. Hypoglycemia also increased the IL-6 response to *M. tuberculosis* in healthy control participants and patients with NAH but had virtually no effect on the IL-6 and IL-1β responses to Pam3Cys or *C. albicans* in any of the three groups (Supplementary Fig. 2). Altogether, hypoglycemia enhanced cytokine responses of PBMCs, with the most prominent increase in TNF-α responses (Fig. 2F).

Of note, cytokine release of stimulated PBMCs from patients with IAH isolated during euglycemia tended to be higher than cells from healthy control participants and patients with NAH, although the differences were not statistically significant.
Figure 2: PBMCs isolated after hypoglycemia produce more proinflammatory cytokines than PBMCs isolated after euglycemia. A-E: IL-6 (A), IL-1β (B), TNF-α (C), MCP-1 (D), and IL-10 (E) production of PBMCs isolated from euglycemic or hypoglycemic conditions and stimulated for 24 h with LPS. F: Fold change in cytokine production (IL-6, IL-1β, TNF-α) by PBMCs upon hypoglycemia vs. euglycemia. The gray dashed line represents euglycemic values. PBMCs were stimulated with the TLR4 agonist LPS, the TLR2 agonist Pam3Cys, C. Albicans, or lysate of M. tuberculosis. Data are mean (continuous lines) ± SEMv(dotted lines). * P < 0.05, ** P < 0.01. HC, healthy control; T1DM, type 1 diabetes mellitus.
**Hypoglycemia does not affect glycolytic metabolism of PBMCs**

We then investigated whether hypoglycemia affected glycolytic metabolism of PBMCs. As expected, stimulation with LPS significantly increased glucose consumption and lactate production of PBMCs in all groups (Fig. 3A and B). However, no difference was found in either glucose consumption or lactate production between cells exposed to hypoglycemic versus euglycemic conditions, regardless of whether stimulated with LPS.

![Figure 3](image)

**Figure 3**: No differences in glycolytic metabolism of PBMCs isolated from hypoglycemia vs. euglycemia. Glucose consumption (A) and lactate secretion (B) measured in the supernatants of PBMCs isolated from euglycemic or hypoglycemic conditions and cultured for 24 h with or without stimulation with LPS. *P < 0.05, **P < 0.01. HC, healthy control; T1DM, type 1 diabetes mellitus.

**Hypoglycemia generally increases expression of markers for demargination and cells with cytotoxic effector potential**

Because adrenaline levels increased markedly in response to hypoglycemia and because adrenaline drives demargination of leukocytes (20), we investigated whether hypoglycemia altered gene expression levels of demargination markers in isolated PBMCs. Hypoglycemia increased the expression of the integrin CD11a in PBMCs of healthy control participants and patients with NAH but not in patients with IAH (Fig. 4A). Hypoglycemia also increased the expression of the chemokine receptor CX3CR1 in PBMCs of healthy control participants (Fig. 4B).

![Figure 4](image)

Next, we assessed the expression of marker genes of various immune cell types in PBMCs exposed to hypoglycemia or euglycemia (Fig. 4C-G). Hypoglycemia increased the expression of CD8 but not of CD4 or CD56 in PBMCs in all groups.
Pro-inflammatory effects of hypoglycemia

Figure 4: Increased expression of markers for demargination and cells with cytotoxic effector potential after hypoglycemia as assessed by qRT-PCR in PBMCs exposed to euglycemia or hypoglycemia. Relative expression of CD11a (A), CX3CR1 (B), CD4 (C), CD8 (D), CD56 (E), CD14 (F) and CD16 (G). * $P < 0.05$, ** $P < 0.01$. HC, healthy control; T1DM, type 1 diabetes mellitus.
Figure 5: CD14+ monocytes isolated after hypoglycemia produce more proinflammatory cytokines than CD14+ cells isolated after euglycemia. A: Percentage of CD14+ cells isolated by MACS. B: Fold change in cytokine production (IL-6, IL-1β, TNF-α) by CD14+ cells isolated from hypoglycemic vs. euglycemic conditions. CD14+ cells were stimulated with the TLR4 agonist LPS, the TLR2 agonist Pam3Cys, C. Albicans, or lysate of M. tuberculosis. Data are mean (continuous lines) ± SEM (dotted lines). HC, healthy control; T1DM, type 1 diabetes mellitus.
Moreover, although hypoglycemia did not alter expression of CD14, it increased the expression of CD16, a marker for the nonclassical monocyte subset that produces more cytokines than the classical monocytes in response to certain stimulations (29).

**Hypoglycemia increases ex vivo cytokine production of CD14^+ cells**

Because monocytes are the major producers of proinflammatory cytokines within the heterogeneous PBMC cell population, we specifically investigated the effect of hypoglycemia on the inflammatory function of CD14^+ monocytes. Hypoglycemia did not affect the percentage of isolated CD14^+ monocytes within the PBMC fraction in any of the three groups (Fig. 5A). When stimulated ex vivo, CD14^+ cells produced more proinflammatory cytokines, particularly TNFα, if isolated after hypoglycemia compared with euglycemia (Fig. 5B). Nevertheless, CD14^+ cells did not have increased gene expression levels of surface markers characterizing proinflammatory monocytes (CD11a, CXCR1, CCR5, CCR2) (Supplementary Fig. 3).

**Discussion**

The present study aimed to investigate the effect of acute hypoglycemia on the composition and inflammatory function of circulating immune cells. We demonstrate that exposure to hypoglycemia leads to demargination of specific immune cell subtypes and enhances the inflammatory response of PBMCs and CD14^+ monocytes. Of note, the hypoglycemic response of PBMCs was partly blunted in patients with type 1 diabetes and IAH, highlighting the role of adrenaline in immune cell recruitment and in the acute inflammatory response to hypoglycemia. The data support the concept that hypoglycemia shifts circulating immune cells toward a more proinflammatory state. When sustained, such an enhanced inflammatory state could contribute to atherogenesis in people with diabetes.

In line with previous findings (15), the results demonstrate that hypoglycemia induces leukocytosis. The strong correlation with adrenaline responses to hypoglycemia suggests a role for adrenaline, which is supported by our observations in patients with type 1 and IAH who have blunted counterregulatory hormone responses to hypoglycemia. These studies now extend previous findings by investigating the inflammatory function of isolated immune cells ex vivo. We observed that TNFα production significantly increases in PBMCs exposed
to hypoglycemia, independent of the pathogenic stimulus (LPS, Pam3Cys, C. albicans or M. tuberculosis), which strongly implies that the hypoglycemic event causes a universal potentiation of inflammatory function of the cells. Because equal numbers of PBMCs were used in stimulations to compare the two glycemic conditions, the increased levels of circulating proinflammatory cytokines found in previous studies (15; 17-19; 30) are not only due to the increase in the number of circulating immune cells in response to hypoglycemia but also likely reflect changes in the functional status of immune cells.

In contrast to TNF-α responses, the effect of hypoglycemia on IL-6 and IL-1β production was less pronounced and more variable between the various stimuli, suggesting that changes in pathogen-specific signalling pathways are involved. Such changes could affect either the expression of pattern recognition receptors on the cell surface or expression of their downstream effectors. If intracellular signalling pathways are indeed affected by hypoglycemia, this could also prime the immune cells to respond differently to other stimuli, such as proatherogenic factors.

PBMCs are a heterogeneous mix of cell populations, and changes in composition could also explain the increased cytokine production observed in response to hypoglycemia. However, measurements of the cellular composition of several PBMC samples did not reveal major changes (percentage of lymphocytes, monocytes, and granulocytes) after hypoglycemia. Of note, CD11a and CX3CR1 gene expression levels were increased in PBMCs exposed to hypoglycemia, suggesting an increase in the number of demarginated cells (20). Recruitment of a distinct cell population with a different phenotype and function likely contributes to the observed change in inflammatory responses after exposure to hypoglycemia. Similar to leukocytosis experimentally induced by adrenaline (20), hypoglycemia increased the number of cells with cytotoxic effector potential, such as lymphocytes expressing CD8. Additionally, hypoglycemia increased levels of CD16, suggesting an increase in circulating CD16+ monocytes and natural killer cells, also secondary to adrenaline-mediated leukocytosis (20). Future studies applying flow cytometric analysis of circulating immune cells to provide additional information on the specific surface expression of selected proteins on certain cell populations would be of particular interest.

Cytokine production was similarly altered in CD14+ cells and PBMCs exposed to hypoglycemia. Although we cannot distinguish between the different monocyte subsets (classical, intermediate, nonclassic) within the population of
isolated CD14⁺ cells, the increased cytokine response of PBMCs is likely based on the enhanced cytokine production capacity of CD14⁺ monocytes because the percentage of monocytes was similar at hypoglycemia and euglycemia.

Another factor that may contribute to an altered inflammatory output of immune cells is a shift in cellular metabolism induced by changes in the metabolic environment. For instance, a highly active glycolytic metabolism has been shown to drive proinflammatory cytokine production in M1 macrophages and is also important for activated effector T cells (31). However, similar glucose consumption and lactate production of cells isolated from hypoglycemic versus euglycemic conditions make it unlikely that changes in inflammatory responses are due to changes in glycolytic metabolism of immune cells. We cannot fully exclude an involvement of glycolytic metabolism because acute changes can occur in vivo, but might be masked at the time point that we measured lactate levels in vitro.

The results revealed abrogation of hypoglycemia-induced leukocytosis and an attenuated inflammatory response to hypoglycemia in patients with IAH, potentially as a consequence of extensive prior exposure to hypoglycemia. The attenuated inflammatory response in patients with IAH underscores the contribution of counterregulatory hormones, especially adrenaline, in hypoglycemia-induced proinflammatory effects. Of note, hypoglycemia increased CD8 and CD16 expression in PBMCs of patients with IAH, indicating an increase in circulating cells with cytotoxic effector potential in these patients even while total leukocyte count did not increase upon hypoglycemia. This might be explained by the minimal, albeit still significant, increase in adrenaline levels in response to hypoglycemia. Leukocyte numbers and cytokine responses during euglycemia appeared higher but were not significantly elevated in patients with IAH compared with healthy control participants or patients with NAH. Although attributing this trend to prior exposure to hypoglycemia is tempting, a larger sample size would be required to address this question.

One could speculate that an attenuated proinflammatory response to acute hypoglycemia as observed in the patients with IAH might provide some protection against harmful effects of subsequent hypoglycemia. Frequent hypoglycemic events, typical for patients with type 1 diabetes, have been reported to protect against hypoglycemia-induced mortality (32) or neuronal damage (33) in rats. These adaptive effects of recurrent hypoglycemia appear to be in line with the reported absence of increased cardiovascular mortality.
in patients with type 1 diabetes and IAH compared with those with NAH (10) but contrasts with studies focusing on vascular effects that reported higher rates of preclinical atherosclerosis in patients with repeated hypoglycemia (23) and greater proatherothrombotic responses and endothelial dysfunction after recurrent hypoglycemia (16). Studies are needed to determine the long-term consequences of repeated hypoglycemia and IAH on inflammation and immune cells, their inflammatory function, and their involvement in atherogenesis.

The strengths of this study include the use of glucose clamps in three matched groups of participants under similar glycemic conditions, which enabled us to differentiate between the impact of diabetes and IAH. A larger sample size would have allowed us to differentiate better between patients with IAH and NAH, especially with regard to baseline values. Although we analyzed gene expression of demargination markers and of specific cell types, flow cytometric analysis would have provided a more detailed characterization of changes in composition and inflammatory status of leukocytes. Future studies should focus on mechanistic studies in lymphocytes and also look into the role of neutrophils to extend the current gene expression data. Another limitation of this study is that participants with and without diabetes were healthy and relatively young. Inflammatory responses to hypoglycemia might differ in older patients, in those with a history of cardiovascular disease, or in those with poor glycemic control.

We conclude that hypoglycemia leads to demargination, an increase in circulating immune cells with cytotoxic effector potential, and an induction of proinflammatory functional changes in PBMCs and CD14⁺ monocytes. Acute inflammatory responses to hypoglycemia were partly blunted in patients with type 1 diabetes and impaired awareness of hypoglycemia, highlighting that counterregulatory hormone responses are key modulators of proinflammatory responses to hypoglycemia. These data indicate that hypoglycemia induces a shift in inflammatory function of immune cells, which could promote a sustained proinflammatory state in patients with diabetes.

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Contributors
JR, HR, BdG and RS designed the study with input from MN and CT. JR, RS, AH and HR performed the experiments. JR and HR analyzed the data. All authors discussed the results and implications and commented on the manuscript at all stages. RS is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
References


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Supplementary material

Table S1: Hormonal responses to hypoglycemia

<table>
<thead>
<tr>
<th>Target</th>
<th>Healthy controls</th>
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<th>T1DM-IAH</th>
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<td>EU</td>
<td>HYPO</td>
<td>EU</td>
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<tr>
<td>Glucagon (pmol/L)</td>
<td>15.8 ± 0.8</td>
<td>33.0 ± 3.7*</td>
<td>13.4 ± 0.8</td>
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<td>Adrenaline (nmol/L)</td>
<td>0.11 ± 0.02</td>
<td>1.90 ± 0.46*‡</td>
<td>0.10 ± 0.01</td>
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<td>Noradrenaline (nmol/L)</td>
<td>1.19 ± 0.17</td>
<td>1.94 ± 0.20*</td>
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<tr>
<td>Cortisol (µmol/L)</td>
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<tr>
<td>hGH (mU/L)</td>
<td>8.4 ± 3.8</td>
<td>32.2 ± 9.5*</td>
<td>5.7 ± 1.6</td>
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Hormonal responses at euglycemia and after 60 minutes of hypoglycemia. Data are mean ± SEM, *P < 0.05 for euglycemia versus hypoglycemia, †P < 0.05 versus healthy controls, ‡P <0.01 versus T1DM-IAH

Table S2: Primer sequences used to assess gene expression by qRT-PCR.

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<th><strong>target gene</strong></th>
<th><strong>forward primer</strong></th>
<th><strong>reverse primer</strong></th>
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<td>CCR5</td>
<td>CAAAGAGAGGCTCTCTTAC</td>
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Figure S1: Correlation between the difference in lymphocyte (A), monocyte (B) and neutrophil (C) numbers during hypoglycemia versus euglycemia and the difference in adrenaline levels between hypoglycemia and euglycemia. Black circles, healthy controls; black squares, T1DM-NAH patients; black triangles, T1DM-IAH patients.

Figure S2: IL-6, IL-1β and TNF-α production of PBMCs isolated from euglycemic or hypoglycemic conditions and stimulated for 24 h with Pam3Cys (A-C), C. albicans (D-F) or lysate of M. tuberculosis (G-I). Open bars, euglycemic values; black bars, hypoglycemic values. * P < 0.05, ** P < 0.01.
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**Figure S3:** Relative expression of CD11a, CX3CR1, CCR5 and CCR2 assessed by qRT-PCR in CD14⁻ (A-D) or CD14⁺ (E-H) cells exposed to euglycemia or hypoglycemia. Open circles, euglycemic values; black circles, hypoglycemic values. *P < 0.05.
Chapter 8

Summary and discussion
Summary

One out of three patients with type 1 diabetes has a reduced ability to perceive the onset of hypoglycemia. This condition is referred to as impaired awareness of hypoglycemia (IAH) and is usually the consequence of a process of habituation to recurrent hypoglycemia. IAH and the associated increased risk for severe hypoglycemia constitute a major burden for patients with type 1 diabetes and their relatives. Alterations in brain metabolism are most certainly involved in the pathogenesis of IAH, but the precise mechanisms underlying IAH have remained unclear. As a consequence, targeted treatment options that do not deteriorate glycemic control are lacking, and more detailed knowledge regarding the pathophysiology of IAH is urgently needed. We hypothesized that the brain of patients with IAH has been conditioned to use an alternative fuel when plasma glucose levels are low. The overall aim of this thesis was to explore the role of lactate as a non-glucose alternative fuel in the development of IAH.

In Chapter 2, we explained which neuroimaging techniques can be used to study brain metabolism in humans in vivo and critically reviewed the results of studies that have used these techniques to assess cerebral metabolism in response to (recurrent) hypoglycemia in individuals with and without IAH. In short, neuroimaging studies have shown that recurrent hypoglycemia and IAH induce cerebral adaptations on many different levels, including alterations in regional cerebral blood flow, neuronal activation patterns, and in the handling of brain fuels. Overall, patients with IAH seem better able in preserving brain metabolism during hypoglycemia than healthy volunteers, probably as a result of these adaptations. Most neuroimaging studies found no evidence of altered brain glucose uptake and concentrations in these patients, suggesting a role for non-glucose alternative fuels, particularly lactate, in preserving brain metabolism.

Brain lactate concentrations can be assessed and quantified in humans in vivo by using proton magnetic resonance spectroscopy (1H-MRS). Throughout this thesis we have employed this non-invasive, MRI-related method to quantify brain lactate concentrations. In a series of experiments, we combined 1H-MRS with the glucose clamp technique, a method that allows plasma glucose levels to be held at specific predetermined targets by using a constant insulin infusion and a variable glucose infusion. The results of the first set of these experiments are described in Chapter 3. Using 1H-MRS for lactate detection, we measured brain lactate concentrations during stepped hyperinsulinemic-
mental glucose counterregulatory responses were reduced compared with the euglycemic condition. We concluded that exercise training may result in glucose counterregulatory adaptation and improved glucose tolerance in patients with diabetes, which may have implications for exercise prescription in diabetes management.

Chapter 6

In Chapter 6, we aimed to investigate the effect of exercise training on brain lactate concentrations and counterregulatory responses in patients with diabetes. We performed a 15 min high-intensity interval training (HIIT) session followed by a hypoglycemic clamp in patients with type 1 diabetes and IAH, patients with type 1 diabetes and normal awareness of hypoglycemia (NAH), and healthy participants without diabetes. Brain lactate concentrations dropped in response to acute hypoglycemia in patients with type 1 diabetes and IAH, whereas concentrations remained unaltered in patients with NAH and in participants without diabetes. This effect could not be explained by differences in plasma lactate levels. We concluded that the fall in brain lactate in patients with IAH most likely reflects increased brain lactate oxidation during acute hypoglycemia, which may preserve brain metabolism and interfere with the brain’s capacity to detect hypoglycemia.

Next, we aimed to investigate the effect of elevated plasma lactate levels, and we studied both the effect of exogenous and endogenous plasma lactate elevations on brain lactate concentrations during hypoglycemia with 1H-MRS. In Chapter 4, we first demonstrate that the administration of exogenous lactate during hypoglycemia, compared with placebo infusion, considerably suppresses counterregulatory responses to hypoglycemia in patients with NAH. These data are in line with studies performed in the late 1990s that demonstrated blunted symptomatic and hormonal responses to hypoglycemia and reduced cognitive dysfunction when lactate was infused during hypoglycemia. While previous studies primarily focused on clinical effects, we also assessed changes in the brain. Lactate administration increased brain lactate levels modestly during both euglycemia and hypoglycemia in patients with NAH and IAH. Since cerebral lactate uptake is driven by a concentration gradient from blood to brain, elevated plasma lactate levels would have been expected to result in an increased brain lactate uptake. As the accumulation of lactate in the brain in response to lactate infusion was rather limited and less than expected, we hypothesized that lactate taken up by the brain is almost immediately oxidized. Such an increase in brain lactate oxidation during hypoglycemia could indeed explain the suppressive effects of lactate on awareness of hypoglycemia observed in patients with NAH.

Next, we studied the effect of endogenous plasma lactate elevations on brain lactate concentrations during hypoglycemia, using high-intensity interval training (HIIT). This relatively new training modality has gained popularity and is increasingly advocated among patients with diabetes, but also markedly elevates plasma lactate levels. Since exercise may affect counterregulatory responses to subsequent hypoglycemia, it is also clinically relevant to examine the effect of HIIT. In Chapter 5, patients with IAH, patients with NAH, and healthy individuals underwent a hypoglycemic clamp after performing a 15 min HIIT-session on a
Summary and discussion

cycle ergometer. We measured brain lactate concentrations before HIIT and continuously during hypoglycemia after HIIT with 1H-MRS. Plasma lactate levels increased similarly in all groups in response to HIIT and resulted in increased brain lactate concentrations during hypoglycemia. However, the increase in brain lactate was most pronounced in patients with IAH. In addition, brain lactate levels decreased below baseline levels in this group during subsequent hypoglycemia, very similar to the observation described in chapter 3. These results further support the concept of increased brain lactate transport and increased brain lactate oxidation in patients with type 1 diabetes and IAH.

Following on our demonstration that HIIT increases brain lactate concentrations, we investigated the clinical effects of HIIT on awareness of subsequent hypoglycemia. We hypothesized that elevated lactate levels in response to HIIT have similar suppressive effects on awareness of hypoglycemia as exogenously infused lactate. To address this question we performed a randomized crossover trial in which patients with NAH, patients with IAH, and healthy individuals underwent a hypoglycemic clamp, either after a HIIT-session as described above, or after seated rest. The results of this trial, reported in Chapter 6, confirmed our hypothesis and showed that a single, short HIIT-session leading to a markedly increased plasma lactate level, reduced symptoms of hypoglycemia in patients with NAH, compared with rest. Furthermore, HIIT attenuated the decline in cognitive function that normally occurs during hypoglycemia, again consistent with previous studies using exogenous lactate administration. In patients with IAH, HIIT had no effect on awareness of hypoglycemia, probably because symptom responses were already low in these patients and could not be suppressed any further. It is likely that elevated (brain) lactate levels after HIIT have mediated the suppressive effects of HIIT, by acting as an alternative brain fuel during hypoglycemia. Clinically, the reduced symptomatic responses may result in an increased risk of hypoglycemia after HIIT in patients with type 1 diabetes.

Hypoglycemia does not only affect the brain, but has also been associated with increased cardiovascular morbidity and mortality. Although hypoglycemia could simply be a marker of underlying vulnerability explaining this association, a direct contribution of hypoglycemia to cardiovascular complications seems likely. It has been hypothesized that inflammation explains this link, because hypoglycemia increases circulating proinflammatory cytokines and systemic inflammation increases the risk for cardiovascular complications. However, the mechanisms leading to increased cytokine production and the effects of
(recurrent) hypoglycemia on the inflammatory function of immune cells are unknown. In Chapter 7, the results of a study into the effect of hypoglycemia on the composition and inflammatory function of immune cells, using ex vivo stimulations of peripheral blood mononuclear cells (PBMCs) obtained during a euglycemic-hypoglycemic clamp in patients with IAH, NAH, and healthy volunteers, are described. We found that hypoglycemia promotes mobilization of specific (more inflammatory) leukocyte subsets and increases the inflammatory response of PBMCs. These data demonstrate that hypoglycemia is able to shift circulating immune cells toward a more proinflammatory profile, which (when sustained) may promote low-grade inflammation and may contribute to the development of atherosclerosis in patients with diabetes. However, acute inflammatory responses to hypoglycemia were related to the adrenaline responses and therefore partly blunted in patients with IAH. This observation highlights the role of counterregulatory hormones in immune cell recruitment and pro-inflammatory responses to hypoglycemia and raises the question as to whether IAH has protective effects against hypoglycemia-induced harm beyond that of the brain.

Discussion

Impaired awareness of hypoglycemia is a fairly prevalent syndrome that constitutes a major burden for patients with type 1 diabetes, but the underlying mechanisms remain insufficiently clarified. The studies described in the present thesis support a role for lactate in the development and persistence of IAH. This is supported by the fact that brain lactate concentrations behave quite comparably in patients with normal awareness of hypoglycemia (NAH) and healthy volunteers, whereas cerebral lactate handling is altered in patients with IAH. In patients with IAH, hypoglycemia decreases brain lactate, most likely due to increased lactate oxidation. After high-intensity interval training, brain lactate concentrations increase more pronounced in these patients, compatible with enhanced cerebral lactate transport into the brain. Both alterations in brain lactate handling may contribute to the preservation of brain metabolism during hypoglycemia that could interfere with the brain’s capacity to detect hypoglycemia, thus explaining the clinical picture of IAH. This concept is in line with our observation that both exogenous and endogenous plasma lactate elevations compromise awareness of subsequent hypoglycemia and reduce hypoglycemia-induced cognitive dysfunction.
**Lactate as an alternative fuel**

We provide further evidence that brain lactate plays an important role in IAH, presumably by acting as an alternative energy fuel for the brain. Measuring brain lactate during hypoglycemia is challenging, but with 1H-MRS we were able to quantify brain lactate concentrations in vivo in a non-invasive and direct manner. However, the current studies cannot fully prove that lactate is indeed oxidized. Although spectra were recorded over time, 1H-MRS was used to measure brain lactate levels and cannot provide information about brain lactate fluxes. Brain lactate concentrations depend on the rates of brain lactate uptake, production, oxidation and export. An decrease in brain lactate transport for example, may also decrease brain lactate concentrations during hypoglycemia as observed in patients with IAH, but we consider this less likely, as plasma lactate levels fall to a similar extent in patients with and without IAH during hypoglycemia, and because lactate transport capacity has been reported to be increased in patients with IAH. Future research, using 13C MRS in combination with infusion of 13C labeled compounds, may provide detailed information on lactate fluxes during hypoglycemia and could be used to confirm increased brain lactate oxidation in patients with IAH. It should be noted that 13C-MRS is technically challenging, involves expensive 13C labeled materials and needs mathematic modeling. Future studies should also address alterations in brain lactate in patients with type 1 diabetes in a longitudinal study and before and after (successful) treatment of IAH. Reversibility of adaptations in brain lactate handling could substantiate a causative role for lactate in the development of IAH.

The present work describes adaptations in brain lactate in a relatively large area in the periventricular region of the brain. Hypotheses that consider alterations in brain fuels as the cause of IAH are based on the premise that hypoglycemia causes a decrease in cerebral glucose metabolism, which in turn causes an increase in sympathoadrenal activity. Metabolism of the alternative fuel (i.e. lactate) needs to be sufficient to raise brain oxidative metabolism. A recent PET study challenges this view, as hormonal counterregulatory responses occurred at milder levels of hypoglycemia than those necessary to produce a reduction in cerebral glucose metabolism (1). While these data suggest that a reduction in global brain metabolism is not required for counterregulatory hormone responses, they do not exclude the possibility that such responses are triggered by local reductions in glucose metabolism in critical brain regions. Regarding lactate, this may implicate that metabolism only needs to be sufficient to increase brain oxidative metabolism in local brain areas. The most important region in the brain for the detection of hypoglycemia is the ventromedial...
hypothalamus (VMH) and it would be interesting to assess changes in lactate handling in patients with IAH in this specific region in future studies.

**Alternative roles for lactate**
Recently, it has been suggested that lactate may exert other roles in the brain, in addition to being used as a fuel (2; 3). Lactate has the potential to act as an intercellular signaling molecule and metabolic regulator. Mechanistic evidence is limited to in vitro data, but may prove relevant in the pathogenesis of IAH. A few observations from hypoglycemia studies support such an additional role for lactate. In a rodent model of IAH, Herzog et al. demonstrated that elevating lactate levels during hypoglycemia resulted in maintenance of brain glucose metabolism rather than in an increase in lactate oxidation (4). A study that evaluated cross-brain arteriovenous concentration differences during hypoglycemia in humans calculated that the increase in brain lactate oxidation during hypoglycemia was able to account for a substantial part (~25%) of the brain glucose energy deficit, but did not completely offset the fuel deficit (5). The proportional contribution of lactate to overall brain energy consumption is likely higher in patients with IAH, due to an increased brain lactate transport capacity (6; 7), but still may not be sufficient to fully substitute glucose.

Lactate may not only support brain metabolism by acting as an energy substrate, but may also act a ‘metabolic regulator’ through various mechanisms. Lactate is taken up by the brain through proton-linked facilitated diffusion by monocarboxylate transporters (8). Entry of large amounts of lactate and co-transported protons may alter intracellular pH, which affects the activity of important glycolytic enzymes, such as phosphofructokinase (9). Inside the brain cell, the enzyme lactate dehydrogenase (LDH) catalyzes the conversion of lactate to pyruvate and back, through a reversible oxidation-reduction (redox) reaction. Even relatively small increases in local brain lactate uptake can shift the LDH reaction toward pyruvate formation (10), which increases NADH/NAD⁺ ratios and triggers several intracellular responses (2). Lactate may also signal through a specific lactate receptor, the G-protein-coupled receptor 81 (GPR81), only discovered in the mammalian brain in 2013 (11), which affects metabolism via the cAMP pathway. Future studies focusing on lactate should further explore these alternative mechanisms.
Other cerebral adaptations in IAH

Given the complexity of the physiological response to hypoglycemia, it is likely that the brain adapts to recurrent hypoglycemia by more than one mechanism. Recent studies (12; 13) report alterations in global and regional cerebral blood flow responses to hypoglycemia in patients with IAH. For example, hypoglycemia increases global CBF in patients with IAH, but not in patients with NAH or healthy volunteers (13). Similar to adaptations in lactate handling, this may be an adaptive response aimed at neuroprotection that simultaneously suppresses counterregulatory responses. In addition, alterations in neurotransmission, in particular increased output of the inhibitory neurotransmitter GABA in the VMH (14), may also contribute to defective counterregulatory responses. Of note, lactate is able to influence both cerebral blood flow and (15) and VMH GABA levels (16).

Clinical implications and future perspectives

The work performed in this thesis contributes to a better understanding of the mechanisms involved in IAH, but also has some direct clinical implications. Antecedent low- and moderate-intensity exercise has previously been shown to suppress hormonal and symptomatic responses to next-day hypoglycemia (17). In chapter 6, we demonstrated that a more intensive, but shorter, exercise modality in the form of a single HIIT-session, is able to rapidly reduce symptomatic responses to a subsequent episode of hypoglycemia in patients with NAH. These results imply that patients with type 1 diabetes should closely monitor their blood glucose levels after high-intensity interval exercise, since this may increase the risk of postexercise hypoglycemia or prolong its duration. Because short-term increments in plasma glucose levels often occur after HIIT, patients may tend to correct this with insulin, but this should only be done with great care.

The central role of lactate in the suppression of counterregulatory responses to hypoglycemia may eventually translate into clinical approaches. First and foremost, the mechanisms by which lactate affects awareness of hypoglycemia require further, detailed mechanistic studies. In animal models of IAH, lactate metabolism and its signaling pathways can be specifically assessed with blockers (e.g. using LDH-blockers, GPR81 antibodies etc.) or studied in rodents with genetically deleted or overexpressed enzymes. In animal models of epilepsy, LDH blockage has already been tested and successfully suppressed seizures (18). It will be challenging to develop targeted clinical interventions, aimed at reducing brain lactate transport or metabolism as lactate is such an important mediator in multiple pathways and interfering with its metabolism may result in untoward effects (19).
While many people with type 1 diabetes experience recurrent hypoglycemia, less than half of them develop IAH. Another future direction would be to investigate genetic variations in lactate receptors or transporters, which may affect susceptibility to develop IAH. It would also be interesting to look into changes in lactate transporters in the brain in response to various types of exercise. Acute exercise has been shown to increase region-specific expression of lactate transporters in rats (20), and this may be a mechanistic link between exercise and the development of counterregulatory defects.

**IAH: the two sides of the coin**

Maintaining a level of glucose control that both prevents complications associated with hyperglycemia and minimizes the problems associated with hypoglycemia is a continuous challenge for patients with type 1 diabetes and their health care providers. Our results contribute to a more balanced view on cerebral and inflammatory effects of hypoglycemia. Recurrent hypoglycemia is regarded by patients and caregivers as unfavorable, because of its potential harmful effects for the brain and the interference with daily life activities, occasionally leading to dangerous situations. Few may realize that recurrent hypoglycemia may also induce some beneficial adaptations that may (partly) protect against harm from subsequent hypoglycemia and perhaps dampen the systemic effects of hypoglycemia. Animal studies have shown that recurrent mild to moderate hypoglycemia protects against severe hypoglycemia-induced neuronal damage, cognitive impairment and death (21; 22). In analogy, the adaptations in brain lactate handling in patients with IAH are likely aimed at protecting the brain against harm from subsequent severe hypoglycemia, by preserving brain metabolism when glucose supply is limited. Paradoxically, these adaptations may simultaneously impair the detection of hypoglycemia by the brain and thus the initiation of protective counterregulatory responses. Altogether, it seems that recurrent episodes of hypoglycemia render a patient more prone to an episode of severe hypoglycemia, but somewhat less vulnerable to its harm.

Hypoglycemia is a systemic stressor with effects beyond that affecting the brain. We found that hypoglycemia induces proinflammatory changes in circulating immune cells in healthy volunteers and patients with NAH, which may contribute to systemic inflammation and aggravate cardiovascular complications in patients with diabetes. In patients with IAH, however, we observed an attenuated proinflammatory response to acute hypoglycemia. Analogously to cerebral adaptations, this attenuated response may provide some protection against
harmful effects of subsequent hypoglycemia. This adaptive effect of recurrent hypoglycemia is in line with recent observations that IAH may not increase all-cause or cardiovascular mortality in patients with type 1 diabetes (23). In contrast, studies focusing on vascular effects report higher rates of preclinical atherosclerosis in patients who experienced recurrent hypoglycemia and greater proatherothrombotic responses after prior hypoglycemia in healthy individuals (24, 25). More research is needed to determine long-term consequences of recurrent hypoglycemia and IAH on inflammation and immune cells, and their role in atherogenesis.

**Closing remarks**
Impaired awareness of hypoglycemia is a prevalent and burdensome syndrome that limits optimal glucose control and substantially increases the risk for severe hypoglycemia in patients with type 1 diabetes. Our studies contribute to the unraveling of its pathophysiology and support an important role for brain lactate handling in the development of IAH. The results of our studies may add towards better understanding of IAH and in the end provide avenues towards therapeutic interventions for IAH. A joint effort of clinical and non-clinical researchers is needed to further disentangle the mechanisms of IAH in order to reduce the burden of severe hypoglycemia, maintain the benefits of good glycemic control, and improve the overall wellbeing of people suffering from type 1 diabetes.
References

Summary and discussion


Chapter 9

Nederlandse samenvatting
Nederlandse samenvatting

Suikerziekte (*diabetes mellitus*) is een steeds vaker voorkomende ziekte. Alleen al in Nederland zijn er ruim een miljoen mensen met diabetes. Er zijn verschillende vormen van diabetes, waarvan de bekendste type 1 en 2 diabetes zijn. Dit proefschrift gaat over type 1 diabetes, de vorm die bij grofweg 10% van de diabetespatiënten voorkomt en vaak op jonge leeftijd ontstaat. Type 1 diabetes is een auto-immuunziekte, dat wil zeggen dat het eigen afweersysteem de insuline producerende cellen in de alvleesklier kapot maakt. Insuline zorgt ervoor dat suiker (*glucose*) uit het bloed wordt opgenomen in spieren en vet, waar het als brandstof gebruikt kan worden. Insuline verlaagt dus de bloedglucosespiegel. Bij patiënten met type 1 diabetes stijgt de bloedglucosespiegel door het gebrek aan insuline. Zonder toediening van insuline leidt dit op korte termijn tot overlijden. Een chronische, mindere, verhoging van de bloedglucosespiegel is ook gevaarlijk, want dit kan leiden tot ernstige complicaties, zoals hart- en vaatziekten, blindheid en zenuw- en nierschade. Om deze complicaties zoveel mogelijk te voorkomen wordt gestreefd naar een zo normaal mogelijke bloedglucosespiegel. Patiënten spuiten hiervoor dagelijks meerdere malen insuline.

De afgelopen decennia zijn er veel ontwikkelingen geweest in de behandeling van diabetes, met verfijndere insulines en nieuwe toedieningsvormen. Desondanks blijft de behandeling met insuline in de praktijk verre van perfect. Bij te weinig spuiten van insuline blijft de bloedglucosespiegel te hoog en bij te veel spuiten van insuline daalt de glucosespiegel te sterk. Een te lage bloedglucosespiegel wordt een hypoglykemie (*hypo*) genoemd. Patiënten met type 1 diabetes ervaren gemiddeld twee hypo’s per week, wat neerkomt op vele duizenden hypo’s gedurende hun leven. Normaal gesproken zorgt een hypo ervoor dat bepaalde stresshormonen vrijkomen (zoals adrenaline), deze hormonen helpen het lichaam om de glucosespiegel te herstellen. Ook ontstaan er herkenbare klachten als zweten, trillen en honger. Echter, bij één op de drie patiënten treden de hormoonreactie en klachten van een hypo niet meer of in verminderde mate op. Dit verminderde gevoel om hypo’s op te merken wordt ‘*impaired awareness of hypoglycemia*’ (IAH) genoemd. Patiënten met deze aandoening lopen een zeer groot risico op ernstige hypo’s. De glucosespiegel is dan zo laag dat een verminderd bewustzijn of een coma optreedt. Patiënten zijn dan niet meer in staat om zelf de glucosespiegel te corrigeren, maar afhankelijk van anderen voor hulp. Ernstige hypo’s kunnen zo gevaarlijke situaties opleveren en leiden tot ongelukken en soms zelfs tot overlijden.
Hoe IAH ontstaat, is niet precies bekend. Men denkt dat oorzaak ergens in het brein ligt, omdat de hersenen een rol spelen in zowel het opmerken van een hypo als in het opstarten en regelen van de hormoon- en symptoomreactie op een hypo. Waarschijnlijk spelen veranderingen in de energiehuishouding van de hersenen, ten gevolge van het ervaren van eerdere hypo’s, een rol in IAH. Normaal gesproken is glucose de belangrijkste brandstof voor het brein, maar de hersenen kunnen ook andere, alternatieve brandstoffen gebruiken. Één van die brandstoffen is melkzuur (lactaat). Lactaat is het zuur wat het lichaam aanmaakt als de spieren ‘verzuren’ door intensief sporten, maar is ook in rust in kleine hoeveelheden in het bloed en brein aanwezig. Onze hypothese was dat de hersenen van patiënten met IAH als het ware zijn getraind om een alternatieve brandstof te gebruiken wanneer de bloedglucosespiegel laag is. In dit proefschrift onderzochten we de rol van lactaat, als alternatieve brandstof voor glucose, in het ontstaan van IAH.

Hoofdstuk 1 geeft uitleg over de achtergrond van IAH, de gevolgen die deze aandoening heeft voor het dagelijks leven van patiënten en de huidige (beperkte) behandelopties. In hoofdstuk 2 bekeken we welke technieken het mogelijk maken om hersenmetabolisme in ‘real life’ (in vivo) te meten in mensen. Ook gaan we dieper in op de resultaten van eerdere studies die deze technieken hebben gebruikt om te kijken naar veranderingen in hersenmetabolisme tijdens een hypo of in reactie op een hypo. Samenvattend laten deze neuroimaging studies zien dat (herhaalde) hypo’s leiden tot aanpassingen in de hersenen op meerdere niveaus, waaronder veranderingen in regionale bloedtoevoer, patronen van hersenactiviteit en gebruik van brandstoffen. Patiënten met diabetes (met name patiënten met IAH) lijken beter in staat om hun stofwisseling (metabolisme) in de hersenen in stand te houden tijdens een hypo dan gezonde vrijwilligers, waarschijnlijk ten gevolge van deze aanpassingen. Opvallend is dat neuroimaging studies geen aanwijzingen hebben gevonden voor een verandering in de hoeveelheid glucose in het brein van deze patiënten. Dit suggereert een rol voor alternatieve brandstoffen, zoals lactaat, in het behoud van brein metabolisme tijdens een hypo.

In de experimenten beschreven in dit proefschrift hebben we gebruik gemaakt van proton (1H) magnetische resonantie spectroscopie (1H-MRS) als neuroimaging techniek. Dit is een niet-invasieve, aan MRI-gerelateerde techniek waarbij concentraties van metabolieten in het brein gemeten kunnen worden. Voor het verrichten van de studies in dit proefschrift werd deze techniek geoptimaliseerd voor het meten van de hoeveelheid lactaat in het brein. In
een serie van experimenten combineerden we 'H-MRS met de zogeheten 'glucose clamp' methode. Middels deze methode is het mogelijk om de bloedglucosespiegel zeer precies te regelen. Met behulp van een insuline-infuus en variabel glucose-infuus wordt de bloedglucosespiegel als het ware 'vastgezet' op een vooraf bepaalde glucosewaarde.

In hoofdstuk 3 worden de resultaten van het eerste onderzoek beschreven, waarin we 'H-MRS gebruikten voor het meten van lactaat in de hersenen tijdens normale bloedglucosewaardes (euglykemie) en tijdens hypoglykemie in drie groepen proefpersonen: 1) proefpersonen zonder diabetes, 2) patiënten met type 1 diabetes en een normale gevoeligheid voor hypo’s (normal awareness of hypoglycemia: NAH) en 3) patiënten met type 1 diabetes en IAH. De concentratie lactaat in het brein daalde gedurende de hypo bij patiënten met diabetes en IAH, maar bleef stabiel in zowel de patiënten met NAH als in de proefpersonen zonder diabetes. De hoeveelheid lactaat in het bloed was gelijk in de drie groepen. We concludeerden dat de daling in brein lactaat in de patiënten met IAH meest waarschijnlijk komt door meer verbruik van lactaat als brandstof (oxidatie) tijdens de hypo. Wanneer lactaat inderdaad als alternatieve brandstof wordt gebruikt, dan zou het zorgen voor behoud van brein metabolisme tijdens een hypo en op deze manier verhinderen dat het brein het glucosegebrek detecteert. Wanneer het brein geen energiegebrek detecteert, zal het ook geen waarschuwingssignaal afgeven naar het lichaam en komt de lichamelijke reactie op de hypo niet op gang.

Vervolgens hebben we gekeken naar het effect van verhoogde lactaatwaardes in het bloed. Er zijn twee manieren om dit te bereiken: door toediening van lactaat via een infuus (exogene lactaat) of door het lichaam zelf meer lactaat te laten aanmaken (endogene lactaat), bijvoorbeeld middels sport. In hoofdstuk 4 laten we zien dat de toediening van exogene lactaat tijdens hypoglykemie, in vergelijking met toediening van een niet werkzame stof (placebo), leidt tot een sterke vermindering van hypoklachten en van de hormonale reactie op een hypo bij patiënten met NAH, patiënten die normaal gesproken een goede gevoeligheid voor hypo’s hebben. In deze studie zagen we een duidelijk toename van lactaat in het bloed, maar de toename van lactaat in het brein was relatief gering. Het is bekend dat het transport van lactaat naar het brein toeneemt bij hogere lactaatwaardes in het bloed. Het ontbreken van een duidelijke stijging van lactaat in het brein bij deze hoge bloedwaardes zou kunnen betekenen dat het extra getransporteerd lactaat in het brein direct wordt gebruikt als brandstof. Dit verhoogde gebruik van lactaat in het brein zou de verklaring kunnen zijn
voor de onderdrukkende effecten van lactaat op hypo symptomen en de hormoonreactie bij patiënten met NAH.

Vervolgens hebben we gekeken naar het effect van een endogene verhoging van bloed lactaat op brein lactaat concentraties tijdens hypoglykemie. Het endogene lactaat werd verhoogd met een hoogintensieve interval training (high-intensity interval training: HIIT). HIIT is een relatief nieuwe, steeds populairdere trainingsmodaliteit die toenemend wordt geadviseerd aan patiënten met diabetes en tevens zorgt voor een sterke stijging van lactaat in het bloed. In hoofdstuk 5 ondergingen vergelijkbare groepen proefpersonen (proefpersonen zonder diabetes, patiënten met diabetes en NAH, en patiënten met diabetes en IAH) een hypo nadat ze een vijftien minuten durende HIIT-training op een fiets hadden uitgevoerd. De bloedlactaatwaardes namen fors toe en waren gelijk in de groepen. Tevens was in iedere groep een toenem in brein lactaat zichtbaar, maar deze toenem was het grootst in patiënten met IAH. Daarnaast daalde de hoeveelheid brein lactaat gedurende de hypo tot onder de beginwaardes in patiënten met IAH, maar niet in de andere twee groepen. Deze bevindingen zijn vergelijkbaar met de resultaten beschreven in hoofdstuk 3 en ondersteunen het idee dat patiënten met IAH een verhoogde capaciteit hebben om lactaat te transporteren naar het brein en om brein lactaat te oxideren (gebruiken als brandstof) tijdens een hypo.

Nadat we aan hadden getoond dat HIIT brein lactaat concentraties verhoogd, onderzochten we het klinische effect van HIIT op de gevoeligheid voor het bemerken van een hypo. Van andere sportmodaliteiten (zoals laag- en middelintensieve training) is bekend dat ze de gevoeligheid voor het bemerken van een hypo kunnen verminderen, maar van HIIT is dit nog niet eerder onderzocht. Onze hypothese was dat verhoogde bloed lactaatspiegels na HIIT vergelijkbare onderdrukkende effecten hebben op de hypo gevoeligheid als exogeen lactaat. Om deze vraag te beantwoorden voerden we een onderzoek uit waarin proefpersonen zonder diabetes, patiënten met NAH en patiënten met IAH op twee ochtenden een hypo ondergingen, op de ene ochtend na een korte HIIT-sessie en op de andere ochtend na een periode van rust (in willekeurige volgorde). Ook bekeken we hoe proefpersonen konden functioneren tijdens de hypo’s met onder andere reken- en geheugentests. De resultaten van de trial worden gepresenteerd in hoofdstuk 6 en bevestigen onze hypothese dat een HIIT-sessie, welke leidt tot hoge lactaatwaardes in het bloed, symptomen van een hypo vermindert in vergelijking met een periode van rust in patiënten met NAH. Daarnaast konden deelnemers beter blijven functioneren tijdens de hypo.
Na HIIT in vergelijking met de hypo na rust. In patiënten met IAH had HIIT geen effect op de gevoeligheid voor hypo’s, waarschijnlijk omdat de hyposymptomen al zeer laag waren in deze groep, zodat ze niet nog verder onderdrukt konden worden. Het is waarschijnlijk dat de verhoogde bloedlactaatspiegels na HIIT de onderdrukkende effecten op hypo gevoeligheid hebben veroorzaakt, doordat lactaat als een alternatieve brandstof functioneert voor het brein tijdens de hypo. Dit zou ook het betere functioneren na HIIT kunnen verklaren. In de praktijk kan de verminderde symptoom- en hormoonreactie op een hypo na HIIT ervoor zorgen dat patiënten met diabetes een groter risico lopen op hypo’s na dergelijke trainingen.

Hypoglykemieën hebben niet alleen effect op het brein, maar zijn ook geassocieerd met een verhoogd risico op (overlijden aan) hart- en vaatziekten. Ontstekingssstoffen in het bloed dragen waarschijnlijk bij aan schade aan bloedvaten bij patiënten met diabetes. Van hoge bloedglucose is bekend dat dit leidt tot meer ontstekingssstoffen in het bloed, onder andere aangemaakt door bepaalde witte bloedcellen, die betrokken zijn bij de afweer tegen ziektes. Wij vroegen ons af of hypo’s op een vergelijkbare manier zouden kunnen bijdragen aan ontsteking. In hoofdstuk 7 presenteren we de resultaten van een onderzoek dat werd gedaan in dezelfde drie onderzoeksgroepen als in hoofdstuk 6. Voorafgaand en aan het einde van een hypo filterden we een deel van de witte bloedcellen uit het bloed van proefpersonen. We zagen dat de hoeveelheid witte bloedcellen bij proefpersonen zonder diabetes en bij patiënten met NAH sterk toenam tijdens de hypo. Dit betrof met name witte bloedcellen die geassocieerd zijn met ontsteking. Deze toename wordt waarschijnlijk veroorzaakt door het stresshormoon adrenaline dat vrijkomt tijdens een hypo. Bij patiënten met IAH, bij wie de stressrespons bijna niet aanwezig is gedurende de hypo, zagen we dan ook geen toename in de hoeveelheid cellen. Wel zagen we in alle deelnemende groepen in het laboratorium dat witte bloedcellen die aan een hypo zijn blootgesteld, meer ontstekingssstoffen aanmaken wanneer ze een nieuwe stressvolle situatie meemaken dan witte bloedcellen die alleen zijn blootgesteld aan ‘normale’ suikers. Deze data laten zien dat een hypo zorgt voor een toename in het aantal witte bloedcellen dat zich in het bloed bevindt, en dat deze cellen ook meer actief zijn (meer ontstekingsstoffen maken). Dit zou het ontstaan van vaatschade kunnen promoten, met name wanneer het effect lang aan houdt. Echter, de inflammatoire reactie was minder uitgesproken in patiënten met IAH, mogelijk ten gevolge van het frequent ervaren van eerdere hypo’s. Voor het brein is het reeds aangetoond dat het herhaald ervaren van milde hypo’s beschermt tegen breinschade tijdens volgende hypo’s. Mogelijk geldt dit beschermende effect ook voor de bloedvaten.
Conclusie

Impaired awareness of hypoglycemia (IAH) is een vaak voorkomend syndroom dat belastend is voor patiënten met type 1 diabetes. Het onderliggende mechanisme blijft onvoldoende verklaard. De studies in dit proefschrift ondersteunen een rol voor lactaat in het ontstaan en persisteren van IAH. Onze bevindingen laten zien dat brein lactaat concentraties zich vergelijkbaar gedragen in proefpersonen zonder diabetes en patiënten met type 1 diabetes met een normale gevoeligheid voor hypo’s, maar dat er veranderingen optreden in brein lactaat concentraties in patiënten met IAH. In deze patiënten daalt de hoeveelheid lactaat in het brein tijdens hypoglykemie, waarschijnlijk ten gevolge van een toename in de capaciteit om brein lactaat te oxideren en gebruiken als brandstof. Na een hoogintensieve intervaltraining nemen brein lactaat concentraties meer toe in patiënten met IAH, wat past bij een verhoogde capaciteit om lactaat van het bloed te transporteren naar het brein. Beide aanpassingen in de verwerking van brein lactaat (meer oxidatie en meer transport) kunnen bijdragen aan behoud van brein metabolisme tijdens een hypoglykemie. Deze aanpassingen zijn waarschijnlijk gericht op bescherming van het brein tegen schade ten gevolge van een mogelijke volgende hypoglykemie, maar belemmeren tegelijkertijd de detectie van de hypo door het brein, waarmee ze bijdragen aan het klinische beeld van IAH. Er zijn in nieuwe laboratorium onderzoeken zelfs aanwijzingen dat lactaat niet alleen direct, maar ook indirect (als een soort van hormoon) invloed zou kunnen uitoefenen op het brein metabolisme. Een rol voor lactaat in het onderdrukken van de hypo reactie wordt bevestigd door onze bevindingen dat zowel een exogene als endogene verhoging van lactaat in het bloed de gevoeligheid voor het bemerken van een hypo vermindert en cognitief disfunctioneren tijdens de hypo beperkt.

Daarnaast hebben we laten zien dat een hypo ook belangrijke effecten heeft buiten het brein. We ontdekten dat het aantal witte bloedcellen toeneemt na een hypo en dat deze cellen ook actiever zijn: ze maken meer ontstekingsstoffen aan bij stress. Het is mogelijk dat deze ontstekingsreactie na een hypo bijdraagt aan het ontstaan van vaatschade bij mensen met diabetes.
Appendices
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List of publications


**Curriculum Vitae**


Hanne Rooijackers is in 2018 gestart met de opleiding tot internist, hiervoor is zij momenteel werkzaam als arts-assistent in het CWZ ziekenhuis in Nijmegen.